Discovery of a Novel Insect Neuropeptide Signaling System Closely Related to the Insect Adipokinetic Hormone and Corazonin Hormonal Systems*[S]

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Neuropeptides and their G protein-coupled receptors (GPCRs) play a central role in the physiology of insects. One large family of insect neuropeptides are the adipokinetic hormones (AKHs), which mobilize lipids and carbohydrates from the insect fat body. Other peptides are the corazonins that are structurally related to the AKHs but represent a different neuropeptide signaling system. We have previously cloned an orphan GPCR from the malaria mosquito *Anopheles gambiae* that was structurally intermediate between the *A. gambiae* AKH and corazonin GPCRs. Using functional expression of the receptor in cells in cell culture, we have now identified the ligand for this orphan receptor as being pQVTFSRD-WNAamide, a neuropeptide that is structurally intermediate between AKH and corazonin and that we therefore named ACP (AKH/corazonin-related peptide). ACP does not activate the *A. gambiae* AKH and corazonin receptors and, vice versa, AKH and corazonin do not activate the ACP receptor, showing that the ACP/receptor couple is an independent and so far unknown peptidergic signaling system. Because ACP is structurally intermediate between AKH and corazonin and the ACP receptor between the AKH and corazonin receptors, this is a prominent example of receptor/ligand co-evolution, probably originating from receptor and ligand gene duplications followed by mutations and evolutionary selection, thereby yielding three independent hormonal systems. The ACP signaling system occurs in the mosquitoes *A. gambiae*, *Aedes aegypti*, and *Culex pipiens* (Diptera), the silkworm *Bombyx mori* (Lepidoptera), the red flour beetle *Tribolium castaneum* (Coleoptera), the parasitic wasp *Nasonia vitripennis* (Hymenoptera), and the bug *Rhodnius prolixus* (Hemiptera). However, the ACP system is not present in 12 *Drosophila* species (Diptera), the honeybee *Apis mellifera* (Hymenoptera), the pea aphid *Acyrthosiphon pisum* (Hemiptera), the body louse *Pediculus humanus* (Phthiraptera), and the crustacean *Daphnia pulex*, indicating that it has been lost several times during arthropod evolution. In particular, this frequent loss of hormonal systems is unique for arthropods compared with vertebrates.

Adipokinetic hormone (AKH)[3] is an insect neuropeptide produced by the corpora cardiaca, a neuroendocrine organ closely associated with the insect brain (1). During high physical activities such as flight and intense locomotion, AKH is released into the circulation and transported to the fat body, where it binds to its G protein-coupled receptors (GPCRs) (2). Activation of these GPCRs stimulates the release of carbohydrates (trehalose) and lipids, which are needed to fuel the high physical activity of the insect (1). AKHs and their GPCRs, however, are also involved in hemolymph carbohydrate homeostasis by releasing trehalose under nonstress conditions, a process that in *Drosophila* larvae is counteracted by insulin-like peptides and the insulin receptor, which decrease hemolymph trehalose concentrations. Thus, AKH and insulin in insects act comparably to glucagon and insulin in mammals during blood sugar homeostasis (3).

The AKHs are a large family of small, structurally related peptides, all being 8–10 amino acid residues long (1). The structure of the AKH peptide produced by the malaria mosquito *Anopheles gambiae* (4) is shown in Fig. 1. AKHs occur in all insects investigated so far, with some insects producing more than one AKH (1, 5). AKH peptides also occur in crustaceans, where they are named red pigment-concentrating hormone, because of their function in color adaptation (1).

The insect corazonins are widespread in insects and are undecapeptides that are structurally related to the AKHs, which is illustrated for the *A. gambiae* corazonin in Fig. 1. The insect corazonin receptors have recently been identified (6, 7). They do not cross-react with AKHs and the AKH receptors do not cross-react with corazonins, showing that these are two independent hormonal systems (2, 6). Phylogenetic tree analyses show that not only the AKH and cora-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and Tables S1 and S2.
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[3] The abbreviations used are: AKH, adipokinetic hormone; Aa, *Aedes aegypti*; ACP, AKH/corazonin-related peptide; ACPR, ACP receptor; AKHR, AKH receptor; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Bm, *Bombyx mori*; CCAP, crustacean cardioactive peptide; CCAPR, CCAP receptor; CHO, Chinese hamster ovary; Cp, *Culex pipiens*; CRZR, corazonin receptor; Dm, *Drosophila melanogaster*; EGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptor; Nv, *Nasonia vitripennis*; qPCR, quantitative PCR; Tc, *Tribolium castaneum*; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; Ag, *Anopheles gambiae*; Bm, *Bombyx mori*; On, *Ostrinia nubilalis*; Rp, *Rhodnius prolixus*.

Neuropeptide name	Amino acid sequence
AKH	pQLTF--TPAW--amide
ACP (AKH/corazonin-related peptide)	pQVTF--SRDWNAamide
Corazonin	pQ-TFQYSRGWTNamide

FIGURE 1. **Comparison between AKH, corazonin, and ACP (AKH/corazonin-related peptide) from the malaria mosquito *A. gambiae*.** Residues that are identical between all three peptides are highlighted in *red*. Amino acid residues that are identical between ACP and corazonin only are highlighted in *green*. A pair of conserved (not identical but very similar) residues between AKH and ACP is highlighted in *blue*.

zonin neuropeptides but also the AKH and corazonin receptors are closely related, suggesting co-evolution of receptors and ligands in a process, where the ancestor receptor and neuropeptide genes have duplicated. This is followed by mutations and evolutionary selection, leading to two independent hormonal systems each with its own physiological role (Fig. 2) (6–9).

The physiological roles of corazonin in insects are, so far, not well understood. The peptide increases heartbeat in cockroaches (10) and induces color change (darkening) associated with starvation, swarming, and migration in crowded locust populations (11), and in some insects, it is involved in the control of motor behavior associated with molting (12). These findings could perhaps mean that corazonin is also involved in the management of stress situations, suggesting that AKHs and corazonins have specialized in the control of different aspects of stress or high physical activities, which would fit well with the receptor/ligand co-evolution hypothesis mentioned above.

In an attempt to understand the function and evolution of AKH and corazonin receptors, we have annotated these and related GPCRs in insects with a sequenced genome (8, 9) and subsequently cloned and characterized many of them. In this process, we have also cloned an AKH and a corazonin receptor from the malaria mosquito *A. gambiae* and characterized them as receptors specifically activated by *A. gambiae* AKH and corazonin, respectively (13). In addition to these two receptors, however, a third *A. gambiae* receptor was annotated and cloned that was closely related to the AKH and corazonin receptors but could not be activated by AKH, corazonin, or any other peptide from our peptide library (13). In this paper, we identify the ligand for this orphan receptor as a peptide that is structurally intermediate between AKH and corazonin, and which we name AKH/corazonin-related peptide (ACP; see Fig. 1). Because the ACP receptor is also structurally intermediate between the AKH and corazonin receptors, this finding is a prominent example of receptor/ligand co-evolution (Fig. 2). Furthermore, we show that the novel ACP/receptor couple is widely occurring in insects, suggesting that it is an important insect signaling system.

EXPERIMENTAL PROCEDURES

Animals—*A. gambiae* (strain G-3) was kindly supplied by Dr. Robert E. Sinden, Imperial College, London, UK. *Bombyx mori*

(hybrid strain of Lyon 200 BA and Lyon 300 AB) was a kind gift from Dr. Søren Wilken Rasmussen (Carlsberg Laboratory, Copenhagen, Denmark); *Tribolium castaneum* (strain GA-1) was a generous gift from Prof. Martin Klingler (University of Erlangen-Nürnberg, Germany). A transgenic *T. castaneum* strain, where the central nervous system is marked with fluorescent color,[4] was kindly supplied by Prof. Gregor Bucher (University of Göttingen, Germany) and Dr. Michalis Averof (Foundation for Research and Technology, Crete, Greece). *Nasonia vitripennis* was a kind gift from Prof. Jack Werren (University of Rochester). All insects have been cultured in our laboratory, using standardized conditions.

cDNA Cloning, PCR, and qPCR—For PCR, total RNA was isolated using TRIzol reagent (Invitrogen) or the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized and amplified using the SMART RACE cDNA amplification kit (Clontech) or, for 5′RACE, the FirstChoice RLM-RACE kit (Ambion).

The cloning of the *A. gambiae* ACP receptor (Ag-ACPR-A) has been published previously (13). The coding sequence of *A. gambiae* ACPR-B was amplified using the sense primer 5′-CCGCTAGCGCCGCCATGTACCTGGCAGCCGGCTTA-3′ (the underlined nucleotides correspond to nucleotide positions 1–21 of supplemental Fig. S1) and the antisense primer 5′-CCGCTAGCCTAAATGAGATGTTTTTCAAGATACTC-3′ (the underlined nucleotides correspond to nucleotide positions 1399–1425 of supplemental Fig. S1).

The coding sequence of the *T. castaneum* ACP preprohormone was amplified using the sense primer 5′-GCTATATAA-AGTTGCTAGTCCGCC-3′ and the antisense primer 5′-TTA-TCGTCGGCCACTGAAAAT-3′. The coding sequence of the *T. castaneum* ACP receptor was amplified using the sense primer 5′-CCGCTAGCCGCGCGATGCAAGCGGTTGGTA-AAATG-3′ (the underlined nucleotides correspond to nucleotide positions 1–21 of supplemental Fig. S3) and the antisense primer 5′-CCGCTAGCCTATAAAACAGCAGAACTGTAA-TAATC-3′ (the underlined nucleotides correspond to nucleotide positions 1348–1374 of supplemental Fig. S3). 3′RACE of the *T. castaneum* ACP receptor was made with the sense primer 5′-GAAAGGGCTAGGAGCAGAACC-3′ (corresponding to the nucleotide positions 799–819 of supplemental Fig. S3) followed by nested sense primer 5′-GCAGAACCTTAAGGATGACCATC-3′ (corresponding to the nucleotide positions 812–834 of supplemental Fig. S3). 5′RACE of the *T. castaneum* ACP receptor was made with the antisense primer 5′-GCCAGTCAATCTCCAGCCAACC-TCG-3′ (corresponding to the nucleotide positions 300–324 of supplemental Fig. S3) followed by the nested sense primer 5′-AATCAAATCAGCGATGGCAAGATGGCG-3′ (corresponding to nucleotide positions 250–277 of supplemental Fig. S3), using the FirstChoice RLM-RACE kit (Ambion).

All PCR products were cloned into pCR4-TOPO (Invitrogen) using the TOPO TA cloning kit (Invitrogen) and sequenced. The PCR product of the receptor coding sequence was subcloned into the pIRES2-EGFP expression vector (Clontech) using the Rapid DNA ligation kit (Roche Applied Science) and sequenced.

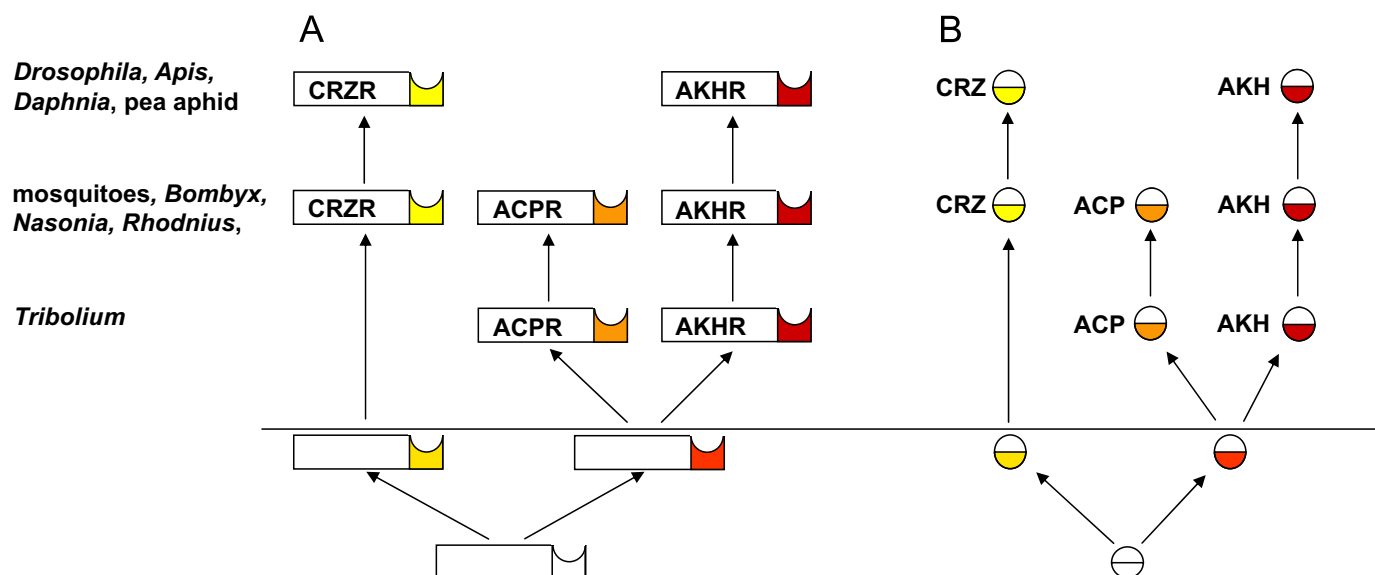[4] M. Averof and G. Bucher, unpublished data.

FIGURE 2. **Proposed scenario for the receptor/ligand co-evolution, leading to receptors specific for either AKH, ACP, or corazonin.** The receptors and ligands *below* the *horizontal line* are ancestral and therefore not identifiable. The *white areas* in the receptors indicate structural relationship. The same holds for the ligands. AKHs and their receptors are highlighted in *red*, corazonins and their receptors in *yellow*, and ACPs and their receptors are marked in *orange*. The ancestral receptors and ligands drawn immediately *below* the *horizontal line* have intermediate colors. *A*, an ancestral receptor (*bottom, white*) duplicates and, after mutations, these duplications give rise to two receptors that are the ancestors of the corazonin and AKH/ACP receptors. Subsequently, the AKH/ACP receptor gene duplicates again, giving rise (after mutations) to the AKH and ACP receptors. This scenario is supported by the branching pattern of Fig. 8. Note that in some present day insects, the corazonin or ACP receptor has been lost (see also Fig. 11), but there always is an AKH receptor (Fig. 8 and Table 1). *B*, similar scenario as in *A* for the ligands. It is unknown whether the ligands or receptors duplicated first. The steps given in *A* and *B* have time windows that overlap (= co-evolution between *A* and *B*).

qPCR was carried out on an MX3000 machine (Stratagene) using a Brilliant® SYBR® Green qPCR Master Mix from Stratagene. qPCR for the *T. castaneum* ACP preprohormone and *T. castaneum* ACP receptor was performed using the primers mentioned in supplemental Table S1. *T. castaneum* genes, coding for actin, rps3, rpl32, elf1, and tubulin, were used as reference genes (see supplemental Table S1 for primer sequences and accession numbers). All primer pairs have been tested for dimerization, efficiency (90–110%), and amplification of only one product. The most stable reference genes and gene normalization expression factors were determined by the software program geNORM (14). All experiments have been repeated at least three times. Water was used as nontemplate control.

Bioassays—Chinese hamster ovary (CHO) cells stably expressing the human G-protein G16 (CHO/G16) were grown as described previously (15) and transfected using FuGENE HD transfection reagent (Roche Applied Science). The bioluminescence assay was performed as described earlier (2). We tested our library of eight biogenic amines and 48 neuropeptides (see supplemental Table S2; all synthesized by GeneMed Synthesis, San Antonio, TX, or Bachem, Bubendorf, Switzerland). For peptide structures, see this study and Refs. 16, 17.

RNA Interference—Double-stranded RNAs were synthesized using the T7 MegaScript kit (Ambion); genes for *Trc*ACPR (1374 bp) and EGFP (358 bp), both flanked by T7 sites, were used as templates. To purify the double-stranded RNA, ethanol precipitation was carried out after DNase treatment, and the pellet was dissolved in 20 μl of H_2O. Concentration of the double-stranded RNA in this sample was determined using Nanodrop. Approximately 2-day-old female pupae were injected with 1 μg/μl double strand RNA (ACPR or EGFP) and subsequently transferred to flour, including 5% yeast, and kept at 32 °C until hatching. 14 days after injection, three groups of 15 or 25 injected virgin female adult beetles were mated with 15 wild type males. Eggs were counted after 3 days. 14 and 21 days after injection, RNA was isolated from three ACPR- or EGFP-injected beetles, and the level of ACPR mRNA was determined by qPCR using 5′-CACCGCCAGGAAT-TACAGAT-3′ as sense primer and 5′-GCGACCTGGGTTCAA-AACTA-3′ as antisense primer.

Immunocytochemistry—Brains and ganglia of first instar *Tribolium* larvae were dissected in a drop of cold phosphate-buffered saline (0.1 M PBS, pH 7.4) using a transgenic line, where the central nervous system is labeled with fluorescent color[4] and fixed subsequently in 4% formaldehyde (Roth, Karlsruhe, Germany) in 0.1 M PBS for 1–2 h on ice. The brains were then rinsed three times for 10 min at room temperature in 0.1 M PBS followed by preincubation overnight at 4 °C in 5% normal goat serum (Jackson ImmunoResearch) in 0.1 M PBS containing 0.3% Triton X-100 (PBST). Brains were then incubated in a solution containing the polyclonal ACP (1:10,000) and the monoclonal synapsin antibody (1:200) in PBST containing 5% normal goat serum for 1 day at 4 °C. The monoclonal synapsin antibody was used to selectively label neuropilar areas in the brain (kindly provided by Dr. E. Buchner, Würzburg, Germany). The polyclonal ACP rabbit antibody was obtained commercially from GeneMed Synthesis. The brains were rinsed three times for 10 min with PBST before they were incubated with a secondary goat anti-rabbit antibody conjugated to Cy3 and a goat anti-mouse antibody coupled to Cy5 (both 1:300, Jackson ImmunoResearch) in PBST and 5% normal goat serum for 1 day at 4 °C. Afterward, the brains were rinsed with PBS three times for 10 min and sub-
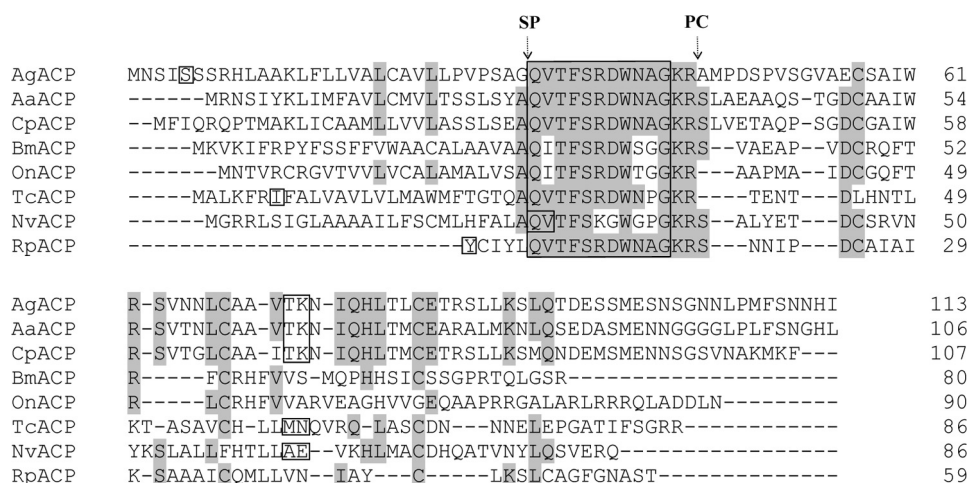
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                                       SP          PC
                                       ↓           ↓
AgACP   MNSISSSRHLAAKLFLLVALCAVLLPVPSAGQVTFSRDWNAGKRAMPDSPVSGVAECSAIW   61
AaACP   ------MRNSIYKLIMFAVLCMVLTSSLSYAQVTFSRDWNAGKRSLAEAAQS-TGDCAAIW   54
CpACP   --MFIQRQPTMAKLICAAMLLVVLASSLSEAQVTFSRDWNAGKRSLVETAQP-SGDCGAIW   58
BmACP   -----MKVKIFRPYFSSFFVWAACALAAVAQITFSRDWSGGKRS--VAEAP--VDCRQFT    52
OnACP   -------MNTVRCRGVTVVLVCALAMLVSAQITFSRDWTGGKRS--AAPMA--IDCGQFT    49
TcACP   -----MALKFRIFALVAVLVLMAWMFTGTQAQVTFSRDWNPGKR----TENT---DLHNTL   49
NvACP   ------MGRRLSIGLAAAAILFSCMLHFALAQVTFSKGWGPGKRS--ALYET---DCSRVN   50
RpACP   ------------------------YCIYLQVTFSRDWNAGKRS---NNIP---DCAIAI    29

AgACP   R-SVNNLCAA-VTKN-IQHLTLCETRSLLKSLQTDESSMESNSGNNLPMFSNNHI        113
AaACP   R-SVTNLCAA-VTKN-IQHLTMCEARALMKNLQSEDASMENNGGGGLPLFSNGHL        106
CpACP   R-SVTGLCAA-ITKN-IQHLTMCETRSLLKSMQNDEMSMENNSGSVNAKMKF---       107
BmACP   R-----FCRHFVVS-MQPHHSICSSGPRTQLGSR--------------------       80
OnACP   R-----LCRHFVVARVEAGHVVGEQAAPRRGALARLRRQLADDLN---------       90
TcACP   KT-ASAVCH-LIMNQVRQ-LASCDN---NNELEPGATIFSGRR-----------       86
NvACP   YKSLALLFHTLIAE--VKHLMACDHQATVNYLQSVERQ----------------       86
RpACP   K-SAAAICQMLLVN--IAY---C----LKSLCAGFGNAST--------------       59
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FIGURE 3. Alignment of the ACP preprohormone from the mosquitoes *A. gambiae* (*Ag-ACP*), *A. aegypti* (*Aa-ACP*), *C. pipiens* (*Cp-ACP*), the silkworm *B. mori* (*Bm-ACP*), the European corn borer *O. nubilalis* (*On-ACP*), the red flour beetle *T. castaneum* (*Tc-ACP*), the parasitic wasp *N. vitripennis* (*Nv-ACP*), and the bloodsucking bud *R. prolixus* (*Rp-ACP*). Amino acid residues that are common in at least four preprohormones are *highlighted*. The immature ACP sequences are framed by a *large box*. The *arrows* indicate the sites where signal peptidase (*SP*) removes the signal peptide or where prohormone convertase (*PC*) liberates immature ACPs from the prohormone. We could not find a complete signal peptide for *R. prolixus* ACP. The mature ACPs have the structures shown in Fig. 4. Intron positions are indicated by small one- or two-amino acid residue boxes, where the splice junction is located between the codons for these two amino acid residues. The preprohormone sequences have been annotated from the sequenced genomes from the above-mentioned insects, except for the *O. nubilalis* sequence (GenBank™ accession number GH989528), which has been derived from an EST library clone; in addition, we have cloned the *T. castaneum* sequence (GenBank™ accession number GQ217536).

Species	Amino acid sequence
A. gambiae	pQVTFSRDWNAamide
A. aegypti	pQVTFSRDWNAamide
C. pipiens	pQVTFSRDWNAamide
B. mori	pQITFSRDWSGamide
O. nubilalis	pQITFSRDWTGamide
T. castaneum	pQVTFSRDWNPamide
N. vitripennis	pQVTFSKGWGPamide
R. prolixus	pQVTFSRDWNAamide
L. migratoria	pQVTFSRDWSPamide

FIGURE 4. Structures of mature ACPs deduced from eight insect prepohormone sequences (*cf.* Fig. 3). *L. migratoria* ACP was isolated from extracts (20). Residues common to at least six ACPs are highlighted in *red*.

sequently mounted in 80% glycerol, 20% PBS. The whole mount preparations were scanned at 1024×1024 pixel resolution with a $\times 63$ glycerol objective (Leica, Germany) using a Leica TCS SP5 confocal laser scanning microscope. Controls included preimmune antiserum (1:10,000) and ACP antiserum (1:10,000) preabsorbed with ACP (10^{-7} M), both of which did not give any staining.

Software—DNA sequence comparisons were done using Vector NTI (Invitrogen). Protein sequence alignments were carried out using ClustalW. The phylogenetic tree of Fig. 8 was generated by the Megalign program of the Lasergene software package (DNASTAR). The receptor proteins were aligned by the ClustalW method using the PAM series protein weight matrix and default multiple sequence alignment parameters. We searched the nucleotide collection (nr/nt) and the non-human and non-mouse ESTs data bases at NCBI using the TBLASTN program (blast.ncbi.nlm.nih.gov).

For the phylogenetic trees shown in supplemental Fig. S5, alignments were performed using ClustalW with the Blosum scoring matrix. Bootstrapping was performed using Phylip protdist to generate 100 replicates. The tree diagrams were drawn using Phylodendron. For the construction of the above-mentioned trees, we used the seven-transmembrane regions of cloned or annotated receptors with the following GenBank™ accession numbers: Nv-AKHR (XP_001599670); Tc-AKHR (DQ422965); Am-AKHR (AAX83121); Aa-AKHR (EAT36594); Ag-AKHR (AY298745); Bm-AKHR (AF403542); Dm-AKHR (AF077299); Aa-ACPR (EAT38429); Cp-ACPR (EDS28711); Ag-ACPR (AY553322 and EU138885, two splice variants); Tc-ACPR (EU138886); Nv-ACPR (XP_001605342); Bm-ACPR-1 (EU138887); Bm-ACPR-2 (BAG68428); Cp-CRZR (EDS44142 and EDS44143, we manually joined the split gene); Ag-CRZR (AY301275); Dm-CRZR (AF373862); Bm-CRZR (BAG68420); Am-CRZR (XP_392570); Bm-CCAPR-1 (BAG68429); Bm-CCAPR-2 (BAG68425); Tc-CCAPR-2 (ABN79652); Am-CCAPR (XP_001122652); Nv-CCAPR (XP_001602277); Ag-CCAPR-2 (XP_321101); Aa-CCAPR (EAT39546); Ag-CCAPR-1 (AAS77205); Cp-CCAPR (EDS26677); Dm-CCAPR (AY219842); Tc-CCAPR (ABN79651); Tc-inotocin receptor (ABX00684); Nv-inotocin receptor (XP_001600203); and Dm-FMRFamide receptor (AAL83921). For the other receptors in Fig. 8 or supplemental Fig. S5, there are no GenBank™ accession numbers available. Prediction of transmembrane helices of the receptor protein was done using the TMHMM server. The signal peptide was predicted by the SignalP server. EC_{50} values were calculated using Prism software.

RESULTS

Identification of the Insect ACPs and ACP Preprohormones— We have previously cloned the *A. gambiae* AKH and corazonin receptors and a third, closely related receptor that, however, remained an orphan (13). To find a candidate ligand for this orphan receptor, we screened the genomic data base from *A. gambiae* (18) with various insect AKH and corazonin sequences, using TBLASTN. This search yielded an *A. gambiae* gene (see also GenBank™ accession number XP_563757), coding for a preprohormone (Fig. 3) that could give rise to a peptide that was related to both AKH and corazonin (Fig. 1) and that we

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Ag-ACPR-A  MYLAAGLLNIMDISLQHEYLQEYLQSAAAMANFSGANPYGLGGFGGLAPNGTGLLGGLDKNGTEVTITAPGHTDSTVAVI   80
Ag-ACPR-B  MYLAAGLLNIMDISLQHEYLQEYLQSAAAMANFSGANPYGLGGFGGLAPNGTGLLGGLDKNGTEVTITAPGHTDSTVAVI   80
Aa-ACPR    -----------------------------------------------------------------MIRGHTETTVAVI   13
Cp-ACPR    ----------------------------------------------------------------MGRKSYTDSTVAVI   14
Bm-ACPR-1  --------------------------------------------------MDESTQMDVTACNDTTCSDTTSTP-EQNFVI   30
Bm-ACPR-2  -------------------------MKMVNLNFDYEDTKEVPTSEIDKKFWEYFDATSTEISNYSSADLVPLDQGPVL   53
Tc-ACPR    --------------------------MQAVGKMGEEHYDEDSKSNFSVLNETLDGFANETVSPDVLFQQNLTVI   48
Nv-ACPR    ---------------------MDQLQGSRMQLLQDFNNHDFRDNMSMAVPTMPPSMTFTRRTLTII   45
Rp-ACPR    ----------------------------------MDPLFSNFTIEFNYTESIYYSPTNNTLYELPKFDDNALIVV   41

                TMI                           TMII
Ag-ACPR-A  IVYCVLFVIAAGGNLSVVITLFRSRRHRRSRVSLMICHLAVADLMVAFIMIPLEVGWRITVQWHAGNVACKVFLFMRAFC  160
Ag-ACPR-B  IVYCVLFVIAAGGNLSVVITLFRSRRHRRSRVSLMICHLAVADLMVAFIMIPLEVGWRITVQWHAGNVACKVFLFMRAFC  160
Aa-ACPR    IVYCVLFIIAAGGNLSVVITLFRSRRHRRSRVSLMICHLAVADLMVAFIMIPLEVGWRITVQWHAGNVACKVFLFMRAFC   93
Cp-ACPR    IVYCVLFIIAAGGNLSVVITLFRSRRHRRSRVSLMICHLAVADLMVAFIMIPLEVGWRITVQWHAGNVACKVFLFMRAFC   94
Bm-ACPR-1  GVYSILLVIGAVGNVAVLISLLRN-RRRKSRVSLLMTHLVIADMIVIFYFIPLEIGWRKTNAWLAGNVACKFLQVFRGFG  109
Bm-ACPR-2  ATYAILLAIGGVCNIAVLVKLAKP-RRRKSRVDMLMTHLALADVCVTCGVIPLEIGWKYTNAWLGGNFLCKLLLVLRAFG  132
Tc-ACPR    LVYSALFVVAAVGNLTVFISLFRS-RHRKSRISLMIRHLAIADLIVTFIMIPIEVGWRLTGKWIAGNVACKVFLFLRAFG  127
Nv-ACPR    IVYCICFLVAAIGNLTVFLTLWRG-RYRKSRISLMICHLSIADLLVAFFTIPIEIGWRLTVQWIAGNYACKLFLFLRAFG  124
Rp-ACPR    IAYSLLFIIAAIGNLTVFITLVRG-RHRKSRISLMITHLAAADLFVTFIMIPLEIGWRLTTQWVAGNIACKLFLFLRAFG  120

            TMIII                                 TMIV
Ag-ACPR-A  LYLSSNVLVCVSLDRCFAVIYPLRVSAARKRGKIMLGGAWFIAFVNAMPQSIIFRVQQHPQVPGFTQCVTFGFFATPGLE  240
Ag-ACPR-B  LYLSSNVLVCVSLDRCFAVIYPLRVSAARKRGKIMLGGAWFIAFVNAMPQSIIFRVQQHPQVPGFTQCVTFGFFATPGLE  240
Aa-ACPR    LYLSSNVLVCVSLDRCFAVIYPLRVSAARKRGKIMLGGAWFIAFANAIPQSIIFRVQHHPNVPDFTQCVTFGFFTTPAME  173
Cp-ACPR    LYLSSNVLVCVSLDRCFAVIYPLRVSAARKRGKIMLGGAWFIAFANALPQSIIFRVQQHPQVPGFTQCVTFGFFRTPGME  174
Bm-ACPR-1  LYLSSNVLVCISVDRFFAIIYPLRLAIARKRSKMMLYVAWAFALLLSLPQSAVFRVMEHPQIPDFKQCVSFEAFSNHQQE  189
Bm-ACPR-2  LYLSSNVLVCISIDRFFAVIYPLRLPEAKRRSRQMLYCAWVGALACSLPQSMVFRVKHHPRVIGFEQCVSFDAFNSYEQE  212
Tc-ACPR    PYLSSNVLVCVSLDRYFAVLHPLRVNDARRRGKIMLAFAWGTSFVYCIPQSFVFRVRAHPKYPNYEQCVSFGFFENTAQE  207
Nv-ACPR    LYLSNNILICVSLDRYFAVLYPLRVNDARRRGKFMLSVAWFSVLYAIPQSIVFHVENHPHHKNFTQCVTFGAFPSDLVE  204
Rp-ACPR    LYLSSNVLVCVSVDRYFAILHPLRVSDARRRGKMMLTMAWIFSLICALPQSVVFHVSQHPQHPDFWQCVTFGFFGSRTQE  200

              TMV
Ag-ACPR-A  TAYNLFCVIAMYFLPLMIISGAYTVILCEISNRSRE---------KETSDSNSTGTMRLRCNDLTHIERARQRTLRLTIT  311
Ag-ACPR-B  TAYNLFCVIAMYFLPLMIISGAYTVILCEISNRSRE---------KETSDSNSTGTMRLRCNDLTHIERARQRTLRLTIT  311
Aa-ACPR    TAYNLFCVVAMYFMPLMVISAAYTVILCEISNRSRE---------KETSDTSHTGGMRLRCNDLTHIERARQRTLRLTIT  244
Cp-ACPR    TAYNLFCVIAMYFMPLMIISAAYTVILCEISNRSRE---------KETSDTSHTGGMRLRCNDLTHIERARQRTLRLTIT  245
Bm-ACPR-1  LAYNVICLSAMYFVPLLVITICYLCIFYKISRNSKQN--------SEKEPPSNSRRVILRRSDQRPLVRARRRTLRMTVT  261
Bm-ACPR-2  VAYNVFCMCAMYFLPLIVITVCYVCIFCEIRKSSKE---------LGDKYHSGLKPVRLRRSDRSLLERARRRTLRMTVT  283
Tc-ACPR    IAYNLMCVMCMYFIPLFVIVAYTAIMCEISKNSKE---------TKGESYRTSNGRMRLRRSDISNIERARSRTLRMTIT  279
Nv-ACPR    NTYNVFCVLTMYFIPLAIICWVYLKILCEISSKSRDNKPAGSNGTLESSNSNQGSRMRLRRSDMSSIERARSRTLKMTII  284
Rp-ACPR    IAYNLFCVMAMYFVPLLVIVIAYTCILLEISKKTKETR-------GHEERT-RGRMRLRRSDMSNIERARARTLRMTVT  271

              TMVI                          TMVII
Ag-ACPR-A  IVVVFVWCWTPYVVMTLWYMFDRESAAKVDVAVQDGLFLMAVSNSCMNPLVYGSYAMKCRLP-----CRRRNTLGGAQTP  386
Ag-ACPR-B  IVVVFVWCWTPYVVMTLWYMFDRESAAKVDVAVQDGLFLMAVSNSCMNPLVYGSYAMKCRLP-----CRRRNTLGGAQTP  386
Aa-ACPR    IVVVFVWCWTPYVVMTLWYMFDRESALKVDGAIQDGLFLMAVSNSCMNPLVYGSYAMKCRRP-----WRRQMAPNGVQTP  319
Cp-ACPR    IVVVFVWCWTPYVVMTLWYMFDRESAIKVDGAIQDGLFLMAVSNSCMNPLVYGSYAMKCRWP-----WKRQNVPNGVATP  320
Bm-ACPR-1  IVTVFACCWFPYATMTLWYMLDWESAMRVPKRLQDFFFIMAVSNSCMDPLVYGSYTVDLRALILALRKIFCIRKEPTVLP  341
Bm-ACPR-2  IVSVFALCWLPYAIMAMWYMVDRESASKVSRRIQDLLFAMAVSNSCMPLVYGSYTLDIRGALRRFLKKCCSSTTPEVKG  363
Tc-ACPR    IVAVYVWCCTPYVIITMWYMFDRASATSLPEWLQDTFFMMVVSNSCMNPIVYGSYVINFQRVNCNCFCFRKTASESHLNV  359
Nv-ACPR    IVVAFIFCWTPYITMNLWYVIDKKSAKEVNEMVQESLFIMAVGNSCANPLVYGSYAIDLKKE-----CFRCFLPCTTTKS  359
Rp-ACPR    IVLAFIWCWTPYVVMTLWYMFDRESAEKVDPRLQDALFIMAVSNSCMNPLVYGSYALNFRRECTTCFCY---LFSSHQQL  348

Ag-ACPR-A  NAAQRRSTGFRCQQRWDPKMNLRFCMSILKNISFSLLLLW                                         427
Ag-ACPR-B  NAAQRRSTGT-YGWWWDCMRVHVLCFPIEQTLEFPRRKGRGHGSRPSFWKLYVCSDYNSLFWKRVSMPTAMGSKNEPSVL  465
Aa-ACPR    NAAQRRSTGK                                                                       330
Cp-ACPR    NAAQRRSTDAVSGMAGPHSDRLTGRDNKDELMYEKERSIKLNQFGLANGAPGQTKIGMNSTGE                  384
Bm-ACPR-1  GIKRPETITLVDQLRISQSRKRVRLSNPRDDLTTPRTSSEPFAYRAHHSFSERAIIMKPTHSCDDFTLSSPKKWYSA    419
Bm-ACPR-2  QAG-SSSNKNANFDTPHITEPKNIRTRLGVRFAETSLTAVPERLEVPRAPRGPA                           417
Tc-ACPR    GSGATRSTAMVHGGAGGNGYTRSPTPKSNLNLTGLLSKSRLPDKPPSVGHISFLSEPRTARNYRSSFHSEPCSRTRMCPDE 439
Nv-ACPR    NADVNLIQRSLGSKFQKPEMKSPGVSKQIVHSVCQAVHGFFKAGSGQTKSTNVCGKVLVPISPRLSVSTTSKGVVVEKLP  439
Rp-ACPR    ---DRRSTGISFNLFIYLYRKE                                                           367

Ag-ACPR-B  HEYLEKHLI                                                                        474
Tc-ACPR    LCLDTSCHSADYYSSAVL                                                               457
Nv-ACPR    LHTIVS                                                                           446
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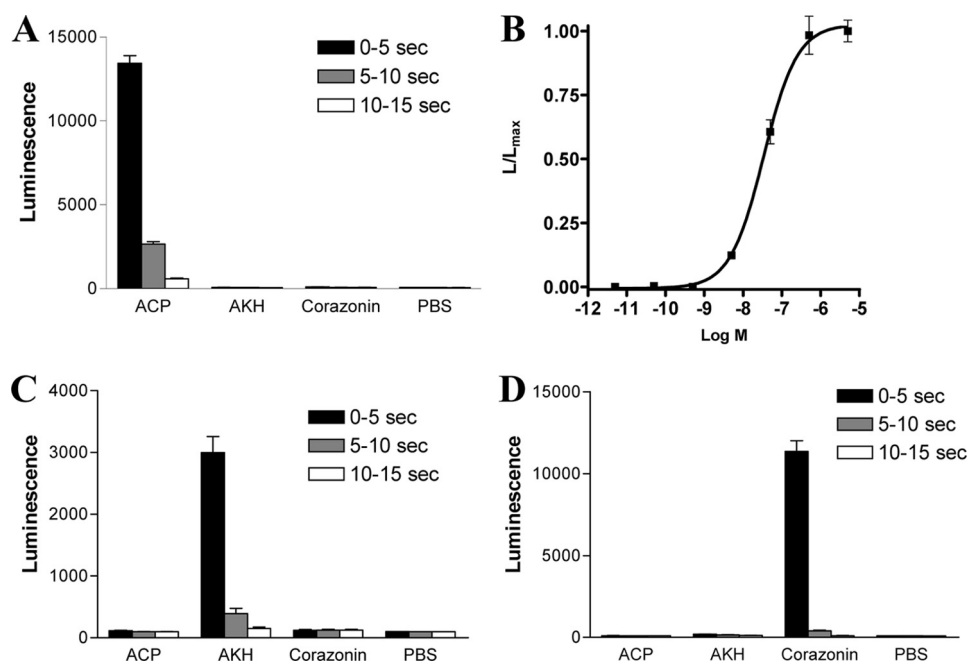
FIGURE 6. **Bioluminescence responses of CHO cells expressing *A. gambiae* neuropeptide receptors.** In all panels, the S.E. are given as *vertical bars*, which are sometimes smaller than the symbols used (*squares* or *lines*). In these cases only the symbols are given. The number of samples (*n*) to calculate S.E. are as follows: *A*, $n = 3$; *B–D*, $n = 4$. *A*, CHO/G-16 cells, expressing the *A. gambiae* ACP receptor variant-A, 0–5 s (*black*), 5–10 s (*gray*), and 10–15 s (*white*), after addition of 10^{-6} M *A. gambiae* ACP. Note that the bioluminescence response after addition of ACP is about 200 times over background (which is 70 bioluminescence units). Addition of *A. gambiae* AKH (5×10^{-6} M), corazonin (5×10^{-6} M) (for structures see Fig. 1), or PBS did not activate the receptor. Also, more than 40 other insect neuropeptides and biogenic amines (a list is given in supplemental Table S2) did not activate the receptor (tested up to 10^{-5} M). *B*, dose-response curve of the effect (during 0–15 s) of *A. gambiae* ACP in CHO/G-16 cells expressing the *A. gambiae* variant-A ACP receptor. The EC_{50} for ACP is 5×10^{-8} M. *C*, cells expressing the *A. gambiae* AKH receptor (13). This receptor is only activated by *A. gambiae* AKH, and not by ACP, corazonin, or PBS (same concentrations tested as in *A*). *D*, cells expressing the *A. gambiae* corazonin receptor (13). This receptor is only activated by *A. gambiae* corazonin and not by ACP, AKH, or PBS (same concentrations tested as in *A*).

named ACP (AKH/corazonin-related peptide). TBLASTN searches of other sequenced insect genomes showed that similar ACP preprohormone genes did occur in the yellow fever mosquito *Aedes aegypti* and in the West Nile virus transmitting mosquito *Culex pipiens* (Fig. 3). Mosquitoes belong to the insect order Diptera, but, surprisingly, other Diptera with a sequenced genome, such as the 12 different *Drosophila* species (19), did not have an ACP preprohormone gene. However, the silkworm *B. mori*, the European corn borer *Ostrinia nubilalis* (both belonging to the Lepidoptera), the red flour beetle *T. castaneum* (Coleoptera), the parasitic wasp *N. vitripennis* (Hymenoptera), and the bloodsucking bug *Rhodnius prolixus* (Hemiptera) did contain ACP preprohormone genes (Fig. 3), whereas we were unable to find such genes in the honeybee *Apis mellifera* (Hymenoptera), the pea aphid *Acyrthosiphon pisum* (Hemiptera), the body louse *Pediculus humanus* (Phthiraptera) and in the water flea *Daphnia pulex,* the only crustacean, whose genome has been sequenced.

Fig. 3 aligns all eight ACP preprohormones identified so far. In all cases, the ACP sequences are located directly after the signal peptide sequence and have dibasic (KR) processing sites at their C termini. The mature ACPs are shown in Fig. 4. It is striking that the first five to eight amino acid residues of these ACPs are nearly identical among all listed insects, whereas the last two residues are somewhat variable.

Two other research groups have found insect ACPs. The peptide was first identified in extracts from the locust *Locusta migratoria* by Siegert in 1999 (20) and in 2006 by Kaufmann and Brown (21) after screening of the *A. gambiae* genomic data base. Both peptides were described as AKH peptides with unknown functions (20, 21). Because the genome from *L. migratoria* has not been sequenced, we could not annotate its preprohormone gene (Fig. 3). Based on its striking similarities with both AKHs and corazonins, the ACPs are good candidates for being ligands for orphan GPCRs that are structurally related to both the AKH and corazonin receptors.

Cloning and Identification of Insect ACP Receptors—We re-cloned the orphan receptor from *A. gambiae* that we published previously (13). This work yielded two transcription variants; variant-A (*Ag-ACPR-A*, Fig. 5) was identical to the published orphan receptor transcript (13), and variant-B was an unpublished splicing variant with a different intracellular C terminus (supplemental Figs. S1 and S2).

Screening of all available insect and arthropod genomes and EST data bases with these *A. gambiae* orphan receptor sequences using TBLASTN yielded nearly identical sequences from the mosquitoes *A. aegypti*, and *C. pipiens* (one gene in each species) and closely related sequences from the silkworm *B. mori* (two genes), the beetle *T. castaneum* (one gene), the wasp *N. vitripennis* (one gene), and the bloodsucking bug *R. prolixus* (one gene) (Fig. 5). However, we did not find ACP receptor sequences in any of the 12 *Drosophila* species with a sequenced genome (19), in the honeybee *A. mellifera* (22), in the pea aphid *A. pisum*, in the body louse *P. humanus*, or in the water flea *D. pulex*.
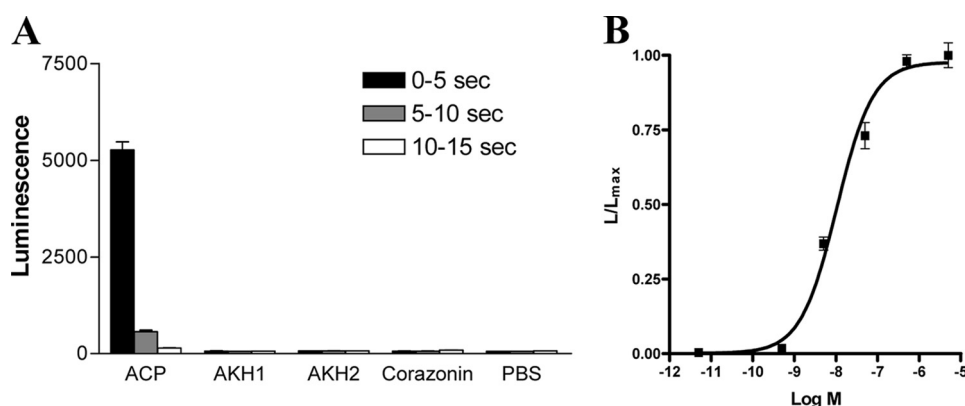
FIGURE 5. **Alignment of nine insect ACP receptor sequences.** The receptors from *A. gambiae* (*Ag-ACPR-A* and *-B*; GenBank™ accession number EU138885) and *T. castaneum* (Tc-ACPR; GenBank™ accession number EU138886) have been cloned, expressed in CHO cells, and identified as ACP receptors (Figs. 6 and 7 and supplemental Figs. S1, S3, and S4). The other receptors have been retrieved from their genomic data bases using TBLASTN and subsequently undergone manual curation. Amino acid residues that are common to at least four receptors are highlighted. Transmembrane (*TM*) helices are indicated by TMI–TMVII. Introns are indicated by *boxes*.

FIGURE 7. Bioluminescence responses of cells expressing the *T. castaneum* ACP receptor. The figure is presented in the same way as in Fig. 6. The number of samples (*n*) used to calculate S.E. are as follows: *A*, (ACP and PBS), $n = 4$; *A* (AKH-1 and -2, corazonin), $n = 3$; *B*, $n = 4$. *A*, *T. castaneum* ACP receptor is only activated by *T. castaneum* ACP but not by *T. castaneum* AKH1, AKH2, corazonin (for structures see Ref. 5), or PBS (same concentrations tested as in Fig. 6*A*). More than 40 other insect or invertebrate neuropeptides and 8 biogenic amines (supplemental Table S2) did also not activate the receptor (tested up to 10^{-5} M). *B*, EC$_{50}$ for *T. castaneum* ACP is 1×10^{-8} M.

To check whether these annotated orphan receptors represent the GPCRs for insect ACPs, we also cloned, in addition to the *A. gambiae* sequences, the *T. castaneum* receptor sequence (supplemental Fig. S3). CHO cells were stably transfected with cDNA sequences coding for the two *A. gambiae* receptor variants, and the *T. castaneum* receptor and clonal cell lines were selected, expressing each receptor effectively. Addition of 10^{-6} M *A. gambiae* ACP to the CHO cells expressing the receptor variant-A (*Ag-ACPR-A,* Fig. 5) resulted in a strong receptor response, which we measured as bioluminescence and which was about 200 times over background (Fig. 6*A*). A dose-response curve showed that this response had an EC$_{50}$ value of 5×10^{-8} M (Fig. 6*B*). The *A. gambiae* ACP receptor could not be activated by *A. gambiae* AKH or corazonin or by a library of more than 40 insect neuropeptides, showing that the receptor was specific for ACP (Fig. 6*A*). Similarly, the *A. gambiae* AKH receptor (13) was specific for AKH and did not react with ACP or corazonin (Fig. 6*C*), and the *A. gambiae* corazonin receptor (13) was specific for corazonin and did not react with ACP or AKH (Fig. 6*D*). The *A. gambiae* ACP receptor variant-B (*Ag-ACPR-B,* Fig. 5) had a much lower affinity for ACP and was, like the *A. gambiae* receptor, not cross-reacting with AKH or corazonin (supplemental Fig. S5). The ACP receptor from *T. castaneum* had the highest affinity for ACP with an EC$_{50}$ value of 1×10^{-8} M for activation by *T. castaneum* ACP (Fig. 7). Also, this receptor was specific for ACP and did not cross-react with the two *T. castaneum* AKHs (5) or corazonin (Fig. 7*A*).

Phylogenetic tree analyses showed that all ACP receptors from holometabolous insects (Fig. 5) cluster closely and that they group between the insect AKH and corazonin receptors (Fig. 8). This strongly suggests that also the *A. aegypti, C. pipiens, B. mori,* and *N. vitripennis* receptors (Fig. 5) are ACP receptors.

In addition to the receptors, phylogenetic tree analyses of the ACP, AKH, and corazonin preprohormones show that also these proteins form separate clusters and that the ACP preprohormones group between the AKH and corazonin preprohormones (supplemental Fig. S5). Thus, the evolutionary patterns of the ACP, AKH, and corazonin preprohormones and ACP,

AKH, and corazonin receptors look the same, supporting a scenario of receptor/ligand co-evolution as given in Fig. 2.

Expression of the ACP Receptor and Peptide Genes in Tribolium—We have used quantitative PCR (qPCR) to assess the expression of the ACP receptor and ACP preprohormone genes in adult *T. castaneum*. We found that in adult animals the receptor was mostly expressed in the head (presumably the brain), being 15 times higher than in the whole beetle and about 30 times higher than in the torso (thorax plus abdomen) (Fig. 9*A*). During development, the ACP receptor gene is prominently expressed shortly before and after hatching (20 times adult animals) (Fig. 9*B*). The ACP preprohormone gene had similar expression patterns (Fig. 9, *C* and *D*).

RNA Interference—To find a physiological role for the ACP hormonal system, we injected *T. castaneum* female pupae with double strand RNA for the ACP receptor. qPCR of treated beetles 14 and 21 days after injection showed a marked suppression of the targeted transcript (55–65% down-regulation compared with EGFP injected controls). However, we were unable to detect any differences in physical appearance, egg number, or mortality between the ACP receptor knockdown and control beetles.

Immunocytochemistry—We stained the first larval stage of *T. castaneum* with antibodies against *T. castaneum* ACP. These experiments showed three strongly stained neurons and one weakly stained neuron in each hemisphere of the larval brain (Fig. 10*A*). These neurons projected to the brain neuropil, the subesophageal ganglion, and the thoracic and abdominal ganglia (Fig. 10, *A* and *B*). Based on the varicose appearance of these projections, we suggest a neurosecretory role for ACP.

DISCUSSION

In this study, we describe ACP and its receptor, which constitute a novel signaling system in insects. The ACPs are structurally intermediate between the AKHs and corazonins (Fig. 1), and the same is true for the ACP preprohormones and the AKH and corazonin preprohormones (supplemental Fig. S5). Furthermore, the ACP receptors are structurally intermediate between the AKH and corazonin receptors (Fig. 8). Already for the AKH and corazonin signaling system, a co-evolution between ligands and receptors has been proposed (6, 7). The existence of a third signaling system intermediate between the AKH and corazonin systems is therefore an even more convincing example of receptor and ligand co-evolution, where an ancestral receptor and ligand gene duplicate several times followed by mutations and evolutionary selection, leading to three signaling systems (Fig. 2). The branching pattern of Fig. 8 suggests that the AKH and ACP receptors originated by duplication of an ancestral receptor that emerged after an earlier dupli-
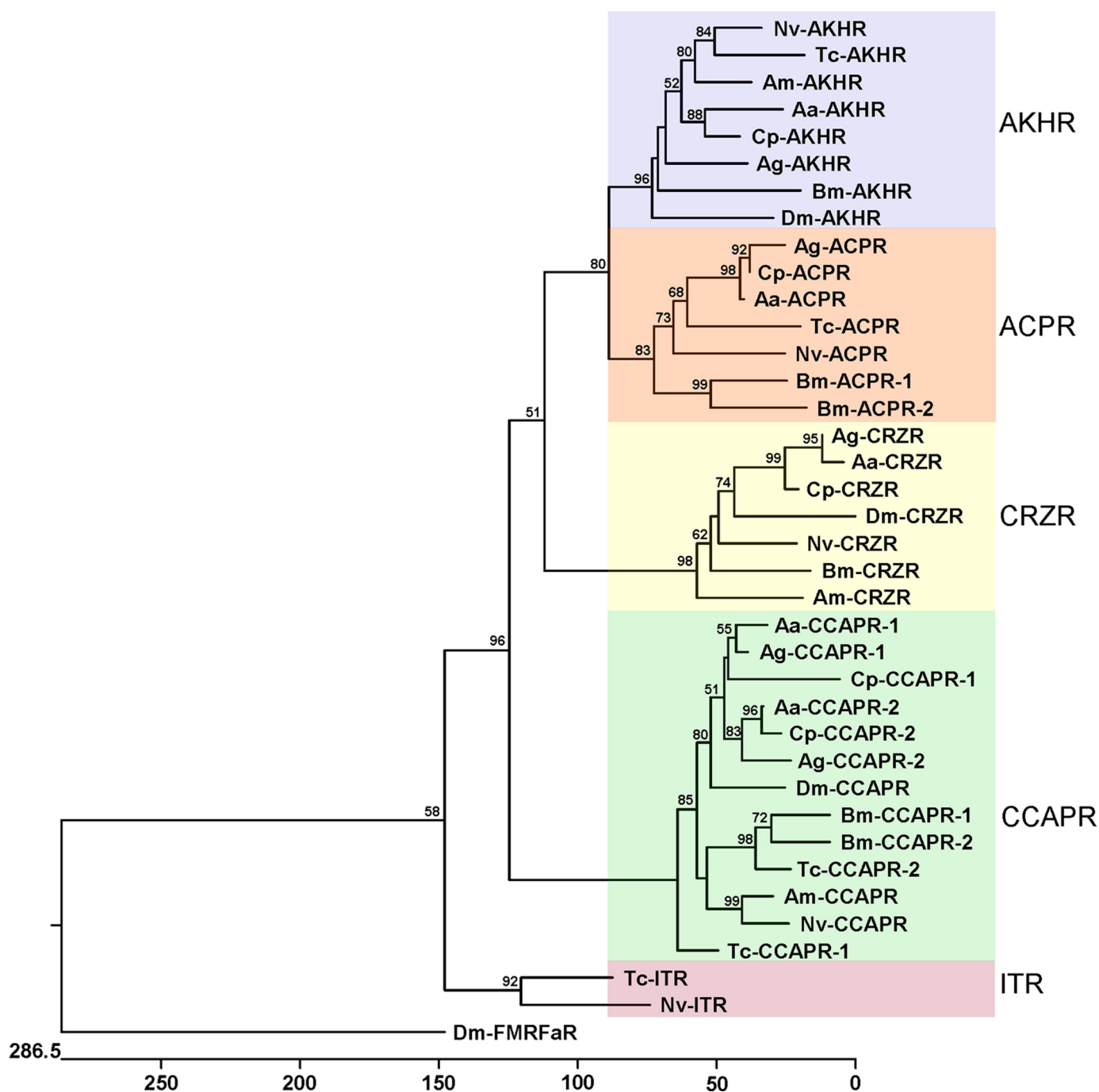
FIGURE 8. **Phylogenetic tree analysis of cloned or annotated neuropeptide receptors from holometabolous insects with a sequenced genome belonging to the cluster of AKH receptors (*AKHR*), ACP receptors (*ACPR*), corazonin receptors (*CRZR*), CCAP receptors (*CCAPR*), or inotocin receptors (*ITR*).** The length of each branch represents the evolutionary distance (measured as amino acid residue exchanges, see *abscissa*) between each receptor and the common ancestor of that receptor and its neighbor. Bootstrap values (only those above 50) are given where the branches split. The tree is rooted by the *Drosophila* FMRFamide receptor (*Dm-FMRFaR*). The GenBank[TM] accession numbers of the receptors used in this figure are given under "Experimental Procedures." Abbreviations are as follows: *Aa* (*A. aegypti*); *Ag* (*A. gambiae*); *Am* (*A. mellifera*), *Bm* (*B. mori*), *Cp* (*C. pipiens*), *Dm* (*Drosophila melanogaster*), *Nv* (*N. vitripennis*), and *Tc* (*T. castaneum*).

cation, leading to the development of the corazonin receptor (see also Fig. 2*A*). Whether this ancestral receptor is more AKH- or ACP-like is uncertain. In branchiopods (crustaceans such as *D. pulex*), which are believed to be the direct ancestor group of insects (23), AKH but not ACP receptors occur, which would suggest that the AKH system is more primordial. Lindemans *et al.* (24) provide a list of gonadotropin-releasing hormone-resembling peptides in lower invertebrates that also show some resemblance to AKHs. However, it is difficult to decide whether these peptides are more AKH- than ACP-like and which of the two peptides is the most ancient.

The ACP receptor cannot be activated by AKH and corazonin, and vice versa, the AKH receptor cannot be activated by ACP and corazonin and the corazonin receptor not by ACP and AKH (Figs. 6 and 7). These results show that the ACP, AKH, and corazonin systems, after they have evolved from their com-
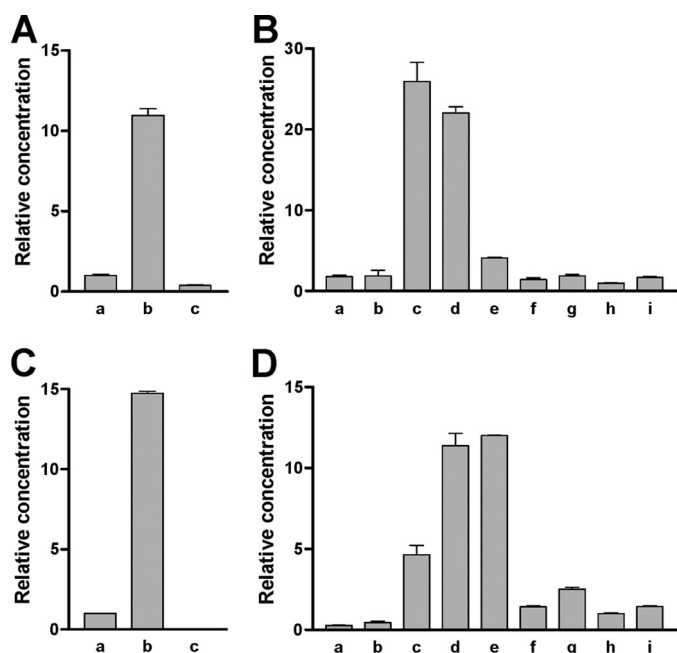
FIGURE 9. **qPCR of the ACP receptor and ACP preprohormone mRNAs in adult *T. castaneum* and in different developmental stages.** In each column at least 60 animals were pooled. The qPCR experiments were run as triplets; the *bars* (which sometimes are smaller than the lines) represent S.E. geNORM (14) has been used to calculate normalization factors of the most stable reference genes for each experiment, which are the genes coding for rps3 and rpl32 in *A* and *C* and rpl32, elf1, and actin in *B* and *D*. *A,* ACP receptor mRNA in adult mixed male and female animals as follows: *a,* whole bodies; *b,* heads; *c,* torsi (body minus head). The receptor mRNA concentrations given are relative to *a* (= 1); *b* is highly significantly different from *a* (*p* = 0.0006). *B,* ACP receptor mRNA in different developmental stages (the sexes were mixed except for adult animals) as follows: *a,* eggs 0–24 h after egg laying; *b,* eggs 24–48 h after egg laying; *c,* eggs 48–72 h after egg laying; *d,* larvae 96–120 h after egg laying (about 0–1 day after hatching); *e,* larvae 15–16 days after egg laying; *f,* larvae 20–21 days after egg laying; *g,* pupae (24–25 days after egg laying); *h,* adult female ; and *i,* adult male animals (27 days or more after egg laying). The receptor mRNA concentrations given are relative to *h* (= 1). The difference between *c* and *h* (*p* = 0.0089) and *d* and *h* (*p* = 0.0002) is highly significant. *C,* ACP preprohormone mRNA in the same tissues as given in *A*. The mRNA concentrations given are relative to *a* (= 1); *b* is highly significantly different from *a* (*p* = 0.00005). *D,* ACP preprohormone mRNA in the same developmental stages as given in *B*. The mRNA concentrations given are relative to *h* (= 1). The difference between *c* and *h* (*p* = 0.0068), *d* and *h* (*p* = 0.0002), *e* and *h* (*p* = 0.0001), and *g* and *h* (*p* = 0.0051) is highly significant.

mon ancestral neuropeptide/receptor signaling system (Fig. 2), are now three independent signaling systems that can co-exist without direct interference.

How far is the neuropeptide receptor/ligand co-evolution, as we have depicted it here for the ACP, AKH, and corazonin hormonal systems (Fig. 2), unique for insects or for animals in general? In vertebrates, there are also some clear examples of neuropeptide receptor/ligand co-evolution. All vertebrates, for example, have an oxytocin- and a vasopressin-like peptide. In mammals, there are one oxytocin and three vasopressin (V1a, V1b, and V2) receptors, and these GPCRs are structurally closely related (16). Also, the oxytocin and vasopressin peptides are structurally very similar, as are their preprohormones each of which contains a similar neurophysin part characterized by seven cystine bridges (16). Within its range of physiological concentrations, oxytocin does not activate the vasopressin receptors, and vice versa vasopressin does not activate the oxytocin receptor (25, 26), so they are, like the insect ACP, AKH,

and corazonin systems, independent hormonal systems that can co-exist without direct interference. Interestingly, although oxytocin and vasopressin have different actions, they also have overlapping roles especially in the control of social and reproductive behaviors (27). This again would be in agreement with a common evolutionary origin of the two hormonal systems, where some aspects of the original function of the ancestral system would have been conserved in both the oxytocin and vasopressin systems. Other examples of receptor neuropeptide ligand co-evolution are the mammalian glycoprotein hormones and their receptors (28, 29) and the opioid/orphanin peptides and their receptors (30).

In insects or protostome invertebrates in general, examples of neuropeptide ligand/receptor co-evolution are quite scarce. Clear examples are first, the glycoprotein hormones and their receptors, simply because their evolutionary origins and diversification (by duplications) occurred long before the split of proto- and deuterostomia (8, 9, 29). The second example is the capa/pyrokinin system, where two structurally related preprohormones produce capa and pyrokinin neuropeptides that act on two or three structurally related receptors (7–9). The third example is our current findings of the co-evolution of the ACP, AKH, corazonin, and their receptor genes. There are no further obvious examples of neuropeptide ligand/receptor co-evolution in insects (8, 9), although additional examples might be discovered in the future, when more insect GPCRs are being deorphanized.

The ACP signaling system is widespread in insects. It occurs in Hemimetabola (insects with an incomplete metamorphosis, where the young insects, called nymphae, resemble the adult animals), such as *R. prolixus* and locusts (Fig. 4) (20), and it occurs in representatives from all four major orders of Holometabola (insects with a complete metamorphosis, from wormlike larvae to mainly flying adults) as follows: Diptera, Lepidoptera, Coleoptera, and Hymenoptera (Fig. 11). However, some dipterans, such as the 12 *Drosophila* species with a sequenced genome (19), hymenopterans, such as the honeybee *A. mellifera* (22), and some hemimetabolous insects, such as the pea aphid *A. pisum* and the body louse *P. humanus*, have lost the ACP/receptor system. This suggests that the ACP signaling system has been abandoned several times during insect evolution (see dead-end signs in Fig. 11) and confirms earlier observations that insects easily can duplicate or abandon hormonal systems (5, 8, 9, 16). We have seen this phenomenon, for example, for the corazonin hormonal system that is widespread, but does not occur in Coleoptera (9), and for the recently discovered inotocin system that does occur in Coleoptera but is absent in Diptera, Lepidoptera, and some Hymenoptera (16).

Fig. 8 illustrates in more detail how common neurohormone receptor gene duplications and losses are in holometabolous insects. This figure shows five receptor types, the AKH, ACP, corazonin, inotocin, and crustacean cardioactive peptide (CCAP) receptors (2, 6, 7, 16, 31). These five receptor types form a distinct branch in phylogenetic tree analyses of insect neuropeptide receptors (8, 9), suggesting that they have a common evolutionary origin. Although, as discussed above, the AKH, ACP, and corazonin peptides are structurally related, the inotocin and CCAP peptide sequences are quite different, both
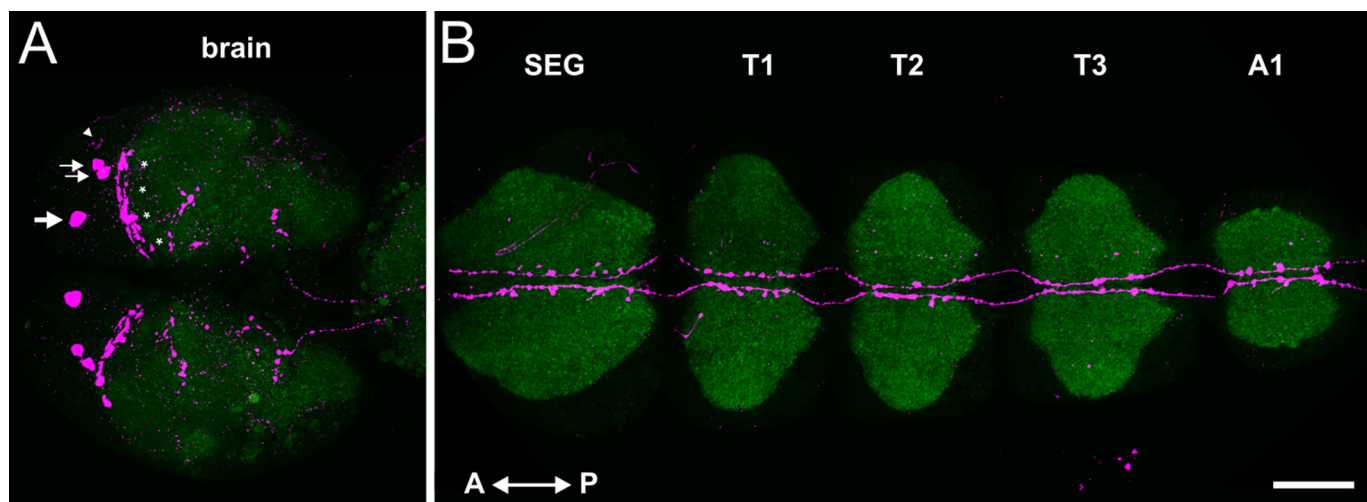
Scale: 25 µm

FIGURE 10. **ACP immunostaining of the central nervous system of first instar larvae of *T. castaneum*.** The images are maximum projections of 166 (*A*) and 156 (*B*) optical sections. *A*, in each hemisphere of the brain, we find one large cell body (*large arrow*), a pair of smaller cell bodies (*two small arrows*), and a peripheral weakly stained neuron (*arrowhead*, not visible on the *left side*). These neurons are lying anterior to the central brain neuropil (stained *green* by a synapsin antibody) and project strongly varicose processes to the anterior border of the central brain neuropil (indicated by *asterisks*). *B*, projections descend from the brain to the subesophageal ganglion (*SEG*) and thoracic (*T1–3*) and abdominal ganglia (only A1 is shown). These projections are strongly varicose, especially within the ganglia, suggesting an endocrine (or paracrine) function of ACP. *A*, anterior; *P*, posterior; the scale for both figures is 25 µm.
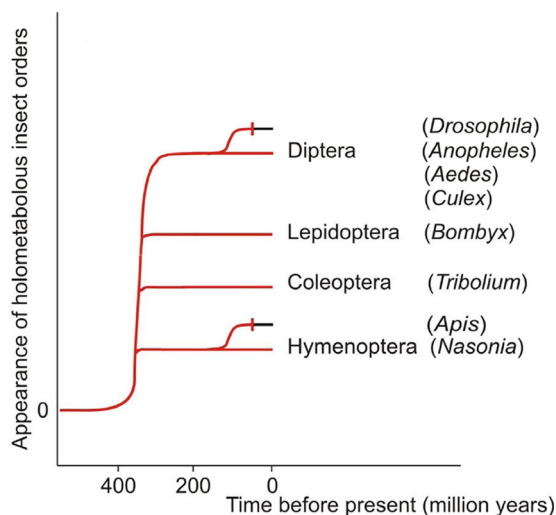

FIGURE 11. **Presence of the ACP/receptor couple in major orders of the Holometabola (highlighted in *red*).** This signaling system occurs in all four major orders but is absent in 12 *Drosophila* species (Diptera) and in the honeybee *A. mellifera* (Hymenoptera), showing that it has been abandoned at least two times during the evolution of holometabolous insects. The ACP/receptor couple is also present in some hemimetabolous insects (*R. prolixus*) but absent in others (*A. pisum* and *P. humanus*), suggesting that also in Hemimetabola this hormonal system has been abandoned several times.

TABLE 1

Number of deorphanized or annotated AKH receptors (AKHR), ACP receptors (ACPR), corazonin receptors (CRZR), CCAP receptors (CCAPR), and inotocin receptors (ITR) in various holometabolous insects with a sequenced genome

Note that for some receptors (indicated by asterisks) only a partial sequence could be identified. Also note that when a receptor is absent its neuropeptide ligand is also absent, meaning that a complete hormonal system is lacking.

	AKHR	ACPR	CRZR	CCAPR	ITR	Σ
D. melanogaster	1	0	1	1	0	3
A. aegypti	1	1	1*	2**	0	5
A. gambiae	1	1	1	2	0	5
C. pipiens	1*	1	1	2**	0	5
B. mori	1	2	1	2	0	6
T. castaneum	1	1	0	2	1	5
A. mellifera	1	0	1	1	0	3
N. vitripennis	1	1	1*	1	1	5

* Indicates a partial sequence.
** Indicates one of the two sequences is partial.

the ACP receptor has been duplicated in *B. mori*, and the inotocin system has been retained in *T. castaneum* and *N. vitripennis*. Thus, *B. mori* has six members and many other insects have five members of this neuropeptide receptor cluster, whereas the different *Drosophila* species and the honeybee have only three (Fig. 8 and Table 1).

Receptor duplications are also common in vertebrates, because most vertebrate neuropeptides have two or more receptors, which are structurally related. However, receptor losses are extremely seldom in these animals, and we are aware of only one example, where three (out of originally seven) neuropeptide Y receptor subtypes have been lost in some teleost fishes (32). To our knowledge, there are no examples of losses of whole hormonal (receptor plus ligand) systems in vertebrates, such as we have seen for the ACP system in branches of the Hymenoptera and Diptera (Fig. 11). Thus, this phenomenon of neuropeptide/receptor losses is quite unique for insects.

How can we explain these hormonal system (receptor plus ligand) duplications and losses in insects (Table 1)? One could

being small cyclic neuropeptides with no sequence homologies to the AKHs, ACPs, and corazonins (1, 16, 17). Some insects such as the 12 *Drosophila* species with a sequenced genome and the honeybee have only one AKH, one corazonin, and one CCAP receptor, whereas they have lost the ACP and inotocin systems (Fig. 8 and Table 1) (8, 9, 16). Other insects, such as the three mosquito species, the silkworm *B. mori*, and the parasitic wasp *N. vitripennis*, are rich in members of the receptor cluster; in addition to AKH, and corazonin, they have retained the ACP system (Fig. 11); moreover, the CCAP receptor gene has been duplicated in many of them (Fig. 8 and Table 1). Furthermore,

imagine that a certain insect species needs a complete palette of a related group of neuropeptide hormonal systems (generated by gene duplications as exemplified in Fig. 8 and Table 1) to survive in its extreme habitat or complex ecological niche, whereas other insects are less exposed and would suffice with fewer receptors. Thus, neuropeptide hormonal system duplications and losses would reflect the life style of a certain insect and in how far a certain physiological process needs multiple control points or back-up systems (= multiple hormonal systems) or not (= basic package). Could these considerations help us to understand Table 1? Unfortunately, we are unable to give an overall explanation of Table 1, which is due, in our eyes, to our incomplete understanding of the actions of the five hormonal systems given in this table.

What is the function of the newly discovered ACP signaling system? We do not know this yet, but gene expression studies using qPCR show that both the receptor and the peptide genes are highly expressed shortly before and after hatching of *T. castaneum* larvae (Fig. 9). This suggests a role of the ACP system in early larval development or physiology. Peptide or receptor gene knockdown experiments in *T. castaneum*, where systemic RNA interference works very effectively (33), should be the method of choice to further unravel the function of the ACP system. So far, however, we have been unable to see a clear effect of ACP receptor down-regulation using RNA interference. This might be due to the presence of multiple hormonal back-up systems (discussed above) or to our inability to see small behavioral changes. Anatomical work in early larvae suggests that ACP is an endocrine or paracrine hormone released into the anterior part of the central neuropil of the brain (Fig. 10*A*) and thoracic and abdominal ganglia (Fig. 10*B*). This might point to a role of ACP as a neuromodulator, perhaps influencing motor behavior.

Finally, our study has clarified a confusion with respect to AKH neuropeptide nomenclature, because two of the peptides that we now know are ACPs (those from *L. migratoria* and *A. gambiae*, Fig. 1) have been described in the literature as AKHs (20, 21). These peptides, however, do not activate the AKH receptor and should not be named AKHs. The corazonins have never been regarded as AKHs and the same should hold for the ACPs.

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