Review

A genome-wide inventory of neurohormone GPCRs in the red flour beetle *Tribolium castaneum* [☆]

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Abstract

Insect neurohormones (biogenic amines, neuropeptides, and protein hormones) and their G protein-coupled receptors (GPCRs) play a central role in the control of behavior, reproduction, development, feeding and many other physiological processes. The recent completion of several insect genome projects has enabled us to obtain a complete inventory of neurohormone GPCRs in these insects and, by a comparative genomics approach, to analyze the evolution of these proteins. The red flour beetle *Tribolium castaneum* is the latest addition to the list of insects with a sequenced genome and the first coleopteran (beetle) to be sequenced. Coleoptera is the largest insect order and about 30% of all animal species living on earth are coleopterans. Some coleopterans are severe agricultural pests, which is also true for *T. castaneum*, a global pest for stored grain and other dried commodities for human consumption. In addition, *T. castaneum* is a model for insect development. Here, we have investigated the presence of neurohormone GPCRs in *Tribolium* and compared them with those from the fruit fly *Drosophila melanogaster* (Diptera) and the honey bee *Apis mellifera* (Hymenoptera). We found 20 biogenic amine GPCRs in *Tribolium* (21 in *Drosophila*; 19 in the honey bee), 48 neuropeptide GPCRs (45 in *Drosophila*; 35 in the honey bee), and 4 protein hormone GPCRs (4 in *Drosophila*; 2 in the honey bee). Furthermore, we identified the likely ligands for 45 of these 72 *Tribolium* GPCRs. A highly interesting finding in *Tribolium* was the occurrence of a vasopressin GPCR and a vasopressin peptide. So far, the vasopressin/GPCR couple has not been detected in any other insect with a sequenced genome (*D. melanogaster* and six other *Drosophila* species, *Anopheles gambiae*, *Aedes aegypti*, *Bombyx mori*, and *A. mellifera*). *Tribolium* lives in very dry environments. Vasopressin in mammals is the major neurohormone steering water reabsorption in the kidneys. Its presence in *Tribolium*, therefore, might be related to the animal's need to effectively control water reabsorption. Other striking differences between *Tribolium* and the other two insects are the absence of the allatostatin-A, kinin, and corazonin neuropeptide/receptor couples and the duplications of other hormonal systems. Our survey of 340 million years of insect neurohormone GPCR evolution shows that neuropeptide/receptor couples can easily duplicate or disappear during insect evolution. It also shows that *Drosophila* is not a good representative of all insects, because several of the hormonal systems that we now find in *Tribolium* do not exist in *Drosophila*.

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1. Introduction

Insects are the largest animal group living on earth (75% of all animal species are insects) and are extremely important for agriculture and ecology, because 70% of all flowering plants depend on insects for their pollination. Insects, however, can also be severe agricultural pests, destroying 30% of our potential annual harvest and can be the vectors for major diseases, such as malaria, which causes 1.5–2.7 million deaths each year.

Because of the importance of insects, 24 insect genome projects have been initiated during the last 8 years and several of them have been completed. These insects with a genome project are listed in Fig. 1 and are either medically or agriculturally important, or are model organisms (such as *Drosophila*). The most recent addition to the list of insects with a fully sequenced genome is the red flour beetle *Tribolium*

castaneum [134]. *Tribolium* belongs to the insect order Coleoptera (or beetles), which is the largest insect order and which comprises about 40% of all insect species. Thus, about 30% of all animal species living on earth are coleopterans.

Insects can be subdivided into Holometabola (insects with a complete metamorphosis, from larvae to pupae to adults) and Hemimetabola (insects with an incomplete metamorphosis, where the larvae, also called nymphae, resemble the adults). So far, only the genomes from holometabolous insects have been fully sequenced (Fig. 1).

The Holometabola consist of four major insect orders, the most basal being Hymenoptera (to which honey bees, wasps and ants belong), followed by Coleoptera, Lepidoptera (butterflies and moths), and Diptera (flies) [139,162]. The evolutionary distance between Hymenoptera and Diptera is about 340 million years [33,139], a distance comparable to that between bony fishes and humans.

Tribolium has been selected for a genome project, because it is agriculturally important and a major global pest of stored grain, flour and many other dried and stored commodities for human consumption. Furthermore, *Tribolium* is a versatile genetic model for studies of eukaryotic development (<http://www.hgsc.bcm.tmc.edu/projects/tribolium/TriboliumWhitePaper.pdf>). The size of the *Tribolium* genome is about 190 Mb and it contains more than 16,400 genes [134].

The genomic data from *Tribolium* are highly relevant not only for our understanding of the biology of *Tribolium*, but also for the understanding of the other coleopterans, which comprise about 600,000 species. One would naively expect that the results from the *Tribolium* Genome Project would be somewhat intermediate between those from the more primitive honey bee (Hymenoptera) and the more advanced fruitfly (Diptera). However, as we can read in this review, each insect group has specialized itself and adapted to its ecological niche. As a consequence, *Tribolium* has obtained (or retained) characteristics that can not be found in the honey bee or in *Drosophila*.

G protein-coupled receptors (GPCRs)¹ are the largest superfamily of transmembrane proteins in metazoans and up to 1–2% of all genes in an animal's genome code for GPCRs [43]. GPCRs have seven α -helical transmembrane domains and are, therefore, also called seven transmem-

Fig. 1. The evolutionary relationships of insects for which genome projects exist. Insects whose genome has been completely sequenced (to at least 8–9 times coverage) are highlighted in green. Insects for which the genome sequence is still incomplete (less than 8 times coverage, as of July 2007) are highlighted in blue. The divergence times (the evolutionary distances in time between each species and the common ancestor with its neighbour in this figure) are given as horizontal bars and are from data presented in [139,162], or in the various relevant “white papers” accessible on the internet. The completed insect genomes are published in [1,62,111,115,133,162,166] (colors in this figure can only be seen in the online version of the article).

¹ Abbreviations used: AKH, adipokinetic hormone; BCM, Baylor College of Medicine, Houston, USA; CCAP, crustacean cardioactive peptide; CHO, Chinese hamster ovary; CRF, corticotropin releasing factor; CT, calcitonin; DH, diuretic hormone; DLGR, *Drosophila* leucine-rich repeats-containing GPCR; ETH, ecdysis triggering hormone; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GPA2, glycoprotein hormone alpha2; GPB5, glycoprotein hormone beta5; ic3, intracellular loop-3; LGR, leucine-rich repeats-containing GPCR; LRR, leucine-rich repeat; MIP, myoinhibitory peptide; MS, mass spectrometry; MYR, million years; NPF, neuropeptide F; na, not applicable or annotated; OT, oxytocin; PDF, pigment dispersing factor; sNPF, short neuropeptide F; TSH, thyroid stimulating hormone; 7TM, seven transmembrane; V1a, vasopressin 1a.

brane, or 7TM receptors. They interact at their cytoplasmic loops with G proteins, leading to second messenger cascades [46]. GPCRs and their G proteins even occur in plants and prokaryotes, showing that these signaling systems are evolutionarily very old [50,101].

Our research group is especially interested in neurohormone GPCRs and their ligands (biogenic amines, neuropeptides and protein hormones), because these molecules occupy a central position in the physiology of animals and steer important processes, such as behavior, reproduction, and development. Neurohormone GPCRs are an important, but only smaller subset of the large GPCR superfamily. These GPCRs belong to two subfamilies, family-A (family-1, or rhodopsin-like) GPCRs, and family-B (family-2, or calcitonin-like) GPCRs [46]. The biogenic amine GPCRs belong to family-A, whereas the neuropeptide and protein hormone GPCRs cover both families. Because the *Drosophila* genome was the first insect genome to be sequenced [1], most information on insect neurohormone GPCRs is available from *Drosophila* [56,57].

In general, after an insect genome has been sequenced and assembled, software programs are applied to predict all the genes present in the genome and, by comparison with genes already identified from other genomes, to propose a function for these genes (annotations). These automated annotations have, of course, to be verified by manual curation and subsequent experimental approaches, such as PCR and cloning. Shortly after the publication of the *Drosophila* genome [1], Hewes and Taghert [59] annotated 21 genes as coding for biogenic amine GPCRs and 44 genes for neuropeptides and protein hormone GPCRs. During the last few years, we have found 5 additional neuropeptide GPCRs, bringing the total number of neurohormone GPCRs in *Drosophila* first to 69 [56,57], and now to 70 (present paper, Figs. 2–4). Already before the publication of the *Drosophila* genomic sequence [1], a few neuropeptide and biogenic amine GPCRs had been cloned and their ligands determined. The publication of the *Drosophila* genome, however, has greatly boosted this line of research and currently the ligands for 17 biogenic amine GPCRs and 31 neuropeptide and protein hormone GPCRs have experimentally been identified [57]. Thus, for 48 or nearly 70% of all *Drosophila* neurohormone GPCRs, the ligands are known (we say that these *Drosophila* GPCRs have been deorphanized). Although the remaining 30% of these *Drosophila* GPCRs are certainly more difficult to deorphanize, we are confident that this will happen and that we will have a rather complete picture of *Drosophila* GPCRs and their ligands within the next few years.

To deorphanize a neurohormone GPCR, its cDNA has to be cloned and its coding region expressed in cultured mammalian cells or *Xenopus* oocytes [25,26,57]. The transformed cells are then exposed to peptides or other ligands from a chemical library or to a tissue extract containing the ligand. When a second messenger response occurs (or an ion current in the case of oocytes), we have a bioassay enabling the ligands to be

purified and identified. There are many ways to measure the various second messenger responses or to manipulate the host cells in such a way that they are optimally reacting to GPCR activation by its ligand. Our research group has especially applied a technique, originally developed by Stables et al. [146], where Chinese hamster ovary (CHO) cells are transfected with both DNA coding for a “universal” (or “promiscuous”) G protein α -subunit, $G\alpha$ -16, and DNA for the insect GPCR. Activation of $G\alpha$ -16 in these cells leads to an increase in intracellular Ca^{2+} concentration, which is measured as a strong (up to 400× over background) bioluminescence response [57]. More than half of the above-mentioned 48 identified *Drosophila* receptors has been deorphanized with this technique by us and other groups.

Our increased knowledge of *Drosophila* neurohormone GPCRs and their ligands strongly contributes, of course, to our understanding of *Drosophila*. This understanding of *Drosophila*'s neurobiology and endocrinology will be further improved by the application of genetic tools such as the knocking-out of GPCR and neuropeptide genes. Since a few years, there has existed a *Drosophila* Gene Disruption Project, where, using P-element or piggyBac insertions, more than half of all *Drosophila* genes have been disrupted (<http://flypush.imgen.bcm.tmc.edu/pscreen/>). The current collection of mutants also contains flies with disrupted neurohormone GPCR and neuropeptide genes and it can be expected that the phenotypic characterization of these *Drosophila* mutants will teach us much about the functioning of the neurohormone/GPCR couples. This will certainly challenge many of the existing paradigms in insect neuroendocrinology.

One can expect that *Drosophila* neurohormone GPCRs will become a reference for our understanding and interpretation of other insect neurohormone GPCRs. For example, in a previous review on honey bee neurohormone GPCRs, we have used the *Drosophila* GPCRs as a “gold standard” to annotate the honey bee orthologues [57]. On the other hand, *Drosophila* can not be regarded as a perfect representative of all holometabolous insects and there are many biological processes absent in *Drosophila* that exist in other insects and *vice versa*. To obtain a better insight into the biology of insects, therefore, it is crucial also to investigate representatives of other insect orders and to study them with the same rigorousness as in *Drosophila*. The first step in this direction is the sequencing of their genomes (Fig. 1), followed by proper annotation and analysis of their genes.

In our current review, we have annotated the neurohormone GPCR genes in the first beetle with a sequenced genome, the red flour beetle *Tribolium*, using mainly *Drosophila* GPCR genes as a reference. Furthermore, we have compared these results with the neurohormone GPCR genes present in the honey bee and fruitfly. *Tribolium* is living in very dry surroundings and it can be expected that its endocrine system is organized in a different way from that of *Drosophila*. Therefore, we have

Fig. 2. Phylogenetic tree analysis of biogenic amine GPCRs from *Drosophila* (indicated by a CG number), *T. castaneum* (Tc 1–Tc 20), and *A. mellifera* (Am 1–Am 19). The tree is rooted by the *Drosophila* FMRamide receptor CG2114. When a ligand has been experimentally identified for a GPCR and the GPCR thus has been deorphanized, this ligand is given in parentheses. We have highlighted identical ligands with the same color. The biogenic amine GPCRs belong to the rhodopsin- (or family A- or -1) type of GPCRs.

not only used *Drosophila* GPCRs for our TBLASTN searches, but also GPCRs from several other animals, including mammals.

In a recent paper, we have determined the occurrence of neuropeptides and protein hormone genes in the genome of *Tribolium* and we have also experimentally

confirmed the presence of many of these neurohormones, using mass spectrometry [100]. A summary of these neurohormone findings is given in Table 1. We found that our current neurohormone GPCR annotations perfectly

fit to the independently annotated neurohormones. The outcome from the two studies is highly relevant for our understanding of the neurobiology of *Tribolium*. They also give us excellent insights into the evolution of

Fig. 3A. (A and B) Phylogenetic tree analysis of neuropeptide and protein hormone GPCRs, belonging to GPCR family A. The *Drosophila* GPCRs are indicated by a CG number, the receptors from *T. castaneum* by Tc 21–Tc 64, and the receptors from *A. mellifera* by Am 20–Am 52. The tree is rooted by the *Drosophila* metabotropic glutamate receptor CG11144. When the *Tribolium* receptor gene has a deorphanized orthologue in *Drosophila* and, thus, a likely ligand, it is highlighted in red. When no ligand can be assigned, it is highlighted in blue. The *Tribolium* vasopressin-like receptor gene Tc 45 is highlighted in green (top Fig. 3B), because it has an orthologue in mammals and not in *Drosophila*.

Fig. 3B. See legend for Fig. 3A.

Fig. 4. Phylogenetic tree analysis of neuropeptide and protein hormone GPCRs, belonging to GPCR family B. The *Drosophila* GPCRs are indicated by a CG number, the receptors from *T. castaneum* by Tc 65–Tc 72, and the receptors from *A. mellifera* by Am 53–Am 56. The tree is rooted by the *Drosophila* metabotropic glutamate receptor CG11144. The receptor genes are highlighted in the same way as in Fig. 3.

neurohormone GPCRs in insects and their co-evolution with the ligands.

2. Annotation strategies for GPCRs present in *Tribolium*

The *Tribolium* genome was sequenced during 2004–2006 [134]. The results from several automated annotation pipelines and *ab initio* prediction programs were combined into a consensus set of 16,404 gene models, using the GLEAN software program in early 2006 [134].

We searched the complete *Tribolium* genome sequence (not the GLEAN set) with protein sequences corresponding to *Drosophila*, honey bee, and mammalian GPCRs, using the TBLASTN software program at the NCBI server http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=insects. The regions that were hit with highest scores were checked for their presence in the GLEAN set, which was not always the case. Subsequently, they were manually curated and annotated at the Baylor College of Medicine (BCM) *Tribolium* annotation server under a GLEAN number.

Our annotation efforts led to the discovery of 20 *Tribolium* genes, coding for biogenic amine GPCRs (there are 21 *Drosophila*, and 19 honey bee biogenic amine GPCRs; Fig. 2), 44 neuropeptide and protein hormone GPCRs, belonging to family-A (there are 40 in *Drosophila* and 31 in the honey bee; Fig. 3), and 8 neuropeptide GPCRs, belonging to family-B (there are 5 in *Drosophila* and 4 in the honey bee; Fig. 4). For clarity, we have named these *Tribolium* neurohormone GPCRs Tc 1 to Tc 72 (Figs. 2–4). The GLEAN numbers, corresponding to these Tc numbers can be retrieved from Tables 2 and 3.

We used three criteria for deciding whether a *Tribolium* gene is the orthologue of a *Drosophila* (or honey bee) neurohormone GPCR gene: (i) its gene product should have high amino acid sequence identity with the *Drosophila* neu-

rohormone GPCR (more than 50% in the 7TM region, see Tables 2 and 3); (ii) in a phylogenetic tree analysis, the *Tribolium* GPCR should lie “close” to the *Drosophila* GPCR (for example, the *Tribolium* receptor Tc 1 and the *Drosophila* octopamine receptor CG31351, top of Fig. 2, are clear orthologues); (iii) the genomic organizations should be similar (various identical intron positions, see Tables 2 and 3). For the other *Tribolium* GPCR genes that did not have a clear *Drosophila* or honey bee orthologue, the criterion for being a neurohormone GPCR gene was that it had a neurohormone GPCR orthologue in an other animal group, for example in mammals.

To obtain a quick overview of the neurohormone GPCRs in *Tribolium*, the reader is referred to the phylogenetic tree analyses (Figs. 2–4), which are quite illustrative. For example (top of Fig. 2), Tc 1 to Tc 4 are clearly octopamine receptors, Tc 5 is clearly a dopamine receptor, etc. These three figures contain, in addition to the *Tribolium* (indicated by Tc numbers) and *Drosophila* (CG-numbered) GPCRs, also the honey bee (Am-numbered) GPCRs for comparison [57]. In the following, we will discuss the GPCRs given in Figs. 2–4 in more detail.

3. Biogenic amine GPCRs

The known biogenic amines in insects that exert their actions via GPCRs are dopamine, tyramine, and octopamine (derived from tyrosine), serotonin (derived from tryptophan), and acetylcholine. The structures and biosynthetic pathways of tyramine and octopamine are given in [23].

Biogenic amines steer numerous behaviors and physiological processes in insects, among them learning, memory, sleep, arousal, honey bee dance, feeding, and diuresis [7,11,14,48,119,135,143,161]. As mentioned earlier, we found twenty biogenic amine GPCRs in *Tribolium*. Fig. 2 shows a phylogenetic tree analysis of these receptors, which

Table 1

A summary of neuropeptides and protein hormones present in *Tribolium*

Name (Trica-)	Structure	Predicted (o)	Confirmed (+)
Adipokinetic hormone-1	pQLNFSTDWamide	o	
Adipokinetic hormone-2	pQLNFTPNWamide	o	+
Adipokinetic hormone-3	pQVTFSRDWNamide	o	
Allatostatin B-1	DWNKDLHIWamide	o	+
Allatostatin B-2	GWNNLHEGWamide	o	+
Allatostatin B-3	AWQSLQSGWamide	o	+
Allatostatin B-4	NWGQFHGGWamide	o	+
Allatostatin B-5	SKWDNFRGSWamide	o	+
Allatostatin B-6	EPAWSNLKGIWamide	o	+
Allatostatin C	pQSRYRQCYFNPISCF	o	
Allatotropin	DAKYPQVVRTPQQLTRGIEALKYHNMDLGTARGYamide	o	+
Antidiuretic factor-1	HDDGSYKPHVYGW	o	
Antidiuretic factor-2	YDDGSYKPHVYGF	o	
Antidiuretic factor-3	YDDGSYKPHIYGF	o	
Antidiuretic factor-4	YDDGSYKPGLYA	o	
Antidiuretic factor-5	YDDGSYKPHIYGH	o	
Bursicon α -subunit	GLEAN_13832	o	
Bursicon β -subunit	GLEAN_13831	o	
Capa-1	EPKEPKRNKLASVYALTPSLRVamide	o	+
Capa-2	RIGKMVSFPRamide	o	+
Crustacean cardioactive peptide	PFCNAFTGCamide	o	
Diuretic hormone-31	GLDLGLGRGFSGSQAACHLMGLAAANFAGGPamide	o	+
Diuretic hormone-37	SPTISITAPIDVLRKTWAKENMRKQMQUINREYLKLNQamide	o	+
Diuretic hormone-47	AGALGESGASLSIVNSLDVLRNRLLEIARKKAKEG-ANRNRQILLSLamide	o	+
Eclosion hormone	GLEAN_00178	o	
Ecdysis-triggering hormone-1	ENYVLKAAKNVPRamide	o	+
Ecdysis-triggering hormone-2	SNTNKNTNIDEMGKFFMKASKSVPRamide	o	+
FMRamide-1	NSNFLRFamide	o	+
FMRamide-2	SGKIEKNDHFIRamide	o	+
FMRamide-3	SKQDFLRFamide	o	+
FMRamide-4	NQPKATTNYLRFamide	o	+
FMRamide-5	DTSNFLRFamide	o	+
Glycoprotein hormone α -2	GLEAN_11941	o	
Glycoprotein hormone β -5	GLEAN_12548	o	
Ion transport peptide/crustacean hyperglycaemic hormone	TC_030007	o	
Myosuppressin	pQDVDHVFLRFamide	o	+
Neuroparsin	GLEAN_13258	o	
Proctolin	RYLPT	o	
Prothoracicotropic hormone	TC_030081	o	
Pyrokinin-1-1	SESNWVPDDNSYGAQRPGANSNGGMWFGPRLamide	o	
Pyrokinin-1-2	TPHESSVPNERNDSDSKETYFWFGPRLamide	o	
Pyrokinin-2-1	HVVNFTPRLamide	o	+
Pyrokinin-2-2	SPPFAPRLamide	o	+
Pyrokinin-2-3	HSSPFSPRLamide	o	+
SIFamide	TYRKPPFNGSIFamide	o	+
Short neuropeptide-F	SGRSPSLRLRFamide	o	+
Sulfakinin-1	pQTSDDYGHLLRFamide	o	+
Sulfakinin-2	GEEPDDDYGHMRamide	o	
Tachykinin-1	APSGFTGVRamide	o	+
Tachykinin-2	APSGFMGMRamide	o	+
Tachykinin-3	APMGFMGMRamide	o	+

(continued on next page)

Table 1 (continued)

Name (Trica-)	Structure	Predicted (o)	Confirmed (+)
Tachykinin-4	APSGFFGMRamide	o	+
Tachykinin-5	MPRQAGFFGMRamide	o	+
Tachykinin-6	YPYQFRGKFVGVRamide	o	+
Vasopressin	CLITNCPRGamide	o	

These sequences are predicted by bioinformatical tools (o) or experimentally confirmed by mass spectrometry (+). For peptide and protein sequences with more than 50 residues, we refer to the GLEAN or gene number [134]. The data are derived from information given in [100].

also includes the *Drosophila* and honey bee biogenic amine GPCRs. Table 2 gives information on the amino acid residue identities between the *Tribolium* and *Drosophila*/honey bee orthologues and also compares their gene structures (intron/exon positions).

3.1. Ligands can be assigned for fourteen *Tribolium* biogenic amine GPCRs

When we used the above-mentioned criteria for orthologues, we found that all twenty biogenic amine GPCR genes in *Tribolium* have clear orthologues in *Drosophila* and honey bee (Fig. 2 and Table 2). Because seventeen *Drosophila* and five honey bee biogenic amine GPCRs have been deorphanized (Table 2), it is easy to assign the likely ligands for the corresponding fourteen *Tribolium* GPCRs (Fig. 2). Thus, Tc 1 to Tc 4 are octopamine receptors, Tc 5 is a dopamine receptor, Tc 7 is an acetylcholine receptor, Tc 10 is a dopamine receptor, Tc 11 is a tyramine/octopamine receptor, Tc 13 to Tc 15 are serotonin receptors, Tc 16 is a dopamine receptor, Tc 17 is a serotonin receptor, and Tc 20 is a dopamine receptor (Fig. 2 and Table 2). These assignments become even more convincing, where both the *Drosophila* and honey bee orthologues have been deorphanized. This is true for Tc 4, where both the *Drosophila* and honey bee orthologues are octopamine receptors; Tc 5, Tc 10, and Tc 16, where both the *Drosophila* and honey bee orthologues are dopamine receptors; Tc 11, where both the *Drosophila* and honey bee orthologues are tyramine/octopamine receptors; and Tc 17, where both the *Drosophila* and honey bee orthologues are serotonin receptors. There are no cases, where the *Drosophila* and honey bee orthologues use different ligands, which illustrates the reliability of our approach.

3.2. No ligands can be assigned for six *Tribolium* biogenic amine GPCRs

Six *Tribolium* GPCRs in Fig. 2 have orthologues in *Drosophila* and honey bee that have not been deorphanized. Thus, no ligands can be assigned for Tc 6, Tc 8, Tc 9, Tc 12, Tc 18, and Tc 19 (Fig. 2).

3.3. The overall picture of biogenic amine GPCRs in *Tribolium*

In terms of numbers, the general picture for biogenic amine GPCRs in *Tribolium* looks very similar to that found

in *Drosophila* and the honey bee. In four out of five (Tc 1, Tc 6, Tc 9, Tc 14, Tc 15) cases, where there was a difference between *Drosophila* and the honey bee, the pattern in *Tribolium* resembled more that of *Drosophila*. For example the honey bee has two paralogues, Am 1 and Am 2, whereas *Tribolium* has only one (Tc 1) just as *Drosophila* (CG31351; top of Fig. 2).

4. Neuropeptide and protein hormone GPCRs

The ligands for the neuropeptide and protein hormone GPCRs in *Tribolium* have been annotated in a companion paper and many of them have been confirmed by mass spectrometry [100]. Table 1 gives an overview of these neuropeptide and protein hormone ligands. As mentioned earlier, their GPCRs belong to two subfamilies, family-A and family-B. Below, we will discuss the two families combined.

4.1. By comparing with *Drosophila*, ligands can be assigned for 30 *Tribolium* neuropeptide and protein hormone GPCRs

4.1.1. Tc 21 is a myosuppressin-like receptor gene

Myosuppressins are neuropeptides that generally inhibit insect muscle contractions [60]. In some insects, the peptide also inhibits the prothoracic gland to secrete ecdysteroids, which are steroid hormones stimulating growth, mating and metamorphosis [167]. Myosuppressins are characterized by the consensus sequence X₁DVX₂HX₃ FLRFamide [117,156]. Also *Tribolium* has a myosuppressin gene and the existence of a *Tribolium* myosuppressin peptide has been confirmed by mass spectrometry (MS) (Table 1) [100]. Tc 21 is probably the myosuppressin receptor: In a phylogenetic tree analysis (top of Fig. 3A) it is the orthologue of CG13803, CG84985, and Am 21, which all three have been identified by us and others as specific myosuppressin receptors [35, 73, Rudolf and Gimmelikhuijzen, unpublished results]. The orthologous relationships between the four receptor genes are corroborated by the presence of a common intron (1 out of 1) with identical intron phasings, and reasonable sequence identities between the receptor proteins (Table 3). The insect myosuppressin receptors are highly selective and need the whole myosuppressin consensus sequence for activation. They do not cross-react with the below mentioned FMRamide-like peptides [35,142].

Table 2
Identification of twenty *Tribolium* biogenic amine receptors

<i>Tribolium</i> receptor no. in the text	CG no. of the <i>Drosophila</i> orthologue	Endogenous ligand for the deorphanized <i>Drosophila</i> receptor	Gene ID no.	Protein region identified	Sequence identity between TM1-7 of the <i>Tribolium</i> and the closest <i>Drosophila</i> / <i>Apis</i> receptor proteins (without ic3)	No. of introns in the <i>Tribolium</i> gene coding region	No. of common introns between the <i>Tribolium</i> and <i>Drosophila</i> / <i>Apis</i> genes (coding regions)	Accession no. for the <i>Tribolium</i> receptor	Reference for the deorphanized <i>Drosophila</i> and/or honey bee receptor
Tc 1	CG31351	Octopamine	GLEAN_12598 GLEAN_12599	Complete	48(83)/72(82)	2	1/2	BK005856	[106]
Tc 2	CG6989	Octopamine	GLEAN_12597	Complete	68(75)/69(75)	0	0/0	BK005857	[106]
Tc 3	CG6919	Octopamine	GLEAN_12600	Complete	66(72)/77(85)	3	2/2	BK005858	[5,106]
Tc 4	CG3856	Octopamine	GLEAN_11639	Complete	51(70)/62(80)	7	2/2	BK005859	[5,51,53]
Tc 5	CG18741	Dopamine	GLEAN_00574	Complete	74(88)/79(88)	2	2/2	BK005860	[38,52,68]
Tc 6	CG7431, CG16766	Tyramine, orphan	GLEAN_12122	Incomplete	na	na	na	BK005868	[23]
Tc 7	CG4356	Acetylcholine	GLEAN_04470	Complete	47(72)/61(73)	5	2/3	BK005871	[118,144]
Tc 8	CG7918	Orphan	GLEAN_00298	Complete	40(72)/50(74)	3	1/1	BK005869	
Tc 9	CG12796	Orphan	GLEAN_03331	Complete	42(48)/na(na)	1/—	0/—	BK005870	
Tc 10	CG9652	Dopamine	GLEAN_12447	Complete	81(89)/76(85)	4	2/2	BK005863	[15,49,151]
Tc 11	CG7485	Octopamine/ tyramine	GLEAN_04545	Complete	52(82)/58(79)	0	0/0	BK005861	[3,16,137]
Tc 12	CG18208	Orphan	GLEAN_11641 GLEAN_11643	Complete	55(73)/68(67)	2	1/2	BK005864	
Tc 13	CG12073	Serotonin	GLEAN_11667	Complete	57(76)/54(76)	1	0/0	BK005867	[140,165]
Tc 14	CG15113	Serotonin	GLEAN_11960	Complete	43(74)/51(72)	4	3/2	BK005865	[138]
Tc 15	CG16720	Serotonin	GLEAN_12297	TMII-end	38(72)/56(70)	2	2/1	BK005866	[138]
Tc 16	CG17004	Dopamine	GLEAN_07490	Complete	62(75)/64(91)	4	4/4	BK005862	[8,58]
Tc 17	CG1056	Serotonin	GLEAN_13979 GLEAN_13980	Complete	39(78)/66(75)	4	2/3	BK005872	[31]
Tc 18	CG7994, CG8007	Orphan	GLEAN_13982	Complete	42(78)/53(83)	6	2/3	BK005873	
Tc 19	CG13597	Orphan	GLEAN_01180	Complete	48(64)/70(82)	5	4/4	BK005874	
Tc 20	CG18314	Ecdysteroids, dopamine	GLEAN_06457	Complete	71(68)/76(72)	1	1/1	BK005875	[145]

ic-3, intracellular loop-3; na, not annotated or applicable.

Table 3
Identification of fifty-two *Tribolium* neuropeptide and protein hormone receptors

Tribolium receptor no. in the text	CG no. of the <i>Drosophila</i> orthologue	Endogenous ligand for the deorphanized <i>Drosophila</i> receptor	Gene ID no.	Protein region identified	Sequence identity between the identified parts of the <i>Tribolium</i> and the closest <i>Drosophila</i> / <i>Apis</i> orthologue (TM1–TM7)	No. of introns in the <i>Tribolium</i> receptor gene (coding region)	No. of common introns between the <i>Tribolium</i> and <i>Drosophila</i> / <i>Apis</i> genes (coding regions)	Accession no. for the <i>Tribolium</i> receptor	Reference for the deorphanized <i>Drosophila</i> receptor
Tc 21	CG8985, CG13803	Myosuppressin	GLEAN_10505	Complete	37/38	1	1/1	BK006094	[35,73]
Tc 22	CG13229	Orphan	GLEAN_11478	Complete	38/47	1	1/1	BK006093	
Tc 23	CG13229	Orphan	GLEAN_15904	Complete	42/49	2	1/1	BK006095	
Tc 24	CG16752	Orphan	GLEAN_02917	Complete	62/na	2	1/na	XM_970169	
Tc 25	CG6986	Proctolin	GLEAN_03492	Complete	28/na	4	2/na	BK006096	[36,74]
Tc 26	CG2114	FMRFamides	GLEAN_01381	Complete	55/51	1	0/1	BK006097	[19,107]
Tc 27	CG5936	Orphan	GLEAN_02524	Complete	51/66	2	1/2	XM_963259	
Tc 28	CG16726	Orphan	GLEAN_07986	TM 1-5	35/50	0	0/0		
Tc 29	CG5911	Edysis-triggering-hormone	GLEAN_12493	Complete	70/60	3	2/2	BK006098	[71,123]
Tc 30	CG8795	Pyrokinin-2	GLEAN_11318	Complete	53/60	3	2/2	BK006099	[122,136]
Tc 31	CG8784	Pyrokinin-2	GLEAN_11320	Complete	50/58	2	2/2	BK006100	[122,136]
Tc 32	CG9918	Pyrokinin-1	GLEAN_11171	Complete	55/60	3	3/2	BK006101	[22]
Tc 33	CG14575	Capa	GLEAN_07170	Complete	47/49	5	3/3	BK006102	[72,122]
Tc 34	CG6857	Sulfakinin	GLEAN_08438	Complete	59/57	4	2/3	XM_967657	[89, Kobberup et al., unpublished]
Tc 35	CG6857	Sulfakinin	GLEAN_07536	Complete	50/79	5	2/3	XM_970133	[89, Kobberup et al., unpublished]
Tc 36	na	na	GLEAN_14211	Complete	na/45	5	na/4	XM_966369	
Tc 37	na	na	GLEAN_09749	Complete	na/50	4	na/4	XM_968645	
Tc 38	CG10823	SIFamide	GLEAN_11156	Complete	72/80	3	3/2	BK005734	[77]
Tc 39	CG5811	Orphan	GLEAN_01056	Complete	49/48	4	4/3	XM_970025	
Tc 40	CG7887	Tachykinin	GLEAN_11198	Complete	66/70	5	2/1	XM_965009	[12,99]
Tc 41	CG6515	Tachykinin	GLEAN_04977	Complete	56/49	8	5/na	XM_966290	[73,112]
Tc 42	na	na	GLEAN_09184	Complete	na/33	4	na/3	XP_972230	
TC 43	CG6111	Crustacean cardioactive peptide	GLEAN_13945	Complete	63/63	7	5/5	BK006103	[21]
TC 44	CG6111	Crustacean cardioactive peptide	GLEAN_02216	Complete	68/65	6	4/5	BK006104	[21]
Tc 45	na	na	GLEAN_16363	Complete	na/na	5	na/na	BK006105	
Tc 46	CG11325	Adipokinetic hormone	GLEAN_09772	Complete	53/70	6	3/6	DQ422965	[55,148]

Tc 47	CG11325	Adipokinetic hormone	GLEAN_01245	Complete	42/46	7	1/3	BK006106	[55,148]
Tc 48	CG8930	Bursicon	GLEAN_08163	TM 1-7	85/85	15	12/11	BK006107	[37,104,108]
Tc 49	CG7665 (DLGR1)	GPA2/GPB5	GLEAN_09127	N-TM 1-7	68/na	13	12/—	BK006111	[54,150]
Tc 50	CG7665 (DLGR1)	GPA2/GPB5	GLEAN_09575	N-TM 1-7	55/na	12	9/—	BK006108	[54,150]
Tc 51	CG4187 (DLGR4)	Orphan	GLEAN_15777	N-TM 1-7	56/na	18	14/—	BK006109	
Tc 52	CG13702	Allatostatin-C	GLEAN_12842	Complete	57/67	1	0/0	BK006110	[88]
Tc 53	CG14593	Orphan	GLEAN_06805	Complete	60/na	4	2/na	XP_974786	
Tc 54	CG30106	Allatostatin-B	GLEAN_05327	Complete	60/57	5	4/3	XP_974772	[73]
Tc 55	CG14003	Orphan	GLEAN_14731	Complete	58/na	6	5/na	XP_975121	
Tc 56	CG30340	Orphan	GLEAN_02497	TM1-7	49/41	3	2/1	XP_970905	
Tc 57	CG7395	Short neuropeptide-F	GLEAN_03151	Complete	68/70	0	0/0	XP_966794	[39,109]
Tc 58	CG7395	Short neuropeptide-F	GLEAN_03150	Complete	56/60	0	0/0	XP_966881	[39,109]
Tc 59	CG1147	Neuropeptide F	GLEAN_11655	Complete	58/na	5	1/na	XP_967689	[45]
Tc 60	CG4322	Orphan	GLEAN_07687	Complete	56/51	2	2/2	XP_974272	
Tc 61	CG3171	Orphan	GLEAN_04716	Complete	56/63	4	3/2	XP_971005	
Tc 62	CG12610	Orphan	GLEAN_04565	Complete	45/na	5	3/na	XP_967232	
Tc 63	na	na	GLEAN_06608	Complete	na/49	4	na/0	XP_974365	
Tc 64	na	na	GLEAN_15120	Complete	na/na	1	na/na	BK005853	
Tc 65	CG8422	Diuretic hormone 44	GLEAN_07104	Complete	58/60	8	7/8	XM_970323	[75]
Tc 66	CG8422	Diuretic hormone 44	GLEAN_12799	Complete	53/52	7	6/6	XM_966084	[75]
Tc 67	na	na	GLEAN_10267	Complete	na/60	9	na/4	XM_964860	
Tc 68	na	na	GLEAN_08110	Complete	na/41	4	na/2	XP_975039	
Tc 69	CG13758	Pigment dispersing factor	GLEAN_13682	TM 2-7	50/53	7	2/2	XP_971738	[69,91,110]
Tc 70	CG32843	Diuretic hormone 31	GLEAN_02694	Complete	54/53	7	7/7	XP_969030	[76]
Tc 71	CG4395	Orphan	GLEAN_13321	Complete	43/na	7	3/na		
Tc 72	na	na	GLEAN_01222	Complete	na	7	na/na	XP_968807	
	CG10626	Kinin							[131]
	CG10698	Corazonin							[19]
	CG2872,	Allatostatins-A							[10,90,95–97]
	CG10001								
	CG4313	Orphan							
	CG12290	Orphan							
	CG13575	Orphan							
	CG13995	Orphan							

na, not annotated or applicable.

4.1.2. *Tc 25 is a proctolin-like receptor gene*

Proctolin is a small myo- and neurostimulatory peptide with the structure RYLPT [117]. Also *Tribolium* has a proctolin preprohormone gene (Table 1). The Tc 25 gene is probably the *Tribolium* receptor gene, because in a phylogenetic tree analysis (Fig. 3A) it is the orthologue of CG6986, which has been identified as the *Drosophila* proctolin receptor gene [36,74]. Moreover, the Tc 25 gene has two introns in common with CG6986 (Table 3). The two receptor proteins, however, have only modest amino acid sequence identities (Table 3). Both proctolin and its receptor are absent in the honey bee [57].

4.1.3. *Tc 26 is an FMRFamide-like receptor gene*

FMRFamides are small invertebrate neuropeptides with the C-terminal sequence FMRFamide or related sequences. In insects, these peptides are typically myoactive and stimulate for example larval body wall muscle contractions, but might also inhibit other muscles, for example the heart [116,117,156]. FMRFamides do also inhibit the production of ecdysteroids by the prothoracic gland [168]. Also *Tribolium* has an FMRFamide peptide gene that produces 5 different FMRFamides, all of which have been confirmed by MS (Table 1). None of these peptides has a myosuppressin consensus sequence and do, therefore, not cross-react with the insect myosuppressin receptors [35,142]. Tc 26 is likely to be the receptor for these FMRF amide peptides, as it is the clear orthologue of CG2114 and Am 22, which both have been identified as FMRFamide receptors [19, 107, Rudolf and Grimmelikhuijzen, unpublished results]. Moreover, Tc 26 has one (out of one) intron in common with the honey bee gene and the *Tribolium*, honey bee and *Drosophila* receptor proteins have high sequence identities (Table 3).

4.1.4. *Tc 29 is an ecdysis-triggering-hormone (ETH)-like receptor gene*

ETHs are small insect neuropeptides that initiate ecdysis behavior [83,84]. Each insect has usually two ETHs, which have the C-terminal consensus sequence KXV/IPRI/L/Mamide [170]. Also *Tribolium* has an ETH gene, encoding two ETHs and the existence of both peptides has been experimentally confirmed by MS (Table 1). Tc 29 is probably the *Tribolium* ETH receptor, because it is a close orthologue of CG5911, which has been identified as an ETH receptor for both *Drosophila* ETH-1 and -2 [71,123]. Furthermore, Tc 29 has two introns (out of three) in common with CG5911 and the two receptor proteins have very high sequence identities (Table 1). CG5911 produces two receptor transcripts (CG5911-A and -B) by alternative splicing [71,123]. A similar splicing is also likely to occur with the Tc 26 transcripts. In *Drosophila* both the CG5911-A and -B encoded receptor variants bind ETH-1 and -2, albeit with different affinities [71]. The two receptors are specific and do not get activated by other related neuropeptides such as the pyrokinins or capa peptides (see below).

4.1.5. *Tc 30, Tc 31 and Tc 32 are pyrokinin-like receptor genes*

The insect pyrokinins are neuropeptides with C-terminal sequences that resemble that of the ETHs: FXPR(L)amide, where X, however, is a small or hydrophilic amino acid residue (X = S, T, K, A, or G) and L is always an L (in contrast to the ETHs) [61,128]. The insect pyrokinins have very diverse actions, including the stimulation or induction of gut motility, sex hormone production, diapause, and pupariation [2,61,63,117,128,158]. The first insect pyrokinin receptors to be identified were those from *Drosophila*, where three pyrokinin receptors occur and two pyrokinin peptides [22,122,136]. Based on their differentiated affinities for the two pyrokinins, one receptor was named pyrokinin-1 receptor (CG9918) activated by pyrokinin-1 (which has the C terminus WFGPR(L)amide), and the two other receptors were named pyrokinin-2 receptors (CG8784 and CG8795) activated nearly exclusively by pyrokinin-2 (which has the C terminus PFKPR(L)amide) [22,136]. Also *Tribolium* has two pyrokinin encoding genes, yielding at least five pyrokinins. Two of them are tentatively named pyrokinins-1 (having the C terminus WFGPR(L)amide) and three are tentatively named pyrokinins-2. The occurrence of the last three pyrokinins has also been confirmed by MS (Table 1). Tc 30, Tc 31, and Tc 32 probably encode *Tribolium* pyrokinin receptors, because they are close orthologues of CG8784, CG8795, and CG9918 (Fig. 3A). In addition, they have two introns (out of two or three) in common with the *Drosophila* pyrokinin receptor genes and their receptor proteins have high sequence identities with the three *Drosophila* receptors (Table 3). It is presently difficult to say which of the three receptors are pyrokinin-1 or -2 receptors. In Table 3, therefore, they have tentatively been assigned to those *Drosophila* receptors with which they had highest amino acid residue identities. Furthermore, the Tc 32 gene had three introns just as the *Drosophila* pyrokinin-1 receptor gene CG9918, arguing that it is the *Tribolium* pyrokinin-1 receptor gene (Table 3).

4.1.6. *Tc 33 is a capa-like receptor gene*

The insect capa peptides resemble both the ETHs and pyrokinins and their C-terminal sequence is usually FPRVamide. It is interesting that both in *Drosophila* and other insects the capa gene encodes a preprohormone containing two capa peptides and one or more pyrokinins-1 [80]. The physiological significance of this is currently unclear, as capa peptides do not stimulate the three *Drosophila* pyrokinin receptors, nor do pyrokinins stimulate the *Drosophila* capa receptor [22,72,136]. Capa peptides have a diuretic effect on the Malpighian tubules of *Drosophila*, but in other insects they might have antidiuretic or myotropic actions [80,120,125,127]. Also *Tribolium* has two peptides that resemble capa peptides (Table 1). That these two peptides are indeed capa peptides is supported by the finding that they are contained in a preprohormone together with a pyrokinin-1 [100]. Tc 33 is probably the *Tribolium* capa receptor, because in a phylogenetic tree analysis, it is the clear ortho-

logue of CG14575, which has been identified as the *Drosophila* capa receptor [72]. Furthermore, Tc 33 has three introns in common with CG14575 and the two receptor proteins have high sequence identities (Table 3).

4.1.7. Tc 34 and Tc 35 are sulfakinin-like receptor genes

Sulfakinins are insect neuropeptides that are structurally related to mammalian cholecystokinin and gastrin and that, like their mammalian counterparts, also have a sulphated tyrosine residue [113]. Just like cholecystokinin and gastrin in mammals, sulfakinins inhibit food intake in insects [105,160]. *Tribolium* has two sulfakinins (Table 1). Tc 34 and Tc 35 (Fig. 3A) are probably *Tribolium* sulfakinin receptor genes, because they are the orthologues of the two *Drosophila* genes CG6857 and CG6881, which have previously been identified as the sulfakinin receptor genes [89, Kobberup and Hauser, unpublished]. The *Tribolium* genes have two common introns with their *Drosophila* orthologues and their receptor proteins have high sequence identities with their *Drosophila* counterparts (Table 3).

4.1.8. Tc 38 is a SIFamide-like receptor gene

SIFamide is a highly conserved neuropeptide with the C-terminal sequence SIFamide that has been isolated from various insects and crustaceans [4,24,159,169]. The physiological role of this peptide has been unknown, but recently it has been proposed that SIF amide is involved in *Drosophila* sexual behavior [153]. Also *Tribolium* has a SIFamide peptide (Table 1). Tc 38 is probably the *Tribolium* SIFamide receptor, because it is the close orthologue of CG10823 (Fig. 3A), which is the gene coding for the *Drosophila* SIFamide receptor [77]. Moreover, the two receptor genes have all three introns in common, and their receptor proteins have very high amino acid sequence identities (Table 3).

4.1.9. Tc 40 and Tc 41 are tachykinin-like receptor genes

Insect tachykinins are short neuropeptides that differ from mammalian tachykinins by their C-terminal consensus sequence, which is $\text{FX}_1\text{GX}_2\text{Ramide}$, rather than FXGLMamide as in mammals. There are many different tachykinin isoforms in each insect, which are all encoded by a single gene, and which play various roles in neuronal signaling and gut activity [117,126]. Also *Tribolium* has six different tachykinins (Table 1). Tc 40 and Tc 41 are probably the *Tribolium* tachykinin receptor genes, because they are the close orthologues of CG7887 and CG6515 (Fig. 3A), which have been identified as the *Drosophila* tachykinin receptor genes [12,73,99,112,124]. Moreover, the two *Tribolium* receptor genes have several introns in common with their *Drosophila* orthologues and the receptor proteins show high amino acid sequence identities with their *Drosophila* counterparts (Table 3).

4.1.10. Tc 43 and Tc 44 are CCAP-like receptor genes

Crustacean cardioactive peptide (CCAP) is a small cyclic neuropeptide, activating heartbeat in crustaceans and

insects [34,40]. In addition, CCAP plays a central role in ecdysis behavior in insects [83,84]. Also *Tribolium* has a CCAP that is fully identical with the crustacean CCAP (Table 1). Tc 43 and Tc 44 are probably the *Tribolium* CCAP receptor genes, as they are close orthologues of CG6111 (Fig. 3B, top), which has been identified as the *Drosophila* CCAP receptor gene [21]. In addition, Tc 43 has 4 (out of 6) and Tc 44 has 5 (out of 7) introns in common with CG6111 (Table 3). The encoded receptor proteins of Tc 43 and Tc 44 have high (63–68%) amino acid sequence identities with their *Drosophila* counterparts (Table 3). It is interesting that *Tribolium* has two CCAP receptors compared to a single CCAP receptor in *Drosophila* and the honey bee (Fig. 3B).

4.1.11. Tc 46 and Tc 47 are AKH-like receptor genes

The insect adipokinetic hormones (AKHs) are a large family of neuropeptides that mobilize lipids and carbohydrates from the insect fat body during intense physical activities such as flight and locomotion [44]. In addition, AKHs play a critical role in carbohydrate homeostasis in *Drosophila* larvae [70,82]. Also *Tribolium* has three peptides belonging to the AKH family (Table 1). Tc 46 and Tc 47 are probably the *Tribolium* AKH receptors, because they are clear orthologues of CG11325 (Fig. 3B), which has been identified as the *Drosophila* AKH receptor [55,122,148]. Also, Tc 46 has three introns in common with CG11325 and 6 (out of 6) with Am 44, which has been identified as the honey bee AKH receptor gene [Haue and Grimmelikhuijzen, unpublished results]. Finally, the Tc 46 receptor protein has high amino acid sequence identities with the *Drosophila* (53%) and especially the honey bee (70%) receptors (Table 3).

Although Tc 47 is a clear orthologue of CG11325 in our phylogenetic tree analysis (Fig. 3B), their gene structures show less resemblance: only 1 intron (out of 7 introns) is in common. When Tc 47 is compared with the honey bee AKH receptor gene, Am 44, it only shows 3 common introns (Table 3). Despite these differences, however, there is a considerable amount of amino acid sequence identities between the Tc 47 receptor and the AKH receptors from *Drosophila* (42%) and honey bee (47%) (Table 3), clearly suggesting that Tc 47 is an AKH-like receptor.

4.1.12. Tc 48 is a bursicon-like receptor gene

Bursicon is a heterodimeric protein hormone, consisting of an α - and β -subunit, which both have cystein-knot structures [104,108,155]. The hormone is responsible for the hardening and tanning of the initially soft cuticle shortly after hatching of an adult fly from its pupa [41,42]. Furthermore, bursicon is responsible for a behavioral motor program, causing wing expansion of the newly hatched adult insect, and it initiates apoptosis of the wing epithelial cells after this wing expansion has been completed [67,85,103]. Also the *Tribolium* genome contains two genes, one coding for a bursicon- α and another for a bursicon- β subunit (Table 1).

Our research group has originally cloned four *Drosophila* GPCRs that were evolutionarily related to the mammalian glycoprotein hormone receptors. Mammalian glycoprotein hormones, to which luteinizing hormone, chorionadotropin, follicle stimulating hormone, thyroid stimulating hormone, and relaxin belong, and their GPCRs are all involved in various aspects of reproduction or development [64,79,157]. Like their mammalian counterparts, the *Drosophila* GPCRs are characterized by having a large, extracellular N-terminus containing a high number of leucine-rich repeats and were, therefore, called *Drosophila* leucine-rich repeats containing GPCRs, or DLGRs [37, 54, Bohn and Williamson, unpublished results]. Recently, one of these receptors, DLGR2 [37], has been deorphanized and identified as the *Drosophila* receptor for bursicon [104,108].

Tc 48 is a very close orthologue of the *Drosophila* DLGR2 gene (CG8930) and, therefore, likely encodes the *Tribolium* bursicon receptor (Fig. 3B). Our phylogenetic tree analysis (Fig. 3B) also shows that the *Tribolium*, honey bee, and *Drosophila* bursicon receptor proteins are highly related, suggesting a strong evolutionary pressure to conserve the structure of these three proteins. In addition, the amount of sequence identities between the three proteins is very high (85%, Table 3) and Tc 48 shares 12 introns (out of 15) with the *Drosophila* bursicon receptor gene (Table 3).

4.1.13. Tc 49 and Tc 50 are glycoprotein hormone (GPA2/GPB5)-like receptor genes

In addition to the above-mentioned established mammalian glycoprotein hormone, thyroid stimulating hormone (TSH), another, novel glycoprotein hormone has recently been found to stimulate the mammalian TSH receptor. This hormone, named thyrostimulin, is a heterodimer of two novel glycoprotein hormone subunits, named GPA2 and GPB5 [114]. Also the *Drosophila* genome has two genes, one coding for a GPA2- and another for a GPB5-cystine-knot glycoprotein-like subunit [65]. Purified recombinant *Drosophila* GPA2/GPB5 heterodimers stimulated one of our previously cloned DLGRs, DLGR1, thereby deorphanizing this *Drosophila* receptor [54,150]. The physiological roles of DLGR1 and its heterodimeric GPA2/GPB5 ligand, however, remain elusive.

Also *Tribolium* has two genes, one encoding the *Tribolium* GPA2 and one encoding the GPB5 subunit (Table 1). Furthermore, *Tribolium* has two genes, Tc 49 and Tc 50, that are the close orthologues of CG7665, which is the *Drosophila* gene coding for DLGR1 (Fig. 3B). Thus, Tc 49 and Tc 50 are likely to be the genes coding for a *Tribolium* GPA2/GPB5 receptor. Tc 49 has 12 (out of 13) introns in common, and Tc 50 has 9 (out of 12) introns in common with CG7665 (Table 3). Furthermore, the two *Tribolium* receptor proteins have high percentage of amino acid sequence identities with the *Drosophila* GPA2/GPB5 receptor (55–68%, Table 3). It is interesting

that *Tribolium* has two paralogues compared to one in *Drosophila*, suggesting an important role for the GPA2/GPB5 system in the beetle.

4.1.14. Tc 52 is an allatostatin-C-like receptor gene

The insect allatostatins are three distinct groups (-A, -B, and -C) of neuropeptides that have originally been isolated, because they could inhibit the synthesis and release of juvenile hormone by the *corpora allata*, two endocrine organs associated with the insect brain [149]. Juvenile hormone is a terpene important for insect development and reproduction. Allatostatin-C is a small cyclic neuropeptide originally isolated from the moth *Manduca sexta* (therefore it is also called *M. sexta*-type or lepidopteran allatostatin) [87] that also occurs in other insects, including *Drosophila* where its action, however, is unknown [149,163]. Also *Tribolium* has an allatostatin-C closely related to the *Drosophila* and *M. sexta* C-type allatostatins (Table 1) [149,163].

The *Drosophila* genes CG13702 and CG7285 have been identified as coding for the allatostatin-C receptors [88]. Tc 52 is a close orthologue of these two *Drosophila* genes (Fig. 3B) and, thus, likely codes for a *Tribolium* allatostatin-C receptor. Tc 52 has one intron in its coding region, which position, however, is different from those in the *Drosophila* and honey bee genes (Table 3).

4.1.15. Tc 53 and Tc 54 are allatostatin-B-like receptor genes

The allatostatic activities of B-type allatostatins were first established in crickets and these peptides are, therefore, also called cricket-type allatostatins [102]. B-type allatostatins occur in various insects, including *Drosophila* [149,164]. The B-type allatostatins are small neuropeptides with the C-terminal W(X₆)Wamide consensus sequence. In addition to their allatostatic actions in crickets, the B-type allatostatins have myoinhibitory properties in other insects, such as the locust *Locusta migratoria* and the moth *M. sexta* and are, therefore, also called MIPs (myoinhibitory peptides) [13,141]. Furthermore, the B-type allatostatins also have prothoracicostatic activity, inhibiting the release of ecdysteroids from the prothoracic gland during molting in lepidopterans [66]. Also *Tribolium* has 6 B-type allatostatins, which are all located on a single prohormone (Table 1) [100].

The *Drosophila* gene CG30106 has been identified as an allatostatin-B receptor gene [73]. This gene has a paralogue, CG14593 (Fig. 3B) that, however, has remained an orphan, although it can be assumed that also this gene codes for an allatostatin-B receptor. Tc 53 and Tc 54 are close orthologues of CG30106 and, thus, likely code for *Tribolium* allatostatin-B receptors. Tc 54 has 4 introns (out of 5) in common with CG30106. The receptor proteins encoded by Tc 53 and Tc 54 have a high percentage of amino acid sequence identities with the *Drosophila* CG30106 and CG14593 receptors (58–60%) (Table 3).

4.1.16. *Tc 57 and Tc 58 are short neuropeptide F-like receptor genes*

Short neuropeptides F (sNPFs) are short insect neuropeptides (less than 10 amino acid residues long) with the C-terminal consensus sequence $PX_1RLRX_2\text{amide}$ (where $X_1 = S, Q, M$; $X_2 = F, W$) [156]. These peptides appear to stimulate food intake and body size in *Drosophila* [93]. Also *Tribolium* has a sNPF (Table 1).

The *Drosophila* gene CG7395 has been identified as the gene coding for the *Drosophila* sNPF receptor [39,109]. Tc 57 and Tc 58 are the close orthologues of CG7395 and are likely to be the *Tribolium* genes coding for sNPF receptors (Fig. 3B). The coding regions of Tc 57 and Tc 58 have no introns just as their *Drosophila* and honey bee orthologues (Table 3). The two *Tribolium* receptor proteins share a high percentage of amino acid sequence identities with their *Drosophila* and honey bee counterparts (56–70%) (Table 3).

4.1.17. *Tc 59 is a neuropeptide F-like receptor gene*

Drosophila neuropeptide F (NPF) is a 36 residues-long peptide with the C-terminal sequence RVRFamide that resembles mammalian neuropeptide Y [17,156]. NPF has various roles in *Drosophila*: it stimulates feeding and sexual behavior, and dampens aggression [32,92]. NPF occurs in many insects other than *Drosophila*, but in *Tribolium* we were unable, so far, to identify the preprohormone gene for this peptide. At the receptor level, however, we find a clear *Tribolium* orthologue (Tc 59) of the *Drosophila* NPF receptor gene CG1147 [45] (Fig. 3B). The *Tribolium* receptor has high amino acid sequence identity (58%) with the *Drosophila* receptor. However, their genes have only 1 intron (out of 5) in common (Table 3).

4.1.18. *Tc 65 and Tc 66 are diuretic hormone 44-like receptor genes*

There are two unrelated diuretic hormone genes in *Tribolium*, one yielding DH31 (also called calcitonin-related DH or CT-DH) and one yielding two DHs (also called corticotropin-releasing-factor-related DH, or CRF-DH), originating from alternative splicing: DH37 and DH47 (Table 1) [100]. This situation in *Tribolium* is somewhat different from that in *Drosophila*, where the CRF-DH gene only yields one DH (DH44) [18]. In general, all insect DHs stimulate fluid secretion in the insect Malpighian tubules [27,28,30]. A DH-44 GPCR gene, CG8422, has been identified in *Drosophila* [75]. This GPCR gene belongs to the GPCR family B (Fig. 4). The *Tribolium* genome contains two orthologues of CG8422: Tc 65 and Tc 66 (Fig. 4). These *Tribolium* receptor genes have 6–7 introns in common with the *Drosophila* receptor gene (Table 3). Also the *Tribolium* receptor proteins have high amino acid sequence identities (53–58%) with their *Drosophila* counterpart, strongly suggesting they are DH-37/47 receptors.

4.1.19. *Tc 69 is a PDF-like receptor gene*

Pigment dispersing factor (PDF) is a circadian transmitter in the insect brain, transmitting signals from the

central circadian pacemaker neurons to downstream neurons and other cells involved in rhythmicity [152]. *Drosophila* and many other insects produce PDF [121,132], but in *Tribolium* we were unable to find a PDF gene [100]. Three research groups have independently identified the *Drosophila* gene CG13758 as the one encoding the PDF receptor [69,91,110]. Surprisingly, the *Tribolium* genome contains a gene, Tc 69, that is the clear orthologue of CG13758 (Fig. 4). This *Tribolium* gene has only 2 (out of 7) introns in common with CG13758 (Table 3). The *Tribolium* receptor protein, however, has high amino acid sequence identity (50%) with the *Drosophila* PDF receptor (Table 3).

4.1.20. *Tc 70 is a DH-31-like receptor gene*

As mentioned above, *Tribolium* has a DH-31 gene (Table 1) [100]. In *Drosophila*, the gene CG32843 has been identified as coding for a DH-31 receptor [76]. The *Tribolium* genome contains a gene, Tc 70, that is the clear orthologue of CG32843 (Fig. 4). Tc 70 has all seven introns in common with CG32843. Furthermore, the *Tribolium* receptor protein has a high amino acid sequence identity (54%) with the *Drosophila* DH-31 receptor, strongly suggesting that Tc 70 is a DH-31 receptor gene (Table 3).

4.2. *No ligands can be assigned for twenty-one neuropeptide and protein hormone GPCRs*

No ligands (highlighted in blue) can be assigned for Tc 22, Tc 23, Tc 24, Tc 27, Tc 28, Tc 36, Tc 37, Tc 39, Tc 42 (Fig. 3A), Tc 51, Tc 55, Tc 56, Tc 60–64 (Fig. 3B), Tc 67, Tc 68, Tc 71, and Tc 72 (Fig. 4). The ligand for Tc 45 (highlighted in green in Fig. 3B) is discussed in paragraph 7.

5. Neurohormone GPCR gene losses in *Tribolium* compared to *Drosophila*

Tribolium has apparently lost the tyramine GPCR gene, present in both *Drosophila* (GC7431) [23] and honey bee (Am 13) (Fig. 2). Furthermore, it has lost the kinin GPCR gene, again present in both *Drosophila* (CG10626) [131] and honey bee (Am 33) (bottom of Fig. 3A). This last loss fits very well with the absence of the kinin peptide in *Tribolium* (Table 1) [100]. *Tribolium* has also lost the corazonin GPCR gene present in both *Drosophila* (CG10698) [20] and the honey bee (Am 45) (top Fig. 3B). Again, this agrees very well with the absence of the corazonin peptide in *Tribolium* (Table 1) [100]. Surprisingly, *Tribolium* has lost the allatostatin-A GPCR gene, of which 2 copies (paralogues) are present in *Drosophila* (CG2872 and CG10001) [10,90,95–97] and one copy in the honey bee (Am 30) (middle of Fig. 3B). Also this fits very well with the absence of allatostatin-A peptides in *Tribolium* (Table 1) [100].

Fig. 5. Upper panel. Phylogenetic tree analysis of the Tc 43–Tc 46 receptor proteins, the mouse oxytocin (OT) receptor, the mouse vasopressin-1a (V1a) receptor, and the *Drosophila* AKH receptor (CG11325), corazonin receptor (CG10698), and CCAP receptor (CG6111) proteins. The analysis clearly shows that the Tc 45 protein is more closely related to the mammalian vasopressin and oxytocin receptors than to its most closely related insect receptor proteins, suggesting that it is a vasopressin/oxytocin receptor. Lower panel. That the Tc 45 protein is a vasopressin receptor is supported by the presence of a clear vasopressin-like peptide in *Tribolium*, whereas an oxytocin-like peptide is absent (Table 1) [100].

Fig. 6. A schematic representation of the evolution of the vasopressin/receptor couple in holometabolous insects. The vasopressin/receptor couple (highlighted in red) is present in primitive holometabolous insects and continues to be so in Coleoptera. In the evolutionary line leading to Hymenoptera, this hormonal system has been abandoned (indicated by the dead-end sign) and the same event has occurred in the line leading to Lepidoptera and Diptera (again indicated by a dead-end sign).

and its receptors play a crucial role in water reabsorption in the kidneys [6,47,86]. It is possible that the vasopressin/receptor couple plays a similar antidiuretic role in *Tribolium*. A strict regulation of water homeostasis (water reabsorption) is essential for *Tribolium* to survive its very dry ecological niche.

A vasopressin receptor has, so far, never been found in insects or other arthropods. A vasopressin peptide, however, with an identical structure as the *Tribolium* vasopressin-like peptide (Fig. 5, lower panel) has previously been isolated from the locust *Locusta migratoria* [130]. *Locusta* is a hemimetabolous insect and it is interesting that, like *Tribolium*, it is adapted to a relatively dry ecosystem. However, a physiological role of this insect vasopressin in water homeostasis in *Locusta* has never been proven [29].

Vasopressin also occurs in other invertebrates, such as annelids and molluscs and vasopressin receptors have also been cloned and characterized from these animals [78,98,154]. These data show that the vasopressin/receptor couple is a very ancient neurohormonal system that has evolved before the split of Proto- and Deuterostomia, 640–760 million years (MYR) ago [33].

We have tried to understand the evolutionary events that have led to the presence of the vasopressin system in *Tribolium*, but to its absence in all the other holometabolous insects with a sequenced genome (Fig. 6). In our opinion, the ancestral insects did possess the vaso-

Fig. 7. A similar schematic representation as in Fig. 6, but now highlighting the corazonin/receptor couple (in red). Ancestral holometabolous insects have this hormonal system, which continues to exist in Hymenoptera, Lepidoptera, and Diptera, but which is abandoned in the evolutionary line leading to modern Coleoptera.

pressin system, but during holometabolous insect evolution it has been lost at least two times (see dead-end signs in Fig. 6).

Another striking difference between *Tribolium* and honey bee/*Drosophila* is the clear absence of certain hormonal systems. For example, corazonin has always been regarded as a very essential neuropeptide, being important for the initial steps of insect moulting by releasing ETH from the tracheal Inka cells, thereby triggering ecdysis [81,171]. Corazonin has been found in nearly all insect orders except for the Coleoptera [129]. Also, the corazonin receptors have been identified in *Drosophila*, *Anopheles*, and the honey bee (Fig. 3B) [9,20,57]. *Tribolium*, however, can apparently live without the corazonin system, as both corazonin and its receptor are lacking (Table 1; Fig. 3B, top) [100]. How is this possible?

The correct answer is currently difficult to give, but it could be that certain evolutionarily related receptor groups overlap in their physiological actions. For example, the insect CCAP, corazonin, vasopressin, and AKH receptors (top Fig. 3B) have apparently originated from one ancestral receptor, having one ancestral ligand. This ancestral hormonal system could have been responsible for a palette of physiological processes. After gene duplications of the ancestral receptor and its ligand, specializations could have occurred, leading to a better control of the various physiological processes, but certain overlaps could still have persisted. When, as in *Tribolium*, numerous receptor duplications have occurred (both the CCAP and AKH receptors have been duplicated compared to *Drosophila* and the

honey bee and also a vasopressin receptor is there, while absent in *Drosophila* and honey bee), a fourth hormone system (corazonin) might have become superfluous and no evolutionary pressure might have existed to preserve it. In other words: one of the *Tribolium* CCAP, vasopressin, or AKH receptors might have overtaken the role that corazonin receptors have in other (non-coleopteran) insects.

Fig. 7 tries to depict the evolutionary events that have led to the absence of the corazonin/receptor couple in *Tribolium* (Coleoptera), but to its presence in all the other orders of the Holometabola. Figs. 6 and 7 are clearly two extremes. We can find other examples in Figs. 3 and 4 of such extremes, for example the absence of the allatostatin-A/receptor couple in *Tribolium*, but its presence in members of the other orders of Holometabola (a situation as depicted in Fig. 7) [10,90,94,97,149]. Again, the allatostatin-A/receptor system has an ancient evolutionary origin and it is present in the ancestor groups of insects, such as crustaceans [149].

We have always been amazed and puzzled why proctolin and its receptor are present in *Drosophila* [36,74], but absent in the honey bee, *Anopheles*, and *Bombyx* [57]. We can now see that the proctolin/receptor couple is, again, present in *Tribolium* (Tc 25, top of Fig. 3A). This finding, therefore, is a somewhat complex variant of Fig. 7, where the evolutionary lines leading to the Hymenoptera, Lepidoptera, and mosquitoes have abandoned the proctolin hormonal system, whereas it has been conserved in the other groups of holometabolous insects. Also the proctolin/receptor couple is an ancient hormonal system already occurring in crustaceans [117,147], but this does apparently not hamper the Hymenoptera, Lepidoptera and mosquitoes to give up the system.

There are two lessons that we can learn from our survey of hormonal systems in holometabolous insects (Figs. 2–4). First, neuropeptide/receptor couples can easily duplicate or disappear during insect evolution. Second, *Drosophila* is not a good representative of all insects, because several of the hormonal systems that we now find in *Tribolium* (for example, vasopressin and its presumed receptor Tc 45) do not exist in *Drosophila*. To understand insect endocrinology, therefore, it is crucial that several insects are studied in detail. It is clear that those insects should be chosen for which a sequenced genome is or will be available (Fig. 1). These insects will be our future models for which, also, the necessary genetic tools will be developed.

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