

# Molecular cloning and functional expression of the first two specific insect myosuppressin receptors

Kristoffer Egerod\*, Eyjólfur Reynisson\*, Frank Hauser, Giuseppe Cazzamali, Michael Williamson, and Cornelis J. P. Grimmelikhuijzen†

Department of Cell Biology, Zoological Institute, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen, Denmark

Edited by John H. Law, University of Arizona, Tucson, AZ, and approved June 18, 2003 (received for review April 15, 2003)

The *Drosophila* Genome Project database contains the sequences of two genes, CG8985 and CG13803, which are predicted to code for G protein-coupled receptors. We cloned the cDNAs corresponding to these genes and found that their gene structures had not been correctly annotated. We subsequently expressed the coding regions of the two corrected receptor genes in Chinese hamster ovary cells and found that each of them coded for a receptor that could be activated by low concentrations of *Drosophila* myosuppressin (EC<sub>50</sub>, 4 × 10<sup>-8</sup> M). The insect myosuppressins are decapeptides that generally inhibit insect visceral muscles. Other tested *Drosophila* neuropeptides did not activate the two receptors. In addition to the two *Drosophila* myosuppressin receptors, we identified a sequence in the genomic database from the malaria mosquito *Anopheles gambiae* that also very likely codes for a myosuppressin receptor. To our knowledge, this paper is the first report on the molecular identification of specific insect myosuppressin receptors.

Most insect myosuppressins are decapeptides with the structure X<sub>1</sub>DVX<sub>2</sub>HX<sub>3</sub>FLRFamide (where X<sub>1</sub> is pQ, P,T; X<sub>2</sub> is D,G,V; X<sub>3</sub> is V,S) (1). These neuropeptides have first been isolated from cockroaches and later from locusts, flies, and moths and it can be anticipated that they occur in all insects (1–11). The myosuppressins obtained their name because they have a general inhibitory action on a variety of visceral muscles from insects (1–3, 6–20). Because the insect myosuppressins also block the visceral muscles involved in the passage of food along the alimentary canal (2, 9, 12, 15–17, 19), they have attracted the interest of researchers that are aiming to develop nonpeptide agonists of myosuppressin receptors to use them as insecticides. On several occasions, one compound, benzethonium chloride, has been claimed to be such a myosuppressin receptor agonist (14, 19, 21). This claim was based on binding studies and physiological experiments carried out with isolated insect muscle preparations (14, 19, 21). However, to be sure that benzethonium chloride is specifically acting on the myosuppressin receptor, or to develop more selective and potent myosuppressin agonists, it is necessary to have the cloned and functional myosuppressin receptor available and expressed, for example, in cell lines plated out on microtiter plates for high-throughput screening of chemical libraries. Here we describe the molecular cloning and functional expression in cell lines of two myosuppressin receptors from the fruit fly *Drosophila melanogaster*. To our knowledge, this article is the first report on the molecular identification of specific myosuppressin receptors from insects.

## Materials and Methods

**PCR.** Total RNA was isolated from adult *D. melanogaster* (Canton S) by using the TRIzol Reagent (Life Technologies, Grand Island, NY), and further treated with DNase I by using the DNA-free kit (Ambion, Austin, TX). cDNA was synthesized, by using the SMART RACE cDNA Amplification Kit (CLONTECH). From the annotated exons of CG8985 [*Drosophila* myosuppressin receptor (DMSR)-1] and CG13803 (DMSR-2) (www.flybase.org) primers were designed for PCR. For DMSR-1, the sense primer 5'-GGCCAGTGGCAACAAT-

GAAACTGAGC-3' and the antisense primer 5'-CAGGACTACT CAGCAGGCGACTG-3' (corresponding to positions 3–28 and 1470–1491 in Fig. 1) were used. The 3' RACE was carried out as a nested PCR with the sense primer 5'-CCTGGACAAGTGGCTGCCGGTG-3' and the sense nested primer 5'-GGTGCCACGGAGAATCAGCTGTAC-3' (corresponding to positions 1284–1305 and 1618–1641 in Fig. 1). The 5' RACE was also nested PCR with the antisense primer 5'-CTGTCCGTCAGGATGTAGTCGTG-3' and the antisense nested primer 5'-CCAGCATAACTGCCAGGTCGGC-3' (corresponding to positions 247–269 and 205–226 in Fig. 1). The PCR program was 94°C for 3 min, then a 21-cycle touchdown, 94°C for 30 s, 73°C for 40 s decreasing 0.6°C per cycle, 72°C for 1 min, followed by 25 cycles at 94°C for 30 s, 59°C for 40 s, 72°C for 1 min. For DMSR-2, the sense primer 5'-CTTCATTGACAC-CATGGTACAG-3' and the antisense primer 5'-GCTCTCT-GCTACACATTTGTC-3' (corresponding to positions –13 to +9 and 1446–1476 in Fig. 2) were used in the initial PCR. The 3' RACE was carried out as a nested PCR with the sense primer 5'-GCACGTTTCGCGTCTCTTC-3' and the sense nested primer 5'-CCATCGATCTCGGGCTGACG-3' (corresponding to positions 1265–1284 and 1427–1446 in Fig. 2). The 5' RACE was also nested PCR with the antisense primer 5'-AGCTGTAGCTGAGCTGCTCTC-3' and the antisense nested primer 5'-CGACAGCCAGACCCGCTGAGTATG-3' (corresponding to positions 274–295 and 177–199 in Fig. 2). The PCR program was 94°C for 3 min, then a 20-cycle touchdown, 94°C for 30 s, 67°C for 45 s decreasing 0.5°C per cycle, 72°C for 2 min, followed by 25 cycles at 94°C for 30 s, 57°C for 45 s, 72°C for 2 min.

**Creation of Stable Cell Lines Expressing the Two Receptors.** The sense 5'-ACAATGGCCAGTGGCAACAATGAAACTGAG-3' and the antisense 5'-CTACAGATTTGTACCTGCGTGATGTTGGTG-3' primers (corresponding to nucleotide positions –3 to +27 and 1407–1437 of Fig. 1) were used in a PCR with cDNA from adult flies to amplify the coding region of DMSR-1. This cDNA was also used to amplify DMSR-2 with the primers sense 5'-TTTGAATTCGCCACCATGGTACGAAACATGTCG-3' and antisense 5'-AAAGAATTCCTACACATTTGTCAGTGGTTCGTCAGC-3' (the underlined nucleotides correspond to positions 1–18 and 1440–1467 of Fig. 2). The coding sequence of DMSR-1 was cloned into the pCR3.1 vector by using the TA cloning kit from Invitrogen, whereas the coding sequence of DMSR-2 was cloned into the pIRES2-EGFP vector (CLONTECH) by using the *EcoRI* restriction sites incorporated in the primers. The inserts were checked for the correct sequence. The two plasmids were stably transfected into Chinese hamster ovary

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CHO, Chinese hamster ovary; Drome-MS, *Drosophila* myosuppressin; DMSR-1 and -2, Drome-MS receptor 1 and 2; G-16, G protein 16.

Data deposition: The data sets for Figs. 1 and 2 have been deposited in the GenBank database (accession nos. AF544244 and AF545042).

\*K.E. and E.R. contributed equally to this work.

†To whom correspondence should be addressed. E-mail: cgrimmelikhuijzen@zi.ku.dk.

ACAGTCTTCATCGCCTCTCGGTGGCGTTC -397

GCATCGTCGGCGCTTCGGTTCCTCCCAATTAATCAGTGAATAAACACATACATAGAAACACATGAATTACAAGAAATCGATAAATATGTAATGAACAAAAT -298

CTGTAAACACCAACCAAGAGTTAAATGATAATATTGCATTAGGCAAAATAAATAATTCGCGAAACAGCTGTTGAATAAATTTGCATAAATCGTCGGTGGGA -199

↓1

ACAGCATTGGGGCTCTAGTTTGCAGGGACACCCCGTTGATAATAAATTAATTAATGCATCTGCTGGGAAGCGGCACATGCACGGCTGCTTGGCAGCAA -100

ATCCAGTTGCAACATCGGTGAGTGACGGGGCCAAATCAAAAGGACTTGCACAGTTGCGCGTTGCTTCTGCTATTGCTGTTGCTGTGCTGCAACA -1

↓2

↓3

ATG GCC AGT GGC AAC AAT GAA ACT GAG CCG CTC TAC TGC GGC AGC GGC ATG GAT AAT TTT CAC ACA AGC TAC AAG 75

Met Ala Ser Gly Asn Asn Glu Thr Glu Pro Leu Tyr Cys Gly Ser Gly Met Asp Asn Phe His Thr Ser Tyr Lys 25

▲

TM I

AAC ATG CAT GGC TAT GTT TCG CTG GTG GTC TGC ATC CTG GGC ACC ATC GCG AAT ACC TTG AAT ATC ATT GTG CTA 150

Asn Met His Gly Tyr Val Ser Leu Val Val Cys Ile Leu Gly Thr Ile Ala Asn Thr Leu Asn Ile Ile Val Leu 50

TM II

ACC CGA CGG GAG ATG CGC TCC CCC ACG AAT GGC ATA CTC ACG GGT CTG GCC GTG GCC GAC CTG GCA GTT ATG CTG 225

Thr Arg Arg Thr Ala Thr Arg Ser Pro Thr Asn Ala Ile Leu Thr Gly Leu Ala Val Ala Asp Leu Ala Val Met Leu Leu 75

GAG TAT ATA CCC TAC ACC ATA CAC GAC TAC ATC CTG ACG GAC AGT TTG CCG CGG GAG GAG AAG CTC ALC TAC AGC 300

Glu Tyr Ile Pro Tyr Thr Ile His Asp Tyr Ile Leu Thr Asp Ser Leu Pro Arg Glu Glu Lys Leu Ser Tyr Ser 100

TM III

TGG GCC TGC TTC ATC AAG TTC CAT TCG ATT TTC GCC CAG GTT CTG CAC ACC ATT TCC ATT TGG CTG ACG GTG ACC 375

Trp Ala Cys Phe Ile Lys Phe His Ser Ile Phe Ala Gln Val Leu His Thr Ile Ser Ile Trp Leu Thr Val Thr 125

CTG GCT GTT TGG CGT TAT ATA GCG GTG GGT TAT CCG CAA AAG AAT CCG GTA TGG TGC GGT ATG AGA ACC ACC ATA 450

Leu Ala Val Trp Arg Tyr Ile Ala Val Gly Tyr Pro Gln Lys Asn Arg Val Trp Cys Gly Met Arg Thr Thr Ile 150

TM IV

ATA ACG ATA ACC ACC GCT TAT GTG GTG TGT GTT CTG GTG GTG TCG CCG TCG CTC TAT TTG ATC ACG GCT ATA ACC 525

Ile Thr Thr Ala Tyr Val Val Thr Cys Val Leu Thr Ser Pro Ser Leu Tyr Leu Ile Thr Ala Ile Thr 175

GAA TAT GTG GAT CAG TTG GAT ATG AAT GGC AAG GTG ATA AAC TCC ATT CCC ATG ACC CAG TAC GTA ATC GAT TAT 600

Glu Tyr Val Asp Gln Leu Asp Met Asn Gly Lys Val Ile Asn Ser Ile Pro Met Thr Gln Tyr Val Ile Asp Tyr 200

CGT AAT GAG TTA ATG AGT GCC CGG ACG GCT GCC CTG AAT GCC ACG CCC ACC AGT GCA CCA CTG AAC GAA ACT GTG 675

Arg Asn Glu Leu Met Ser Ala Arg Thr Ala Ala Leu Asn Ala Thr Pro Thr Ser Ala Pro Leu Asn Glu Thr Val 225

TGG TTA AAT GCG AGC ACC TTG CTG ACA TCG ACA ACC ACC GTT GCA CCA CCC ACG CCA TCG CCA GTG GTG CGA AAT 750

Trp Leu Asn Ala Ser Thr Leu Leu Thr Thr Thr Thr Ala Ala Pro Pro Thr Pro Ser Pro Val Val Arg Asn 250

▲

GTT ACT GTC TAT AGG CTA TAC CAC AGC GAT TTG GCG TTG CAT AAT GCC TCG CTG CAA AAT GCC ACA TTT CTC ATA 825

Val Thr Val Tyr Arg Leu Tyr His Ser Asp Leu Ala Leu His Asn Ala Ser Leu Gln Asn Ala Thr Phe Leu Ile 275

TM V

TAC AGT GTA GTG ATT AAG CTG ATA CCA TGC ATA GCA CTC ACC ATT CTG TCG GTT CGA TTG ATC CTG GCC TTA CTG 900

Tyr Ser Val Val Ile Lys Leu Ile Pro Cys Ile Ala Leu Thr Ile Leu Ser Val Arg Leu Ile Leu Ala Leu Leu 300

GAG GCC AAG CGG CGG CGG AAG AAG CTC ACC AGC AAG CCC GCC ACT CCG GCT GCC AGT AAT GGA ACC AAA TCA CCG 975

Glu Ala Lys Arg Ala Ser Thr Leu Thr Ser Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 325

GCC AAT GGA AAA TCA GCC GAT AGG CCC CGG AAA AAT AGC AAA ACT CTG GAA AAG GAA AAG CAG ACG GAT CGC ACC 1050

Ala Asn Gly Lys Ser Ala Asp Arg Pro Arg Lys Asn Ser Lys Thr Leu Glu Lys Glu Lys Gln Thr Asp Arg Thr 350

TM VI

ACG AGA ATG CTG CTG GCG GTG CTA CTC CTC TTT CTC ATC ACT GAA TTT CCA CAA GGG ATT ATG GGT CTG CTG AAT 1125

Thr Arg Met Leu Leu Ala Val Leu Leu Leu Phe Leu Ile Thr Glu Phe Pro Gln Gly Ile Met Gly Leu Leu Asn 375

↓4

TM VII

GCT GTG CTC GGA GAT GTC TTC TAT CTG CAG TGC TAC CTA AGA CTG AGT GAC CTG ATG GAT ATC TTG GCC CTG ATC 1200

Ala Val Leu Gly Asp Val Phe Tyr Leu Gln Cys Tyr Leu Arg Leu Ser Asp Leu Met Asp Ile Leu Ala Leu Ile 400

AAC TCC AGC ATC AAC TTC ATT TTG TAC TGC TCC ATG AGC AAG CAA TTC CGC ACC ACG TTC ACG CTG CTC TTT CGT 1275

Asn Ser Ser Ile Asn Phe Ile Leu Tyr Cys Ser Met Ser Lys Gln Phe Arg Thr Thr Phe Thr Leu Leu Phe Arg 425

CCA AAA TTC CTG GAC AAG TGG CTG CCG GTG GCG CAG GAC GAA ATG GCA GCT GCT CGA GCT GAA CGC TCT GCG GTG 1350

Pro Lys Phe Leu Asp Lys Trp Leu Pro Val Ala Gln Asp Glu Met Ala Ala Ala Arg Ala Glu Arg Ser Ala Val 450

GCA CCG GTC CTG GAA AAG GGA CGA CAG CAG CCG CAG GTG GTG ATG GCC AGC ACG ACC ACC AAC ATC ACG CAG GTG 1425

Ala Pro Val Leu Glu Lys Gly Arg Gln Gln Pro Gln Val Val Met Ala Ser Thr Thr Thr Asn Ile Thr Gln Val 475

ACA AAT CTG TAG CACAGGAGGATCGTGTGCGCGAACTTTGCTCAGTGCCTGCTGAGTGTCTGAAAACGCGGCAGAGGCGCTCTCCGGCGA 1521

Thr Asp Leu \* 478

AGGAGGAGGCGTGGGAGGAGGTGGCCCGTTGGCCGGCAACGATGCGGTGGAACCGGTTCCAGGCCATCGTGGTGGTGGGACAGGTGAGCGG 1620

TGCCACGGAGAATCAGCTGTACACCGCCGAGCAAGCTCGTATTGTGACGTAGTAAACCTGATAGTACATATTATATGCTAGTTATCCTTGTAAAA 1719

AGTAAACTAGTTGTGTAAGAACCGCAGATAACCAAGTTATGTGCATACCTTTGGTGGGAAATCGTAAAACCCGTTATAGTCATAATAAATAAATGCTGG 1818

TTCACTACTCATAACGACCCGTTTGACTTCACTCTATTCACATTACAGTCAAGTAGGAGCTGATAAAATGCTGTGTATAAACGAGCTCGTATAAGCTA 1917

CAGTTGGCGTCAACAATAATAGTTACCATTTGGTGGTTCACAAATAGATCAAAATCAAAAATATATAGTTTGTAAAAATGTTTCAATAATCTATCT 2016

TCATATTTAACTAACACAATTCAGAACTTTCGCTTGCATGGAAAAATGTTTATTTTCCCACTTGACCCAATTTGGGCACTGTTGAGCTTTTCGGC 2115

ATGAATCAAATCAAATATAGTAACTTTTGCATCTTTCCACTTTGATCCCATCGGTTTTTCATGTTGATTTTCATTCGCAATAAAGTATGAGCAGCACAGTA 2214

CTCACTCAAACACGAAATTTTCGGCAACGATTTGAGCAGAATTTGCCATTTGTTGACAGATTATATATACACACACACACACTTATGTCGCTATC 2313

TTTAATTAAGCAATTAAGTCTAGAAATTTGACCTGATATTGATGAGCAGCATTTGAAAAAATAGTGGCAGTTGCACTGCGGTGCGTAGGTAATGAA 2412

ATATTTTGGCAATCTATTTATGGAATTCGCAATTTGGGCTGTGAATGAAATATAGTTTTTCTTGAACGCACTTGGGAATTCAGTTCCGTTTCGTGGGT 2511

TACTGAATTTATGGGAAATAATTTAAGGCAGTCACTGCAGCTGACCGAATAATCCAATGAAACATTGCATAAACAGTTTGCAGTTTTTCCCACTTGGC 2610

AAAAATGTAATGCAATTCGCTTAAGCCCAACGAGCAATAACAAATGCCCAGCGAACGATAAATATAGTATGAAATTTGCACTTGAAGCTTGAAGCTTGA 2709

CAAAATATTTCAATCTCGTTAATTAATGAACACATAAATCAAATACGAATAGGTACATGTGAATACCAACTATATTGTATGTAACATACTGATGTT 2808

GCTTGAACCAACGATTAATAGCTGCTAAACAAAAAATAAAAAAAAAAAAAAAAAA 2861

**Fig. 1.** cDNA and deduced amino acid sequence of the corrected gene CG8985. The nucleotides are numbered from 5' to 3' end and the amino acid residues are numbered, starting with the first ATG (start) codon in the open reading frame. The introns are marked by arrows and numbered 1–4. The two nucleotides, bordering each of the four introns, are highlighted in gray. The stop codons in the 5' untranslated region are underlined. The putative polyadenylation sites in the 3' untranslated region are underlined twice. The translation termination codon is marked by an asterisk. The seven transmembrane domains of the receptor protein are boxed and marked TMI–VII. The potential N-glycosylation site in the extracellular N terminus (obeying the NXS/T consensus sequence), and four such sites in the second extracellular loop are marked by filled triangles.

(CHO) cells that also stably expressed the human  $\alpha$  G protein subunit G-16 (CHO/G-16) (22, 23) by using FuGENE 6 reagent (Roche Diagnostics). The CHO/G-16 cells and the bioluminescence assay are described in refs. 22 and 23. All wells in a bioassay have about the same number of cells.

**Northern Blot Analyses.** Northern blots were prepared by using the NorthernMax formaldehyde kit (Ambion) and BrightStar-Plus membranes (Ambion). cDNA probes (nucleotide positions 1284–2216 of Fig. 1 and nucleotide positions 1445–2072 of Fig. 2) were labeled by using the Strip-EZ kit (Ambion).



11 GTTGCATTGAATTTTTCGTAATAAAGTTTTCGCGATT -199  
 TGTGTAGCAGGTAGCACTTTTGCCACGCGAGCCTGCATATTTGAGTGTGGAAATTTCCGAAAAGGGAATTTAAATGAAACGCCAGAGGAGCAGCGGGCGC -100  
 CACTGAGTTGGCAGCCGGACCCCGGTCGAATCAAACGGAGTCTCTGCCAGGACATCCCACCACCGACTCCATCTGACCATCTGGCCCTCATTTGACACC -1  
 ATG GTC ACG AAC ATG TCG CAG CCG CAT TAT TGC GGC ACC GGC ATC GAT GAT TTC CAC ACA AAC TAC AAA TAC TTT 75  
 Met Val Thr Asn Met Ser Gln Pro His Tyr Cys Gly Thr Gly Ile Asp Asp Phe His Thr Asn Tyr Lys Tyr Phe 25  
 CAC GGT TAC TTC TCG CTG ATT GTC TGT ATC CTG GGA ACC ATC GCG AAT ACC CTA AAT ATC ATA GTG CTG ACC CGA 150  
 His Gly Tyr Phe Ser Leu Ile Val Cys Ile Leu Gly Thr Ile Ala Asn Thr Leu Asn Ile Ile Val Leu Thr Arg 50  
 CGG GAG ATG CGC TCC CCC ACG AAT GCC ATA CTC ACG GGT CTG GCT GTC GCC GAT TTG GCT GTG ATG CTG GAA TAT 225  
 Arg Glu Met Arg Ser Pro Thr Asn Ala Ile Leu Thr Gly Leu Ala Val Ala Asp Leu Ala Val Met Leu Glu Tyr 75  
 ATA CCC TAT ACG GTG CAC GAC TAT ATC CTC AGT GCA AGG CTG CCG CGA GAG GAG CAG CTC AGC TAC AGC TGG GCG 300  
 Ile Pro Tyr Thr Val His Asp Tyr Ile Leu Ser Ala Arg Leu Pro Arg Glu Glu Gln Leu Ser Tyr Ser Trp Ala 100  
 TGC TTC ATC AAG TTT CAC TCG GTA TTT CCC CAG GTG CTG CAC ACC ATC TCC ATT TGG CTA ACA GTG ACG CTG GCA 375  
 Cys Phe Ile Lys Phe His Ser Val Phe Pro Gln Val Leu His Thr Ile Ser Ile Trp Leu Thr Val Thr Leu Ala 125  
 GTT TGG CGG TAC ATA GCG GTA AGC TAT CCG CAA AGG AAT CCG ATC TGG TGT GGA ATG CGT ACT ACT CTG ATC ACC 450  
 Val Trp Arg Tyr Ile Ala Val Ser Tyr Pro Gln Arg Asn Arg Ile Trp Cys Gly Met Arg Thr Thr Leu Ile Thr 150  
 ATA GCC ACA GCC TAT GTT GTC TGT GTC CTG GTG GTG TCA CCT TGG CTG TAC CTA GTC ACA GCC ATT GCC AAG TTC 525  
 Ile Ala Thr Ala Thr Val Val Cys Ile Leu Thr Val Val Ser Pro Trp Leu Tyr Leu Val Thr Ala Ile Ala Lys Phe 175  
 CTG GAG ACT TTG GAT GCC AGT GGC AAG ACG ATC GCC TCA GTG CCA TTG AGT CAA TAC ATT CTG GAC TAC AAT CGG 600  
 Leu Glu Thr Leu Asp Ala Ser Gly Lys Thr Ile Ala Ser Val Pro Leu Ser Gln Tyr Ile Leu Asp Tyr Asn Arg 200  
 CAG GAT GAG GTG ACC ATG CAG GTC ATG TCG AGT ACA ACG CCA GAT GTT TCC TGG CCG ATA CCA AGT GAT TCG GCC 675  
 Gln Asp Glu Val Thr Met Gln Val Met Ser Ser Thr Thr Pro Asp Val Ser Trp Pro Ile Pro Ser Asp Ser Ala 225  
 AAT GGA ACT GCA GTT AGC TTG CTA AGT CTA ACC ACA GTG ATA CCC CTA ACC ACA TTA AGC ACT GGA GTA ACC ACA 750  
 Asn Gly Thr Ala Val Ser Leu Leu Ser Leu Thr Thr Thr Leu Ser Thr Gly Val Thr Thr Thr 250  
 TTC TCG TCG TTG GGT GAG CGC AAT GTG ACT GTC TAT AAG CTG TAT CAC AGC GCA CTG GCG CTG CAT GAT CGG CAG 825  
 Ser Ser Ser Leu Gly Glu Arg Asn Val Thr Val Tyr Lys Leu Tyr His Ser Ala Leu Ala Leu His Asp Arg Gln 275  
 TTC AGG AAT GCG ACC TTC CTT ATA TAC AGT GTC CTG ATC AAG TTG ATA CCC TGC TTC GCA CTG ACC ATT CTG TCT 900  
 Phe Arg Asn Ala Thr Phe Leu Ile Tyr Ser Val Leu Ile Lys Leu Ile Pro Cys Phe Ala Leu Thr Ile Leu Ser 300  
 GTG CGG CTC ATA GGG GCG CTG TTG GAG GCC AAA AGG AGG AGG AAG ATC CTA GCC TGC CAT GCA GCC AAC GAT ATG 975  
 Val Arg Leu Ile Gly Ala Leu Leu Glu Ala Lys Arg Arg Lys Ile Leu Ala Cys His Ala Ala Asn Asp Met 325  
 CAG CCA ATT GTC AAT GGA AAG GTG GTG ACT CCG ACG CAA CCC AAG AGC TGT AAA CTG CTA GAG AAG GAG AAG CAG 1050  
 Gln Pro Ile Val Asn Gly Lys Val Val Thr Pro Thr Gln Pro Lys Ser Cys Lys Leu Leu Glu Lys Glu Lys Gln 350  
 ACC GAT CGC ACG ACG AGG ATG CTT CTT GCG GTA CTG CTG CTC TTC CTG GTC ACT GAG TTT CCA CAG GGC ATT ATG 1125  
 Thr Asp Arg Thr Thr Arg Met Leu Leu Ala Val Leu Leu Leu Phe Leu Val Thr Glu Phe Pro Gln Gly Ile Met 375  
 GGT CTG CTG AAT GTG CTC CTG GGC GAC GCC TTC TTT CTG CAA TGT TAC TTA AAG CTG AGT GAC CTT ATG GAC ATC 1200  
 Gly Leu Leu Asn Val Leu Leu Gly Asp Ala Phe Phe Leu Gln Cys Tyr Leu Lys Leu Ser Asp Leu Met Asp Ile 400  
 TTG GCG CTT ATT AAT TCG AGC ATC AAC TTC ATC CTG TAC TGT TCG ATG AGC CGC CAG TTC CGG AGC ACC TTC GCG 1275  
 Leu Ala Leu Ile Asn Ser Ser Ile Asn Phe Ile Leu Tyr Cys Ser Met Ser Arg Gln Phe Arg Ser Thr Phe Ala 425  
 CTC CTC TTC CGT CCG CGC TGG CTG GAC AAA TGG CTG CCG CTG TCG CAG CAC GAC GGC GAA GGG AGG GTG GGC GGA 1350  
 Leu Leu Phe Arg Pro Arg Trp Leu Asp Lys Trp Leu Pro Leu Ser Gln His Asp Gly Glu Gly Arg Val Gly Gly 450  
 AGT GGC GGC CTG GGC GGC TAC GGC GGA TAT GGA CGG CAG CCG TTG CTG CAC ACG GAT GCC GTT AGC AAG AGC ATG 1425  
 Ser Gly Gly Leu Gly Gly Tyr Gly Tyr Gly Arg Gln Arg Leu Leu His Thr Asp Ala Val Ser Lys Ser Met 475  
 GCC ATC GAT CTC GGG CTG ACG ACC CAA GTG ACA AAT GTG TAG CAGGAGAGCAGCGCGGGCGGGCGGATGTGACCGCAACCGCGC 1510  
 Ala Ile Asp Leu Gly Leu Thr Thr Gln Val Thr Asn Val \* 488  
 GAGCAGCTGCATCGTGGCTCTGGCCCTGGCAGCCACTGATGTTGATGGATGTCGGCTGCCACCGATGCTGCTGTTTCCACTAACGACATCAGCCTGG 1609  
 TCGAAGAGCTACATTTGAGCCAGTCAAAGGGGGACTGCGATATCCAGTGGCCAGCATCGAAGGCGGCGCAGTGGGAGTGGCACCAGTGCATCTGGC 1708  
 CCACGACGCTGGCTGAGAAAGCTGCGTAACAGAAAGCTAGAGAAACGGAGCAATCCTCCGACAGGATATAGAGCTGGGCAAGAGCTCCATCAACA 1807  
 GCGCCAGCAGTGTCTTCTAATGGTATTACTAAGCAGCTCTGATGAGTTAAAGCCAGGCGGTTTGGTCAGTGAACAGCCCGGAGTCCAGCGGAG 1906  
 AGGATGTGGAAAGCCATCGACGCCCTCTGGCTGTGAGACGACCCCTCACTTTGTCAGCCCTGTAATATCCCAAGTACCCACTAATATTTA 2005  
 ACTACCCTGTACAGACCAATATTGAGACAAATTTGATGCGCTGATCCATTTATTTCCCGCAGTGTGTCTGGCGAATCGAGACCCCTTTCGCT 2104  
 GAGACTTTGCTTGTGGAAATAGTGGATTTTCCCTGAAAGGACTCCCTGGAAGCAGCCTGCAACATTTTGTGACCACTTTGATAAGTGATATACT 2203  
 TGACTAAGTTTCCCCCTTAATAGTTGTGTAAGTGTAAAGCGCTCTCTAGATATAAACGAATTAGTCATAGTCTAACTATTAAATTAAGATATATTGAT 2302  
 GAAATAATTCCGAAGCTAAGAGATTAATTTCTTTTATATTGATTAATGTTTTTCGAAAAAATAAAAAAAAAAAAAAAAAA 2383

Fig. 2. cDNA and deduced amino acid sequence of the corrected gene CG13803. Data are displayed in the same way as Fig. 1.

The ribosomal protein-49 probe was generated as described in ref. 24.

**Sequence Analyses, Software Programs, and Peptides.** DNA sequence comparisons were performed by using the LASERGENE software package (DNASTAR). For Fig. 4, CLUSTALW was used. The TMHMM v.2.0 prediction server was used for locating transmembrane helices of the proteins (www.cbs.dtu.dk). PRISM v.3 software (GraphPad, San Diego) was used for plotting of the bioluminescence data, including normalization of the dose-response curves (these curves were plotted as nonlinear regression). Peptides were synthesized by Genemed Synthesis (San Francisco) or Bachem (Bubendorf, Switzerland).

## Results

The database from the *Drosophila* Genome Project consortium contains the sequences of two annotated genes (CG8985 and CG13803) that are supposed to code for two structurally related G protein-coupled receptors (www.flybase.org) (25). We cloned the cDNA of these two genes by using PCR and primers based on the annotated exons. Subsequently, we carried out 3' and 5' RACEs to obtain the complete cDNAs. During this process, we found that CG8985 had not been correctly predicted. In the prediction, it contained four exons and three introns, but from the cDNA sequence it became clear that intron 3 was an exon (containing a new "corrected" stop codon). Furthermore, we found two additional exons flanking a large intron at the 5' end of the predicted gene. The

**Table 1. Intron/exon boundaries of the corrected CG8985 receptor gene**

Intron	5' donor	Intron size, bp	3' acceptor	Intron phase
1	CAA gtaaataact...	546	...tatccacag TAA	-
2	GCA gtaagtgtc...	15,252	...tattttgcag GGG	-
3	AG gtaatcgac...	3,657	...ttttttcag C	2
	Ser		Ser	
4	A gtaagtaaa...	100	...gccaccag GT	1
	Ser		Ser	

corrected gene CG8985, therefore, has five exons and four introns (Table 1 and Fig. 1). Fig. 1 shows the cloned cDNA of the corrected gene CG8985, which is 3,286 nt long. It contains a putative polyadenylation site at its 3' end and numerous stop codons preceding the start codon in its 5' untranslated region. The cDNA codes for a protein of 478 amino acid residues, which contains seven transmembrane domains. The extracellular N terminus contains one potential N-glycosylation site, and four such sites exist in the second extracellular loop (Fig. 1).

Comparison of the cDNA of Fig. 1 with the genomic sequence in the database (www.flybase.org) showed 13 nucleotide differences. In four cases, these differences led to different amino acid residues, which, however, were all conserved residues (Table 3, which is published as supporting information on the PNAS web site, www.pnas.org).

We also cloned the cDNA of gene CG13803 and, again, found that its gene had not been correctly predicted. The annotated gene had four exons and three introns. We found that the predicted intron 2 was, in fact, an exon. Furthermore, we discovered an additional large intron and an exon, both lying in front (5') of the predicted exon 1. The corrected gene CG13803, therefore, has four exons and three introns (Table 2 and Fig. 2). Fig. 2 shows the cloned cDNA of the corrected gene CG13803, which is 2,618 nt long. The cDNA contains a putative polyadenylation signal at its 3' end, and several stop codons preceding the start codon in its 5' untranslated region. It codes for a protein of 488 amino acid residues, which has seven transmembrane domains, one potential N-glycosylation site in its extracellular N terminus, and three such sites in the second extracellular loop (Fig. 2).

Comparison of the cDNA of Fig. 2 with the corresponding genomic sequence revealed 37 nucleotide differences. Five of these differences led to changes in amino acid residues, of which four were conserved residues (Table 4, which is published as supporting information on the PNAS web site).

We stably transfected CHO cells with the cDNAs corresponding to the coding regions of either the corrected gene CG8985 or CG13803 and established cloned cell lines, expressing these genes effectively. These cells were also stably expressing the  $\alpha$  subunit of the promiscuous G protein, G-16. Two days before the assay, the cells were transiently transfected with DNA, coding for apoaequorin; coelenterazine was added to the culture medium

**Table 2. Intron/exon boundaries of the corrected CG13803 receptor gene**

Intron	5' donor	Intron size, bp	3' acceptor	Intron phase
1	TGT gtaagtgtc...	14,623	...tattttgcag GTA	-
2	AA gtgagtaga...	429	...ccccaacag C	2
	Asn		Asn	
3	A gtaagtatt...	489	...tcgtttcag GT	1
	Ser		Ser	

3 hours before the assay. Addition of receptor ligands and activation of the receptors in these pretreated cells would lead to an inositol 1,4,5-trisphosphate (IP<sub>3</sub>)/Ca<sup>2+</sup>-mediated bioluminescence response that could easily be measured and quantified (22, 23, 26–32). We tested a peptide library (consisting of 24 *Drosophila* or other invertebrate neuropeptides), and seven monoamines on these cells and found that the two receptors were activated by low concentrations of *Drosophila* myosuppressin (Drome-MS) (5, 11). We also found that both receptors had the same EC<sub>50</sub> for Drome-MS ( $4 \times 10^{-8}$  M) (Fig. 3 E and F). The other tested peptides and amines (Fig. 3) did not activate the receptors, not even peptides that resembled Drome-MS (TDVDHVLFLRFamide) in their C termini, such as FMRFamide, *Drosophila* short neuropeptide F-1 (AQRSPSLRLRFamide), or perisulfakinin [EQFDDY(SO<sub>3</sub>H)GHMRFamide]. These results showed that the two receptors are specific for Drome-MS. Another remarkable feature was that the two receptors did not appear to be quickly desensitized. When activated by Drome-MS, they continued to be active for 30 sec or longer ( $t_{1/2} \approx 20$  sec for both receptors), which is a kinetics quite different from that of all of the other insect neuropeptide receptors characterized by us so far, where nearly full desensitization occurred within 5 sec (23, 26–32). Finally, we found that the two receptors were not activated by benzethonium chloride (in concentrations up to  $10^{-4}$  M), which was claimed to be a myosuppressin receptor agonist (14, 19, 21). Concentrations  $>10^{-4}$  M gave bioluminescence responses in both the nontransfected and transfected CHO cells, showing that the actions of benzethonium chloride can be nonspecific.

The two Drome-MS receptors (DMSR-1 and -2) strongly resemble each other (65% overall amino acid residue identity; 71% identity in the transmembrane region) (Fig. 4). A comparison of the two receptors with the other known *Drosophila* neuropeptide receptors or other proteins from the GenBank database revealed no proteins with significant structural similarities. However, the genomic database from the recently sequenced malaria mosquito *Anopheles gambiae* (33), contained a gene sequence coding for a putative G protein-coupled receptor that strongly resembled DMSR-1 and -2, both with respect to amino acid sequence (55–58% overall amino acid residue identities; 65–67% identical residues in the transmembrane region) and gene structure (two shared introns between the three genes with identical intron phasings) (Fig. 4). All of these data strongly suggest that the *Anopheles* receptor is a myosuppressin receptor. Whether *Anopheles* also has a second myosuppressin receptor could not be confirmed at present.

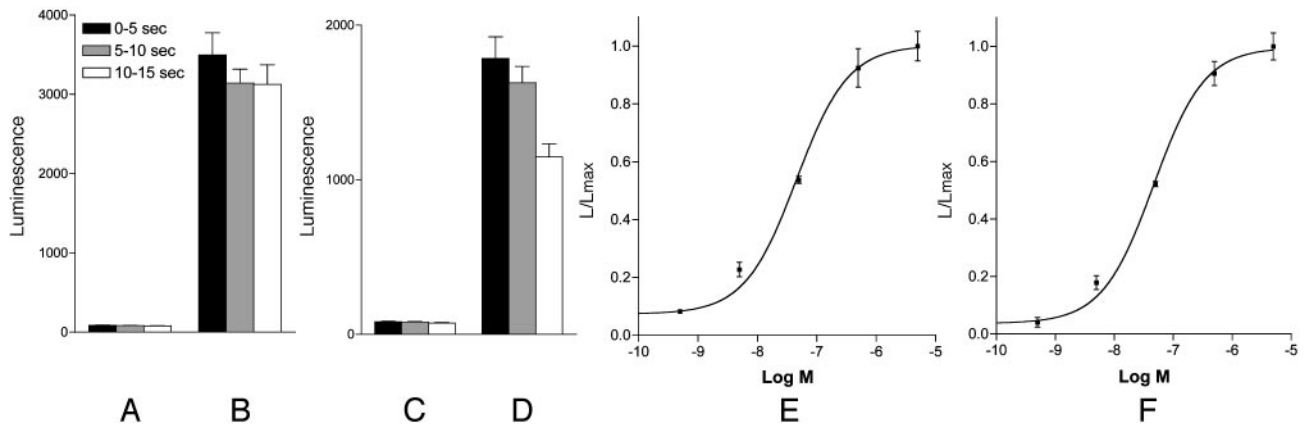
Northern blots revealed that the two Drome-MS receptors were only very weakly expressed in embryos, larvae, and pupae. In adult flies, DMSR-1 was strongly expressed in the head, but virtually absent in the body (thorax/abdomen) (Fig. 5A), whereas DMSR-2 was present in both adult head and body (Fig. 5B). The DMSR-2 mRNA displayed a double band, of which the smaller one corresponded to the size of the cloned cDNA (Fig. 5B).

## Discussion

In this paper we have cloned and characterized two *Drosophila* receptors that are specific for Drome-MS and that do not react with other *Drosophila* neuropeptides, not even with peptides that have C-terminal structures resembling Drome-MS, such as the sulfakinins, the FMRFamides, or peptides belonging to the neuropeptide-F family (Fig. 3). Interestingly, the two receptors are not activated by benzethonium chloride, which was claimed to be a myosuppressin receptor agonist (14, 19, 21), but whose actions, therefore, must be quite different.

The two Drome-MS receptors have an EC<sub>50</sub> for Drome-MS of  $4 \times 10^{-8}$  M. This value compares well with the concentrations of Drome-MS needed in pharmacological or physiological ex-

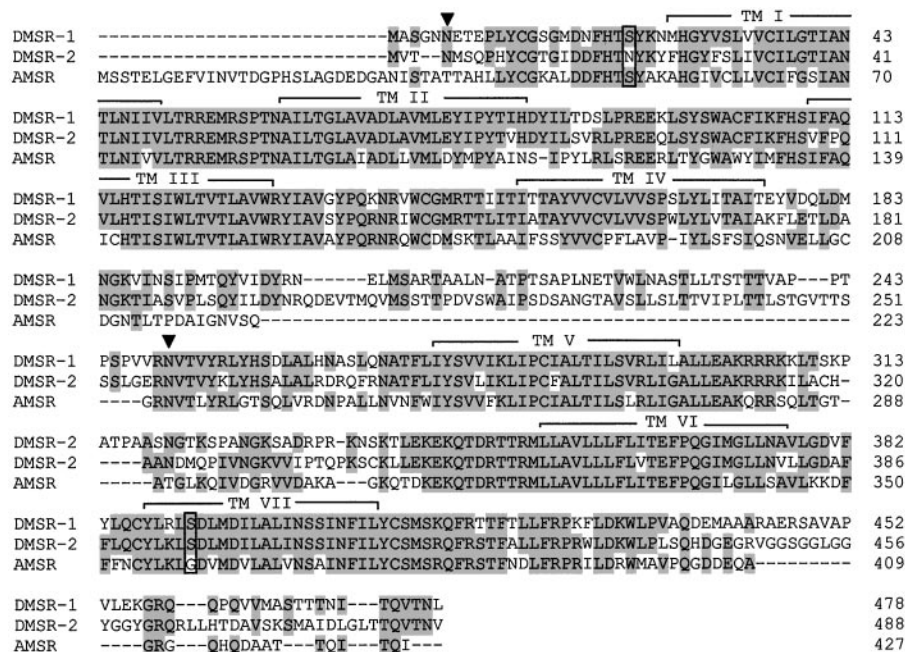




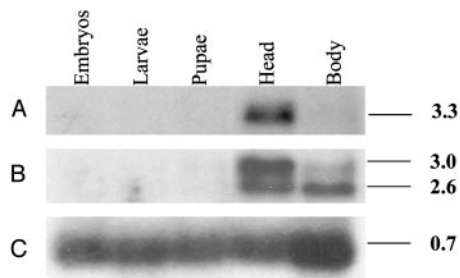
**Fig. 3.** Bioluminescence responses of nontransfected CHO/G-16 cells and of CHO/G-16 cells transfected with DNA coding for the coding region of either the corrected gene CG8985 or CG13803. The vertical bars represent SEM, which are sometimes lower than the symbols (filled squares) used. In these cases, only the symbols are given. (A and C) Bioluminescence responses of CHO/G-16 cells after addition of  $5 \times 10^{-7}$  M Drome-MS. (B) Bioluminescence response of CHO/G-16/CG8985 cells after addition of  $5 \times 10^{-7}$  M Drome-MS. (D) Bioluminescence response of CHO/G-16/CG13803 cells after addition of  $5 \times 10^{-7}$  M Drome-MS. (E) Dose-response curve of the bioluminescence responses of CHO/G-16/CG 8985 cells induced by Drome-MS. The responses 0–5, 5–10, and 10–15 sec after addition of the peptide were added and counted as one response. (F) A similar curve for the CHO/G-16/CG13803 cells. The following peptides did not activate the two receptors (tested up to  $10^{-6}$  or  $10^{-5}$  M): crustacean cardioactive peptide; capa-1, -2, and -3; corazonin; *Drosophila* adipokinetic hormone; *Drosophila* tachykinin-3; *Drosophila* short neuropeptide F-1; *Drosophila* ecdysis triggering hormones-1 and -2; *Drosophila* pigment dispersing hormone; *Drosophila* pyrokinin-2; drostatins-A4–B2, and -C; FMRFamide; *Heliothis zea* hypertrehalosaemic neuropeptide; hug- $\gamma$ ; leucopyrokinin; leucokinin-III; perisulfakinin; and proctolin. For peptide structures see refs. 1, 10, and 11. The following amines did not activate the two receptors (tested up to  $10^{-5}$  M): adrenaline; dopamine; histamine; noradrenaline; octopamine; serotonin; and tyramine.

periments to inhibit *Drosophila* heartbeat ( $EC_{50}$ ,  $10^{-7}$  M) or contractions of the crop ( $10^{-6}$  M) (20), and with the concentrations of other insect myosuppressins that were used to inhibit the blowfly crop ( $4 \times 10^{-8}$  M) (19), the cockroach fore- and hindgut (between  $10^{-10}$  M and  $3 \times 10^{-8}$  M) (9, 14), the locust heart ( $EC_{50}$ ,  $5 \times 10^{-8}$  M) (13), or the salivary gland from *Rodnius prolixus* (between  $5 \times 10^{-9}$  and  $5 \times 10^{-7}$  M) (18). These data, therefore, suggest that the two cloned Drome-MS receptors are the physiologically relevant myosuppressin receptors.

In addition to the two Drome-MS receptors characterized in this paper, we have earlier identified the first insect FMRFamide receptor in *Drosophila* (28). This receptor is activated by very low concentrations of *Drosophila* FMRFamides ( $EC_{50}$ ,  $9 \times 10^{-10}$  M), but also by higher concentrations of Drome-MS ( $EC_{50}$ ,  $2 \times 10^{-7}$  M) and *Drosophila* short neuropeptide F-1 ( $EC_{50}$ ,  $9 \times 10^{-8}$  M). These findings were recently confirmed by another research group (34). We have previously considered the activation of the *Drosophila* FMRFamide receptor by the other two peptides as



**Fig. 4.** Amino acid sequence comparison between the DMSR-1 and -2 encoded, respectively, by the corrected CG8985 and CG13803 genes and the putative myosuppressin receptor from *A. gambiae* (AMSR; present in the clone with accession no. gb/AAAB01008900.1). Amino acid residues that are identical between at least two receptors are highlighted in gray. The seven transmembrane domains are indicated by TM I–VII. The two common introns between the three receptor genes are indicated by vertical boxes. The filled triangles indicate common potential N-glycosylation sites. Gaps are introduced to optimize the alignments.



**Fig. 5.** Northern blots of mRNA isolated from various developmental stages from *Drosophila*. The sizes of the transcripts are given at the right (in kb). Each lane contained  $\approx 5 \mu\text{g}$  of mRNA from either embryos (0–24 h), mixed first- to third-instar larvae, pupae, and heads or bodies (thorax + abdomen) from adult flies. (A) The lanes were hybridized with a cDNA probe, corresponding to CG8985. (B) The Northern blot from A was stripped and subsequently hybridized with a cDNA probe, corresponding to CG13803. (C) The Northern blot from B was stripped and subsequently hybridized with a cDNA probe, coding for ribosomal protein 49. This blot gives the loading efficiency of each lane.

cross reactions caused by the similarities of their C-terminal peptide structures (28). However, the FMRFamide receptor's  $\text{EC}_{50}$  value for Drome-MS is only five times higher than the  $\text{EC}_{50}$  values found for the two Drome-MS receptors identified in the present study. This finding would imply that the *Drosophila* FMRFamide receptor, under certain conditions, possibly also might function as a third Drome-MS receptor. It also implies that high ("pharmacological") concentrations of Drome-MS (e.g.,  $10^{-6}$  M, see above) could effect the *Drosophila* FMRFamide receptor instead of the presumed Drome-MS receptor. However, similarly to the two Drome-MS receptors, the *Drosophila* FMRFamide receptor is not activated by benzethonium chloride (G.C., unpublished observations).

Northern blots showed that DMSR-2 is expressed in both the head and body (thorax and abdomen) of adult flies (Fig. 5B). These results agree very well with the actions of the myosuppressins on numerous visceral muscles present in both the body and head of

insects (see above). However, the very strong expression of both receptors in the head (compare, for example, the loading efficiencies in the last two lanes of Fig. 5C), also suggests the presence of myosuppressin receptors in the brain. In fact, anatomical studies have shown that myosuppressin-like material was abundant in various parts of the insect brain (19, 20, 35–37). These two findings together, then, suggest a so far unknown role of the myosuppressins in the insect central nervous system.

It is interesting that two transcripts were observed with DMSR-2 (Fig. 5B), of which the smaller one corresponded to the cloned receptor (Fig. 2), whereas the somewhat larger transcripts probably corresponded to a mRNA species, having an alternative polyadenylation signal, lying  $\approx 400$  bp downstream from our cloned polyadenylation signal. This longer transcript has not been cloned by us, but can be deduced from the genomic sequence in the database (www.flybase.org). It is also interesting that the smaller transcript was mainly present in the body, whereas the longer transcript was mainly in the head (Fig. 5). This could mean that nerve cells predominantly produce the larger transcript and visceral muscle cells produce the smaller one.

The identification in this paper of two specific myosuppressin receptors in *Drosophila* opens the possibility of finding similar receptors in other insects or arthropods. That this is a realistic option is shown by our discovery of a probable myosuppressin receptor in the malaria mosquito *A. gambiae* (Fig. 4). The identification of myosuppressin receptors in other model insects or other arthropods will certainly contribute to our understanding of the endocrinology and physiology of these animals. Furthermore, the availability of myosuppressin receptors in recombinant cell lines (grown in 96- or 384-well plates) will also make it possible to screen large chemical libraries for agonists, which could be used as leads to develop specific and environmentally safe insecticides.

We thank Drs. S. Rees and J. Stables (Glaxo Wellcome, Stevenage, U.K.) for supplying cell line CHO/G-16, Birgitte Paulsen for typing the manuscript, and Lundbeck Foundation and Fabrikant Vilhelm Pedersen og Hustrus Mindelegat (Manufacturer Vilhelm Pedersen and Wife Memorial Legacy; this support was granted on recommendation from the Novo Nordisk Foundation) for financial support.

1. Nässel, D. R. (2002) *Prog. Neurobiol.* **68**, 1–84.
2. Holman, G. M., Cook, B. J. & Nachman, R. J. (1986) *Comp. Biochem. Physiol.* **C 85**, 329–333.
3. Robb, S., Packman, L. C. & Evans, P. D. (1989) *Biochem. Biophys. Res. Commun.* **160**, 850–856.
4. Kingan, T. G., Teplow, D. B., Phillips, J. M., Riehm, J. P., Rao, K. R., Hildebrand, J. G., Homberg, U., Kammer, A. E., Jardine, I., Griffin, P. R., et al. (1990) *Peptides* **11**, 849–856.
5. Nichols, R. (1992) *J. Mol. Neurosci.* **3**, 213–218.
6. Fonagy, A., Schoofs, L., Proost, P., Van Damme, J., Bueds, H. & De Loof, A. (1992) *Comp. Biochem. Physiol.* **C 102**, 239–245.
7. Schoofs, L., Holman, G. M., Paemen, L., Veelaert, D., Amelinckx, M. & De Loof, A. (1993) *Peptides* **14**, 409–421.
8. Peef, N. M., Orchard, I. & Lange, A. B. (1994) *Peptides* **15**, 387–392.
9. Predel, R., Rapus, J. & Eckert, M. (2001) *Peptides* **22**, 199–208.
10. Gäde, G., Hoffmann, K. H. & Spring, J. H. (1997) *Physiol. Rev.* **77**, 963–1032.
11. Vanden Broeck, J. (2001) *Peptides* **22**, 241–254.
12. Cook, B. J. & Wagner, R. M. (1991) *Comp. Biochem. Physiol.* **C 99**, 95–99.
13. Robb, S. & Evans, P. D. (1994) *J. Exp. Biol.* **197**, 437–442.
14. Nachman, R. J., Olender, E. H., Roberts, V. A., Holman, G. M. & Yamamoto, D. (1996) *Peptides* **17**, 313–320.
15. Lange, A. B. & Orchard, I. (1998) *Peptides* **19**, 459–467.
16. Fuse, M. & Orchard, I. (1998) *Regul. Pept.* **77**, 163–168.
17. Duttlinger, A., Berry, K. & Nichols, R. (2002) *Peptides* **23**, 1953–1957.
18. Orchard, I. & Te Brugge, V. T. (2002) *Peptides* **23**, 693–700.
19. Richer, S., Stoffolano, J. G., Yin, C. M. & Nichols, R. (2000) *J. Comp. Neurol.* **421**, 136–142.
20. Nichols, R. (2003) *Annu. Rev. Entomol.* **48**, 485–503.
21. Lange, A. B., Orchard, I., Wang, Z. & Nachman, R. J. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9250–9253.
22. Stables, J., Green, A., Marshall, F., Fraser, N., Knight, E., Sautel, M., Milligan, G., Lee, M. & Rees, S. (1997) *Anal. Biochem.* **252**, 115–126.

23. Staubli, F., Jørgensen, T. J. D., Cazzamali, G., Williamson, M., Lenz, C., Søndergaard, L., Roepstorff, P. & Grimmelikhuijzen, C. J. P. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 3446–3451.
24. Hauser, F., Nothacker, H.-P. & Grimmelikhuijzen, C. J. P. (1997) *J. Biol. Chem.* **272**, 1002–1010.
25. Hewes, R. S. & Taghert, P. H. (2001) *Genome Res.* **11**, 1126–1142.
26. Lenz, C., Williamson, M., Hansen, G. N. & Grimmelikhuijzen, C. J. P. (2001) *Biochem. Biophys. Res. Commun.* **286**, 1117–1122.
27. Secher, T., Lenz, C., Cazzamali, G., Sørensen, G., Williamson, M., Hansen, G. N., Svane, P. & Grimmelikhuijzen, C. J. P. (2001) *J. Biol. Chem.* **276**, 47052–47060.
28. Cazzamali, G. & Grimmelikhuijzen, C. J. P. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 12073–12078.
29. Cazzamali, G., Saxild, N. P. E. & Grimmelikhuijzen, C. J. P. (2002) *Biochem. Biophys. Res. Commun.* **298**, 31–36.
30. Iversen, A., Cazzamali, G., Williamson, M., Hauser, F. & Grimmelikhuijzen, C. J. P. (2002) *Biochem. Biophys. Res. Commun.* **299**, 628–633.
31. Iversen, A., Cazzamali, G., Williamson, M., Hauser, F. & Grimmelikhuijzen, C. J. P. (2002) *Biochem. Biophys. Res. Commun.* **299**, 924–931.
32. Cazzamali, G., Hauser, F., Kobberup, S., Williamson, M. & Grimmelikhuijzen, C. J. P. (2003) *Biochem. Biophys. Res. Commun.* **303**, 146–152.
33. Holt, R. A., Subramanian, G. M., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., Wincker, P., Clark, A. G., Ribeiro, J. M. C., Wides, R., et al. (2002) *Science* **298**, 129–149.
34. Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., Nachman, R. J., Huybrechts, R., De Loof, A. & Schoofs, L. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 15363–15368.
35. Meola, S. M., Wright, M. S., Holman, G. M. & Thompson, J. M. (1991) *Neurochem. Res.* **16**, 543–549.
36. McCormick, J. & Nichols, R. (1993) *J. Comp. Neurol.* **338**, 278–288.
37. Lu, D., Lee, K. Y., Horodyski, F. M. & Witten, J. L. (2002) *J. Comp. Neurol.* **446**, 377–396.