Chapter

Neuropeptide Biology in *Drosophila*

Elke Clynen, Ank Reumer, Geert Baggerman, Inge Mertens and Liliane Schoofs*

Abstract

Drosophila melanogaster is since decades the most important invertebrate model. With the publishing of the genome sequence, *Drosophila* also became a pioneer in (neuro)peptide research. Neuropeptides represent a major group of signaling molecules that outnumber all other types of neurotransmitters/modulators and hormones. By means of bioinformatics 119 (neuro)peptide precursor genes have been predicted from the *Drosophila* genome. Using the neuropeptidomics technology 46 neuropeptides derived from 19 of these precursors could be biochemically characterized. At the cellular level, neuropeptides usually exert their action by binding to membrane receptors, many of which belong to the family of G-protein coupled receptors or GPCRs. Such receptors are the major target for many contemporary drugs. In this chapter, we will describe the identification, localization and functional characterization of neuropeptide-receptor pairs in *Drosophila melanogaster*.

Introduction: *Drosophila* as a Model to Study Neuropeptide Signaling

Drosophila has revolutionized biology more than any other organism. The entire genus contains about 1,500 species and is very diverse in appearance, behavior and breeding habitat. One species in particular, *Drosophila melanogaster*, has been heavily used in research in genetics and developmental biology. Also for neuropeptide research *Drosophila* is a very suitable model organism, especially since its genome has been nearly fully sequenced and is publicly accessible.[1]

Neuropeptides form the largest class of signaling molecules in animals. They transmit and regulate bio-information in the circulatory as well as the neuronal system and exert their role mostly by acting on G-protein coupled receptors or GPCRs. As such, neuropeptides play critical roles in regulating most biological processes. Neuropeptides are diverse in structure, localization and function. Their only common feature is that they are all synthesized as peptide precursor proteins, also called preproproteins. Besides the neuropeptides themselves, also the receptors they act on are structurally diverse and the resulting signaling cascades are also highly varied, so there is a tremendous potential of different effects on living cells. Therefore, peptides are attractive for pharmaceutical and agro-industrial companies because they represent (lead) compounds that can be further exploited for diverse practical applications. Peptides as such cannot be used as therapeutics or as insecticides, because they are usually broken down before they reach their target. Therefore, small compounds called peptidomimetics that mimic or block the interaction of the peptide with its receptor are being developed. As more genomes become available, the findings in *Drosophila* can be readily expanded to other species, including those of economic interest.

*Corresponding Author: Liliane Schoofs—Department of Animal Physiology and Neurobiology, K.U. Leuven, Naamsestraat 59, 3000 Leuven, Belgium. Email: liliane.schoofs@bio.kuleuven.be

Neuropeptide Systems as Targets for Parasite and Pest Control, edited by Timothy G. Geary and Aaron G. Maule. ©2009 Landes Bioscience and Springer Science+Business Media.

In this chapter we will successively discuss the prediction of neuropeptides from the genome by specialized bioinformatics programs, their biochemical characterization by neuropeptidomics, deorphanization of neuropeptide GPCRs and localization and functional characterization of neuropeptides in *Drosophila*.

Bioinformatics

The *Drosophila melanogaster* genome sequence was published in 2000.[1] Currently, twenty-two other *Drosophila* species are undergoing or have completed whole genome shotgun sequencing. These genome sequencing projects gave a new impulse to (neuro)peptide research, as putative peptides can now be mined from the genome. This, however, presents a major challenge as neuropeptide precursors share little common features. Precursors encoding multiple structurally related peptides, as well as precursors encoding multiple, unrelated peptides and precursors encoding just a single bioactive peptide occur. The only common feature is the presence of an amino-terminal signal peptide that directs the ribosomes synthesizing neuropeptide precursors to the endoplasmic reticulum (ER). However, this is not a unique criterion as all proteins synthesized and sorted in the secretory pathway possess such a signal peptide. In the ER, the precursor is posttranslationally processed into bioactive peptides by a series of enzymatic steps. A typical feature for neuropeptide precursors is the presence of cleavage sites. Neuropeptides are typically cleaved from the precursor at dibasic sites. However, not all dibasic sites are actually used as a cleavage site and other, unconventional sites also occur.

Two motif-finding programs, MEME and Pratt, were used to search for common motifs in all known neuropeptide precursor proteins from *Drosophila*.[2] No general pattern or motif was found, only a very degenerative one in the area of the signal peptide sequence. Only in smaller subpopulations of datasets, common motifs could be found, corresponding to the conserved sequences of various peptide families. These conserved sequences mostly correspond to the biologically active core that interacts with the receptor proteins that mediate their action.

Based on sequence similarity to known peptide genes from other organisms, the *Drosophila melanogaster* genome was screened by means of BLAST (Basic Local Alignment Search Tool) analysis. This way, 43 peptide precursors could be annotated.[3,4] Peptide precursor genes are, however, poorly predicted by the BLAST algorithm as it is not very efficient for finding similarity to short sequences when they are scanned against the whole genome sequence. Most peptide precursors are between 50 and 500 amino acids in length and in general only a small part of the precursor consists of the actual active peptide(s). Also, putative peptide sequences for which no orthologous peptide has been identified will not be revealed this way.

Recently, an alternative searching program was developed to scan predicted proteins for the structural hallmarks of a neuropeptide precursor.[5] This program started from a protein database of *D. melanogaster* and selected all proteins less than 500 amino acids in length that contain an amino-terminal signal peptide. The resulting 5096 proteins were, after removal of the signal sequence, in silico split into short subsequences at cleavage sites typical for neuropeptide precursors. A second database comprised all known peptide precursor subsequences from Metazoa known to date. These were also split into subsequences. Next, a BLAST analysis was conducted on these two databases. Because similarity not necessarily implies homology, the output was further screened. The resulting proteins had to comply to one of following criteria: or the proteins had to contain at least two similar subsequences (based on the principle that multiple peptides encoded by a single invertebrate peptide precursor gene are often highly related) or they should contain a well-conserved motif (these putative peptide precursor genes encode multiple nonrelated peptides or only a single putative peptide). In addition, the motif should be close to a cleavage site. Motifs for neuropeptides in Metazoans have recently been catalogued.[6] In this way, 76 additional putative secretory peptide genes were predicted, which brings the total of predicted peptide precursor genes in *Drosophila melanogaster* to a total of 119.

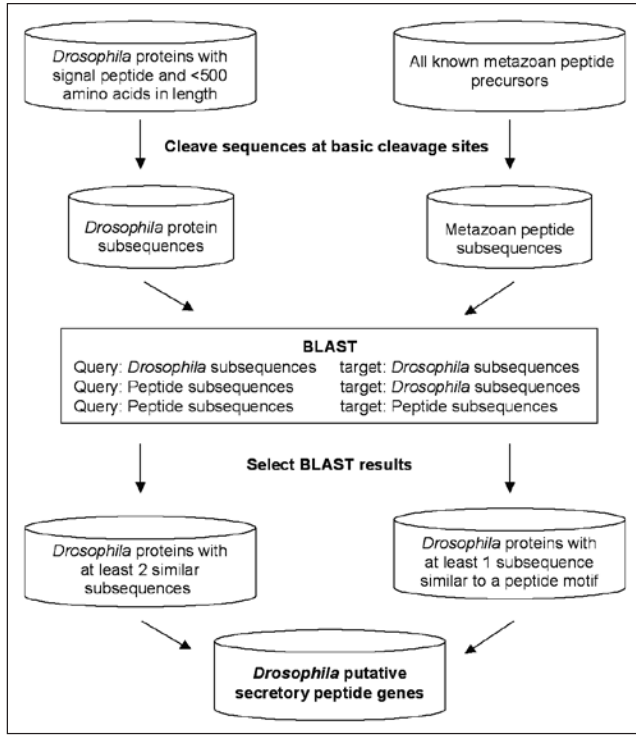

Figure 1. Schematic representation of the in silico neuropeptide searching program developed by Liu et al.[5]

Neuropeptidomics

Bioinformatic predictions do not reveal which peptides are ultimately expressed. Conventional cleaving sites are not always used and there have also been reports on unconventional cleaving sites, meaning that it is hard to reveal the sequences corresponding to the biologically active peptides starting from the precursor sequence. Moreover, the processing of a common precursor can differ during development or between tissues. And, the nucleotide sequence does not give information about posttranslational modifications, which are often essential for neuropeptide stability and activity. Therefore, a biochemical characterization of neuropeptides remains necessary. This sequence information used to arrive slowly, due to the huge efforts required for tissue collection and purification to ultimately isolate and sequence a peptide, until in 2001 the concept of peptidomics was introduced. Peptidomics presents a global strategy, by which all peptides present in a biological sample that can be derived from a cell, tissue, body liquid or even the whole organism, are simultaneously visualized and identified.[7-9] Peptidomics complements proteomics, the study of proteins, which are long chains of amino acids. There are several possible peptidomic methods, all based on mass spectrometry. The most common tool is a combination of nanoscale liquid chromatography, tandem mass spectrometry and database mining, which allows the detection and sequencing of low concentrations of peptides from complex mixtures with a high degree of automation. This way, the *Drosophila* neuropeptidome was investigated starting from only 50 larval CNS, allowing the identification of 47 neuropeptides derived from 19 different precursors (Table 1).[10-12] Only 7 of these peptides had been biochemically characterized before. Four of the precursors were not identified or predicted as neuropeptide precursor before.

Table 1. List of neuropeptides identified in Drosophila melanogaster[10,11]

Peptide	Sequence
FMRFamide	
FMRFamide 1	DPKQDFMRFa
FMRFamide 2	TPAEDFMRFa
FMRFamide 3	SDNFMRFa
FMRFamide 4	PDNFMRFa
FMRFamide 5	SVQDNFMHFa
FMRFamide 6	MDSNFIRFa
MS (myosuppressin)	
MS	TDVDHVFLRFa
MS^{2-9}	DVDHVFLRFa
SK (sulfakinin)	
SK 1	FDDY(SO_3)GHMRFa
SK 2	GGDDQFDDY(SO_3)GHMRFa
sNPF (short neuropeptide F)	
sNPF 1	SPSLRLRFa
sNPF-AP	SDPDMLNSIVE
sNPF 2^{1-10}	WFGDVNQKPI
AKH (adipokinetic hormone)	
AKH	pQLTFSPDWa
CRZ (corazonin)	
Crz	pQTFQYSRGWTNa
Crz^{3-11}	FQYSRGWTNa
AST (allatostatin)	
AST 2^{1-11}	AYMYTNGGPGM
AST 3	SRPYSFGLa
AST 4	TTRPQPFNFGLa
MIP (myoinhibiting peptide)	
MIP 2	AWKSMNVAW
MIP 5	DQWQKLHGGWa
DIM 2 (immune induced peptide 2)	
Dim 2	GNVVINGDCKYCVNGa
DIM 4 (immune induced peptide 4)	
Dim 4	GTVLIQTDNTQYIRTa
CAPA (cardio acceleratory peptide)	
Cap 1	GANMGLYAFPRVa

continued on next page

Table 1. Continued

Peptide	Sequence
Cap 2	ASGLVAFPRVa
MT	TGPSASSGLWFGPRLa
$MT^{2\text{-}15}$	GPSASSGLWFGPRLa
LK (leucokinin)	
LK	NSVVLGKKQRFHSWGa
LK-AP	SPEPPILPDY
TK (tachykinin)	
TK 1	APTSSFIGMRa
TK 2	APLAFVGLRa
TK 3	APTGFTGMRa
TK 4	APVNSFVGMRa
TK 5	APNGFLGMRa
HUG (hugin)	
MT 2	SVPFKPRLa
IFamide	
IFamide	AYRKPPFNGSIFa
NPLP1 (neuropeptide-like precursor 1)	
NAP	SVAALAAQGLLNAP
MTYamide	YIGSLARAGGLMTYa
VQQ	NLGALKSSPVHGVQQ
IPNamide	NVGTLARDFQLPIPNa
GVQ	GALKSSPVHGVQ
NPLP2 (neuropeptide-like precursor 2)	
NEF	TKAQGDFNEF
LTK	EESNPAQEFLTK
KLK	AQGDFNEFIEKLK
NPLP3 (neuropeptide-like precursor 3)	
SHA	VVSVVPGAISHA
VVIamide	SVHGLGVVIa
NPLP4 (neuropeptide-like precursor 4)	
YSY	pQYYYGASGGYYDSPYSY

The lack of identification of more (predicted) peptides may be due to several reasons. First, their concentration in the conditions used may be below the sensitivity of the instrumental setup. In this respect one has to consider that the concentration of a peptide may vary during development and is dependent on the physiological condition of the organism(s). Second, not all peptides are extracted or ionized with the same efficiency. Third, in contrast to peptides obtained after tryptic digestion of a protein, endogenous peptides in most cases do not contain a basic amino acid at their

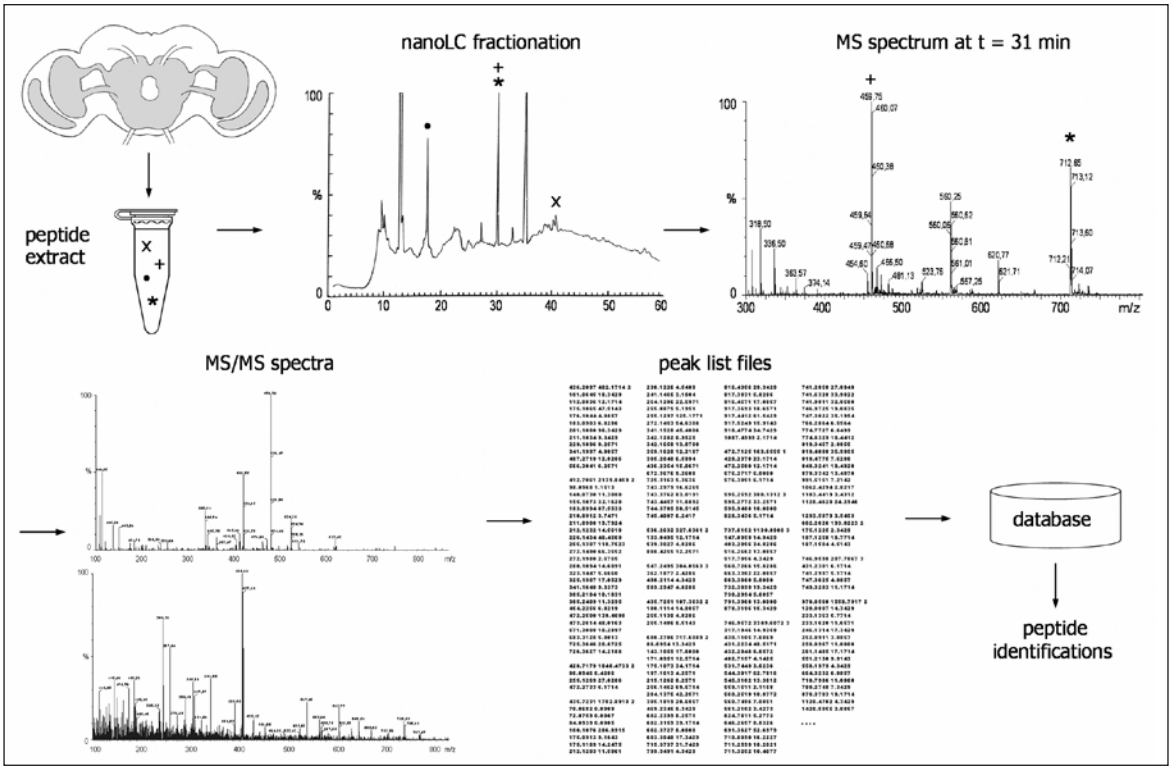

Figure 2. Flowchart of the neuropeptidomics setup used to analyze the peptidome of the *Drosophila* central nervous system. Nervous tissue was homogenized and the extract was separated on a nanoLC system directly coupled to a Q-TOF mass spectrometer. At each moment in the LC run, the mass spectrometer software uses the MS spectrum to decide on peak selection for fragmentation. Typically, ions with 2-4 positive charges are automatically selected for MS/MS analysis through collision induced dissociation (CID). The obtained fragmentation spectra are converted to peak list files, which are then used for querying a database in order to identify the peptides.[8]

C-terminus and will therefore yield fragmentation spectra that are hard to interpret. Fourth, the presence of a putative peptide sequence derived from the genome sequence does not necessarily mean that this peptide is present in vivo. Fifth, the predicted peptides may be present in other tissues than the ones analyzed so far.

Deorphanization of *Drosophila* Neuropeptide GPCRs

Most neuropeptides interact with G-protein coupled receptors (GPCRs), thereby generating an intracellular response.[13] G protein-coupled receptors constitute the largest family of cell surface proteins. They have a typical 7 transmembrane spanning structure and upon ligand activation, they signal via G proteins, composed of an α, β and γ subunit. The α- and βγ-subunits dissociate from each other and separately activate several classical effectors, including adenylyl cyclases and phospholipases and regulate the activity of ion transporters, several kinases and ion channels. GPCRs can be stimulated by a diverse array of external stimuli, including bioactive peptides, chemoattractants, neurotransmitters, hormones, phospholipids, photons, odorants and taste ligands.[14] Based on shared sequence motifs, all GPCRs are categorized into six subfamilies or classes.[15] Neuropeptide GPCRs all belong to the rhodopsin-like (class A) or the secretin-like (class B) subfamily (Table 2).

In 2003, seven of the twenty most prescribed drugs interact with GPCRs, representing a total sales number of 14.3 billion US dollars.[16] Overall, GPCRs are the molecular target for over 30% of all currently marketed drugs, making the GPCR superfamily one of the most valuable target molecules for drug development.[17] Likewise, GPCRs can also be used as pesticide targets in agricultural applications.

Because the structure and function of most neuropeptide GPCRs is conserved in the phylum of the Arthropoda, *Drosophila* neuropeptide GPCRs can be used to find new lead compounds that bind the receptor, thereby inhibiting their function. The identified lead compounds can then be further engineered to real pesticides. However, to reach that goal, it is important that all *Drosophila* GPCRs are linked with their naturally occurring bioactive ligand and that the function(s) of this receptor-ligand pair is revealed. Only then, neuropeptide GPCRs can be selected as candidates for pest control.

Since the publication of the *Drosophila* genome, a large number of orphan GPCRs were characterized (Table 2), but about half of the neuropeptide GPCRs in *Drosophila* remain orphan to date because deorphanization is not straightforward for some GPCRs.[1] When a cellular assay platform is used, several difficulties can hinder the deorphanization process. These can be situated on the level of cloning, expression or signal transduction pathway. An overview of all cellular assays that can be used in receptor deorphanization is given in Mertens et al.[18] Although cell-based assays are adequate for many GPCRs and even new GPCR biosensors and imaging technologies have recently been developed that hold promise for the development of functional GPCR screens in living cells, it is likely that these cell-based formats will limit the development of higher density GPCR assays.[19] Therefore it is not surprising that recently the focus is on further miniaturized assays, ultra-high throughput assays and, eventually microarray/bioship assay formats. Stable, robust, cell-free signaling assemblies comprising receptor and appropriate molecular switching components will form the basis for such future GPCR assay platforms.[20]

Functional Role of Neuropeptides: Localization, Reverse Genetics and Bioassays

Localization

The CNS of *Drosophila* larvae contains approximately 10000 neurons, about 200 of which are peptidergic. The majority of neuropeptides has been demonstrated in brain and VNC interneurons of various types and/or neurosecretory cells. The latter are cells that have axon terminations in neurohemal release sites in contact with the circulation system. In addition, they commonly have varicose processes in the brain, which could act as interneuronal segments.

Table 2. List of characterized and/or predicted G-protein coupled receptors and corresponding ligands in Drosophila: original classification by Hewes and Taghert and update.[4] The receptors depicted in italic were annotated incorrectly and the ones depicted in bold were not annotated at all.

Gene	Receptor	Ligand(s)	Year	Reference
Family A/Group II-B: gastrin/cholecystokinin receptors				
CG6881/CG6894	DSK-R1	Drm-SK-1 and -2	2002	57
CG6857	orphan			
CG14593	orphan			
CG30340	orphan			
Family A/Group III-B: neurokinin receptors				
CG10626	DLKR	Dromyokinin/drosokinin	2002	58
CG6515	DTK-R	DTK	2003	59
CG7887	DTK-R	DTK	2003,2005	59,60
CG8784	Drm-PK-2 receptor	Drm-PK-2	2003	61
CG8795	Drm-PK-2 receptor	Drm-PK-2	2003	61
CG10823	SIFa-R	Drm-SIFa	2006	62
Family A/Group III-B: neuropeptide Y receptors				
CG1147	NPFR-1	NPF	2002	63
CG7395	sNPF-R	Drm-sNPF-1, -2, -3 and -4	2002	64,65
CG5811	orphan			
CG12610	orphan			
CG13995	orphan			
Family A/Group III-B: growth hormone secretagogue, neurotensin, neuromedin U and thyrotropin releasing hormone receptors				
CG2114	FMRFaR	Drm-FMRFamide 1-8	2002	66
CG14575	Capa-1 and -2 receptor	Drm-capa-1 and -2	2002	67,68
CG5911-A and -B	ETH receptors	Drm-ETH-1 and -2	2002	69
CG6986	*Proctolin receptor*	*Proctolin*	*2003*	70
CG8985	*DMS-R*	*Drome-MS*	*2003*	71,59
CG13803	*DMS-R*	*Drome-MS*	*2003*	71,59
CG9918	*pyrokinin-1*	*PK-1*	*2005*	72
CG33639 (CG5936)	orphan			
CG13229	orphan			
CG13575	orphan			
CG14003	orphan			

continued on next page

Table 2. Continued

Gene	Receptor	Ligand(s)	Year	Reference
CG16726	orphan			
Family A/Group V: galanin/allatostatin and opoid/somatostatin receptors				
CG2872	AlstR-2/DAR-1	Drostatin-1	1999	73,74
CG10001	AlstR-2/DAR-2	Drostatin-2	2001	73,74
CG7285	Drostar-1	Drostatin-C	2002	75
CG13702	Drostar-2	Drostatin-C	2002	75
CG14484 (CG30106)	AstB-R	Ast-B	2003	59
CG4313	orphan			
Family A/Group V: gonadotropin releasing hormone, vasopressin and oxytonin receptors				
CG11325	AKH-R1	Drm-AKH	2002	68,76
CG10698	Cor-R	Drm-COR	2002	68,77
CG6111/ CG14547	CCAP-R	Drm-CCAP	2003	78
Family A/Group V (type Ic): glycoprotein hormone receptors				
CG8930 (DLGR-2)	LGR-2 (rk)	bursicon	2005	79,80
CG7665	DLGR-1	GPA2/GPB5	2005	81
CG4187	orphan			
CG5042 (CG31096/ DLGR-3)	orphan			
Family A: unclassified orphan receptors				
CG3171	**orphan**	**neuropeptide?**		
CG4322	**orphan**	**neuropeptide?**		
CG12290	**orphan**	**neuropeptide?**		
Family B/Group I: calcitonin and diuretic hormone receptors				
CG8422	CG8422	DH44	2004	82
CG13758	PDF-R	PDF	2005	83,84,85
CG17415	DH31-R	DH31	2005	86
CG4395	orphan			
CG12370	orphan			
Family B: methuselah-like receptors				
CG6936	**Mth receptor**	**Sun A and B**	**2004**	87
Family B: unclassified orphan receptors				
CG11318	**orphan**	**neuropeptide?**		
CG8639 (CIRL)	**orphan**	**neuropeptide?**		
CG15556	**orphan**	**neuropeptide?**		

The localization of a neuropeptide reveals whether it plays a role as circulatory hormone (present at neurohemal release sites) or as neuromodulator (present in interneurons) and possibly cotransmitter (colocalized with a classical neurotransmitter). Double-immunolabeling experiments have shown that neuropeptides are often colocalized with conventional fast-acting transmitters, like GABA, biogenic amines and nitric oxide that act on an ion-channel-type of receptor. The localization is also often essential for the design of experimental approaches to determine the function.

Representatives of most of the characterized neuropeptide families have been localized in *D. melanogaster*. Each neuropeptide precursor displays a unique neuronal distribution pattern.[21] Some neuropeptide precursor genes encode multiple peptides, which mostly seem to be co-expressed. Commonly, each type of neuropeptide is localized to a relatively small number of neurons, typically a specific subset of 6 to 20 cells. Two or more different neuropeptides can be present in partly overlapping cell populations.

For a precise and comparable morphological description of peptidergic neurons, one needs an anatomical reference system. The segmental nerves and regularly distributed transverse and longitudinal fasciclin-2 expressing fibers provide a convenient 3D-coordinate system in which peptide neurons can be mapped using peptide-specific GAL4-driven expression of GFP markers or immunostaining.[22] This system also enables to identify presynaptic neurons providing inputs onto peptidergic interneurons and neurosecretory cells.

Neuropeptides are produced by a series of enzymatic steps that sequentially cleave and further modify larger precursor molecules. The synthesis and secretion of neuropeptides has to be strictly regulated in order to properly execute a complex behavior. All factors that are involved in the regulation hereof represent possible targets for parasite and pest control. Therefore, (co)localization studies that map proteins critical for neuropeptide signaling, including transcription factors regulating cell-specific neuropeptide expression, peptide-processing enzymes, G-protein coupled receptors and neurosecretory proteins that are required for exocytosis, are important.

There are several possible tools to study the localization of a neuropeptide. The most popular being immunocytochemistry and in situ hybridization. The latter localizes the precursor mRNA and hence gives no information on the translated peptides.

In *Drosophila*, peptidergic neurons can also be easily identified without staining methods based on the GAL4/UAS binary system. The GAL4 protein activates transcription of only those genes containing GAL4 binding sites or upstream activating sequences (UAS). When a certain promoter (or enhancer) directs expression of the transcriptional activator GAL4 in a particular pattern, GAL4 in turn directs transcription of the GAL4-responsive (UAS) target gene in an identical pattern. This system can be used to visualize neuropeptide neurons, using the promoter of a certain neuropeptide precursor gene to drive GAL4 expression and a UAS—reporter gene to reveal the pattern. The green fluorescent protein (GFP) is often used as reporter, as its expression can be visualized by virtue of its natural fluorescence in live specimens throughout development. The GAL4/UAS system is often used in combination with immunostaining to perform colocalization studies. It does, however, not reveal posttranslational (differential) processing of the precursor.

Also mass spectrometry has become an important tool to study the localization of neuropeptides. It enables the rapid and accurate identification of the almost complete neuropeptide identity profile from small numbers of tissues, cell groups or even single cells. Thereby it can confirm, refine and extend data from immunostaining. While using immunostaining only one (or a few) peptide(s) can be studied at a time, mass spectrometric profiling identifies the complete neuropeptide profile of a single neuron, nerve or neurohemal organ at a given moment. This gives us more insight in the coordinated action of neuropeptides. Also the problem of cross-reactivity between structurally related peptides inherent to immunostaining is not posed by MS methods. Here, neuropeptides that differ by only one amino acid (peptide isoforms) can be easily distinguished based on their exact mass and also posttranslational modifications can be identified. This way, one can uncover differential peptide processing and get new insights into the posttranslational processing of peptide precursors.

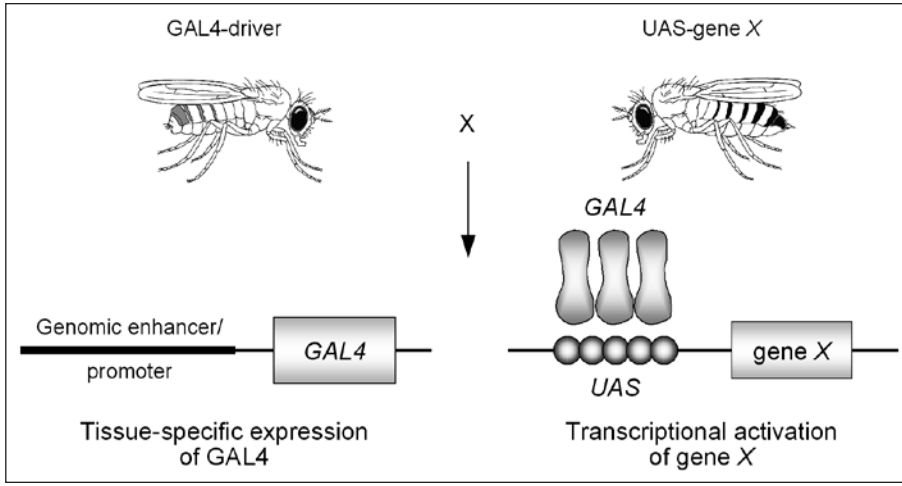

Figure 3. GAL4/UAS system: flylines are generated in which the expression of a transcriptional activator of yeast, the GAL4 protein, is under the influence of different genomic enhancers or promoters. In this way, a plethora of different spatial and/or temporal patterns of GAL4 expression is available. This transcription factor acts on a GAL4 responsive promoter or 'upstream activating sequence' UAS. Flylines can be generated in which any DNA sequence is placed downstream of a UAS sequence. The cross between a transgenic UAS line and a GAL4 line can thus result in spatio-temporal controlled GAL4 driven expression of this sequence.

The peptidome of the major neurohemal organs, i.e., specialized organs where the hormonal release of neuropeptides takes place, of *D. melanogaster* was characterized by direct MALDI-TOF mass spectrometry.[23,24] Peptides present at these sites might classify as (putative) hormones. The ring gland, dorsal sheath of the ventral nerve cord, the epitracheal organs, thoracic perisympathetic organs and abdominal transverse nerves 1-3 were studied. All detected masses up to 2.5 kDa in the neurohemal organs could be assigned to bioactive neurohormones or intermediates of prohormone processing.

Recently, the first single-cell mass analysis in *Drosophila* has been reported. The large (± 20 μm) lateral ventral neurons of the optic lobes (LNvs), known to express the neuropeptide pigment-dispersing factor (PDF), were visualized by (UAS) GFP-expression using pdf-GAL4 flies and subsequently isolated from adult flies and directly analyzed by MALDI-TOF MS.[25] The resulting spectra showed strong mass signals for PDF, but no evidence for other colocalized neuropeptides. The same single cell analyses were performed in larvae for the (smaller) hugin-neurons (± 15 μm), which are expressed in a group of neurosecretory cells of the suboesophageal ganglion.[26]

Reverse Genetics and Bioassays

The advantage of *Drosophila* as a model system for neuropeptide research is that a lot of genetic tools are already available and continuously new techniques are being developed that can be used to probe the in vivo function of neuropeptide precursor genes. For many years the emphasis has been on a forward genetic approach to unravel the function of genes that were discovered on the basis of their mutant phenotype.[27] However, since the publication of the genome sequence of *Drosophila*, the development of reverse genetic methods to search for a gene's function, starting from its DNA sequence and its location in the genome, has boosted. These reverse genetic approaches can be divided into two groups.

The first group focuses on the acquisition of mutations in specific genes of interest by means of modified forward genetic screens. Chemical mutagenesis, making use of DNA-damaging agents like ethyl methanesulphonate (EMS) and ethylnitrosourea (ENU), as well as transposable ele-

ment mutagenesis are often the workhorse of this class.[28] Since the development of a *P*-element transgenesis method to restore the wild type function of the *rosy* gene by Rubin and Spradling in 1982, the *P*-element has become the most utilized transposable element for studying gene function in *Drosophila melanogaster*.[29,30] This enormously powerful tool unfortunately suffers from some constraints, like the *P*-elements target site specificity that makes it difficult to obtain insertions in some regions of the genome called "cold spots". As an alternative, one can either make use of the *Drosophila melanogaster hobo* elements, although they are a rarely used transposon, or one can utilize transposable elements from other species that have been adapted for use in *Drosophila*, like the Lepidopteran derived *piggyback* elements, the housefly-derived *Hermes* element and the *Minos* and *mariner* elements from *Drosophila hydei* and *Drosophila mauritiana* respectively.[31]

The second group of reverse genetic approaches emphasizes on the altering of the function of a gene of interest. The main strategies in this group are site-specific recombination and gene targeting.

The principle of site-specific recombination relies on the discovery of a class of enzymes that recognize specific DNA sequences and carry out reciprocal recombination between two copies of that sequence. They were subsequently called site-specific recombinases.[32] In *Drosophila*, the FLP recombinase from the yeast *2μ* plasmid and the Cre recombinase from the P1 bacteriophage are the two most used site-specific recombinase enzymes.

Gene targeting can be divided into two main strategies: targeted gene replacement by homologous recombination and RNA interference. For targeted gene replacement, the homing endonuclease enzyme strategy is used. These enzymes recognize and cut at a specific and long sequence in double stranded DNA leaving behind a double stranded break (DSB). This method makes it possible to precisely modify an endogenous gene sequence by homologous recombination between an introduced DNA fragment and the homologous target gene. In this way, mutations that reduce, destroy or alter a gene's function in a defined manner can be made or even a complete replacement of the endogenous gene with for example a marker gene can be obtained. In *Drosophila*, the I-*Sce*I and the I-*Cre*I endonucleases are used.[33,34]

Andrew Fire and Craig Mello (1998) were the first to describe their observation of the endogenous RNA interference (RNAi) mechanism for which they were awarded (2006) with 'The Nobel Prize in Physiology or Medicine'.[35] The RNA interference process is initiated by the presence of double stranded RNA (dsRNA) which induces an efficient sequence-specific silencing of gene expression. In *Drosophila*, RNAi can be used for cultured *Drosophila* cells as well as for in vivo experiments. The first in vivo experiments indicated that upon injection dsRNA could be a strong antagonist of gene function in precellular embryos.[36] To overcome the limitations associated with dsRNA injection e.g., the repetition of the injection regarding the quantity, the location and timing of the injection; methods were developed to stably express dsRNA in vivo. Hereby, the place and time of expression can be controlled using the GAL4/UAS binary system. A general overview can be found in '*Drosophila*: a laboratory handbook' written by Scott Hawley, Kent Golic and Michael Ashburner.[37] Although most methods have proven to be effective, they all have their limitations owing to their specific experimental approach.

In a short overview we will demonstrate the use of these reverse genetic approaches in *Drosophila* neuropeptide research.

Transposon mutagenesis can be seen as the umbrella upon which the use of most of the reverse genetic approaches relies, chemical mutagenesis excluded. The hunt for the in vivo functional characterization of a gene mostly starts with a survey of the extensive collections of transposable element insertions in search for hits into or near a gene of interest. Flybase maintains links to the websites with information about these collections. When this survey does not give the desired outcome, one can make use of a collection of *P*-element (or other transposon) transformation vectors, which make it possible to bring any desired piece of DNA into the fly's genome.

Mobilization and imprecise excision of existing *P*-element insertions have already proven to be useful for the elucidation of the function of the pigment dispersing factor or *pdf* and the ecdysis-triggering hormone or *eth* neuropeptide genes. Mobilization of a *P*-element residing in the

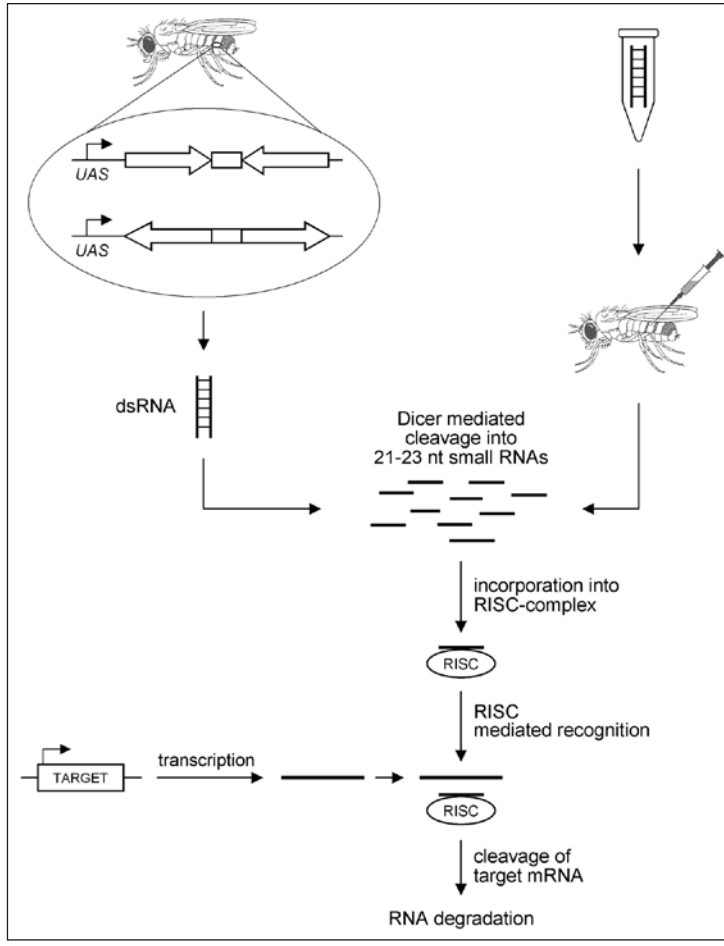

Figure 4. The RNA interference mechanism in the fruitfly. Double stranded RNA can be delivered into *Drosophila* by injection of in vitro transcribed RNA (upper right), or by stable expression of dsRNA in vivo through GAL4 driven expression of inverted repeats (upper left). The ribonuclease Dicer acts upon this dsRNA trigger and cleaves it into 21-23 nt small RNAs.[52] RISC is a multiprotein complex in which a single stranded part of a small RNA is incorporated. This then guides the sequence specific cleavage of the target mRNA.[53]

pdf gene gave rise to a fly strain bearing a nonsense mutation (pfd^{01}). Video recording of locomotor events of this *pfd*-null mutant showed that these flies are arrhythmic in constant darkness and this was one reason for Renn et al to suggest that pdf acts as the principal transmitter in circadian behavior.[38] A deletion of *eth*, the gene encoding ecdysis-triggering hormone (ETH) in *Drosophila*, was obtained by imprecise excision of a *P*-element. In video recorded ecdysis behavior assays, these null mutants (eth^{-}) failed to inflate the new respiratory system on schedule, did not perform the ecdysis behavioral sequence and exhibited the phenotype *buttoned-up*, which is characterized by incomplete ecdysis and 98% mortality at the transition from first to second larval instar.[39]

Targeted ablation of neurons expressing a neuropeptide precursor of interest is generally used to investigate the phenotypical, behavioral and physiological consequence of the absence of its peptides. Neuropeptide enhancer driven GAL4 expression combined with UAS-linked cell death

Table 3. P-element insertion stocks available from stock centers, Bloomington—(http://flystocks.bio.indiana.edu/; Szeged...)Author's Note: The Szeged stock center unfortunately had to close doors on June 30th 2009.

P-Element Insertions	Symbol	Transposon	Reference
Bloomington	EP	P{EP}	88
		P{EPg}	89
	EY	P{EPgy2}	90,91
	BG	P{GT1}	92
	PlacW	P{lacW}	93,94
	LA	P{Mae-UAS.6.11}	95
	PZ	P{PZ}	96
	KG and KV	P{SUPor-P}	90
	XP	P{XP}	97
	DG	P{wHy}	98
Szeged	RS	P{RS3} and P{RS5}	99
	EP	P{EP}	88
	l(3)Sxxxxxx, Sxxxxxx, l(2)SHxxxx	P{lacW}	100,101,102
Exelixis	XP	P{XP}	97
DGRC	NP	P{GawB}	103
	GS	P{GS}, P{Mae-UAS.GS}	104
	LA	P{Mae-UAS.6.11}	95

genes is the most used experimental strategy. Therefore, the first step is transposon transformation of the desired GAL4 and UAS constructs. In this respect, *dAkh* (Drosophila *adipokinetic hormone*)-GAL4,[40] *CCAP* (*crustacean cardioactive peptide*)-GAL4,[41,42] *EHups* (*eclosion hormone upstream sequence*)-GAL4[43] and *pdf*-GAL4[38,44] were used in combination with UAS-*reaper* (UAS-*rpr*), *npf* (*neuropeptide F*)-GAL4 combined with UAS-*DTI* (an attenuated *diphteria toxin* gene)[45,46] and *pfd*-GAL4 combined with UAS-*head involution defective* (UAS-*hid*),[38,44] for the targeted ablation of the respective neuropeptide precursor expressing neurons.

Spatio-temporal controlled or ectopic expression of a neuropeptide gene of interest also has been proven to be a useful approach. Ectopic expression of the *hugin* gene, for example, resulted in larval death predominantly at or shortly after ecdysis from second to third instar, a pattern reminiscent of *ETH* mutants, suggesting that at least one of the posttranslational cleavage products affects molting of the larvae by interfering with the regulation of ecdysis.[47] *Actin5C*-GAL4 driven expression of the *proctolin* gene in the CNS and midgut resulted in a 14% increase in the heart rate in pupae, providing evidence in support of a cardioacceleratory endocrine function for proctolin in *Drosophila*.[48]

Although recently developed, gene targeting and transgenic expression of dsRNA in particular, has already demonstrated to be an excellent tool in the quest for a gene's function.

Winther et al specifically eliminated tachykinin related peptides (TKRPs) in the nervous system of *Drosophila* using targeted RNAi of the *dtk* (Drosophila *tachykinin*) gene to examine odor

perception with a larval olfactory test and locomotor activity with the 'Buridan's paradigm'.[49,50] They found that the gene silencing of these peptides resulted in a loss of sensitivity towards specific odorants and concentrations and also in hyperactivity.

In most cases however, more insight into the function(s) of neuropeptides is gained by using a combination of different reverse genetic approaches and multiple bioassays.

With a food response assay, a glucose sensing and motivational feeding assay and a social behavior and burrowing assay Wu et al demonstrated that transgenic larvae deficient in *Drosophila* neuropeptide F (*d*NPF) signaling through targeted gene silencing of the *dNPF* gene precociously exhibited the phenotypes of food aversion and social behavior normally displayed by older nonfeeding larvae.[46] Conversely, *dNPF* overexpression in the larval CNS prolonged the feeding activity and suppressed the social behavior of older larvae. Whereas Wen et al used a behavioral assay and an ethanol content assay to demonstrate that these *d*NPF (or its receptor *d*NPFR1) RNAi flies showed a decreased sensitivity to ethanol sedation and that overexpression of *d*NPF increased alcohol sensitivity.[45] Also the controlled functional disruption (*npf-GAL4* driven *diphteria toxin* (*DTI*) expression) of *d*NPF and *d*NPFR1 neurons rapidly triggered acute resistance to ethanol sedation, suggesting that the NPF pathway tonically controls acute alcohol response.

The use of gain-of-function and loss-of-function transgenic flies for the short form of NPF, *ds*NPF (Drosophila *short neuropeptide F*), in a feeding assay led to the suggestion that *ds*NPF regulates food intake and body size. Overexpression of *ds*NPF in the CNS of *Drosophila* larvae promots food intake and results in bigger and heavier flies whereas the targeted knock down results in a suppression of food intake. In contrast to *d*NPF, the *ds*NPF did not prolong the feeding behavior suggesting that they are involved in different aspects of the mechanisms controlling feeding.[51]

As proven above, a clever use of reverse genetics combined with a wise selection of bioassays greatly enhances our knowledge about neuropeptides and their functions.

Other Neuronal Molecules as Potential Targets for Insecticides

Neurotransmitter-receptors can be used as targets for insecticides, as they combine the extreme specificity of the ligand-receptor recognition process and fast mode of signaling with a great physiological relevance. Mainly the nicotinic acetylcholine receptor and the GABA-receptor are considered as important targets for insecticides because of their importance in the neurotransmission within the insect nervous tissue, acetylcholine being the most important excitatory and GABA the most important inhibitory neurotransmitter.[54,55] Unfortunately, the pharmacology of these two receptors is closely related to that of the homologous vertebrate receptors, which results in a high toxicity for vertebrates of the respective insecticides. Lindane for instance, is an insecticide that is currently widely used (e.g., in shampoos for lice). Lindane acts through a GABA-A receptor-chloride channel complex. The problem with Lindane, however, is that insects have become resistant and that it has toxic neurologic effects. Therefore, these receptors are not ideally suited to serve as targets for the development of new insecticides. For a review on ion channels as molecular targets for neuroactive insecticides see ref. [56]

GPCRs with insect-specific ligands are a good alternative for the development of new biodegradable, safe, specific and nontoxic insecticides. Besides the receptors, also enzymes that are involved in the formation of bioactive peptides from their inactive precursor proteins represent possible targets for insecticides.

Conclusion

The publishing of the *Drosophila* genome has revolutionized its use for studying a plethora of often conserved physiological systems, and for exploring neuropeptide biology in particular. Despite all this, little still is known about the function(s) of many annotated (neuro-)peptides as well as the receptor(s) they act on. In addition, the use of novel bioinformatic tools has recently led to the prediction of even more peptide precursor genes of which the in vivo peptide expression was explored using neuropeptidomic techniques and other localization studies. The genetic methods that have been used for unraveling the function of some neuropeptides, however, are most promis-

ing. In the near future, quite a lot of functions thus presumably will be elucidated when adequate bioassays are available. Since most of the genetic tools used for examining *Drosophila* genes are not (yet) at one's disposal in other insects of which the genome has been sequenced, *Drosophila* thus is by far the most suitable organism for investigating neuropeptide, as well as other peptide/protein functioning. Before generalizing neuropeptide function(s), one naturally first has to explore there possible conservation since some peptides do not occur in *Drosophila*, or in other insects, and are therefore regarded to be insect family specific. Some of the latter thus can not be examined in the fruit fly. *Drosophila* neuropeptide research nonetheless has enhanced, and will further increase the knowledge of important conserved signaling systems in the future.

Acknowledgments

E. Clynen, G. Baggerman and I. Mertens are postdoctoral researchers of the Fund for Scientific Research-Flanders (FWO-Vlaanderen). Special thanks to J. Puttemans for figure design.

References

1. Adams MD, Celniker SE, Holt RA et al. The genome sequence of Drosophila melanogaster. Science 2000; 287:2185-2195.
2. Baggerman G, Liu F, Wets G et al. Bioinformatic analysis of peptide precursor proteins. Ann NY Acad Sci 2005; 1040:59-65.
3. Vanden Broeck J. Neuropeptides and their precursors in the fruitfly, Drosophila melanogaster. Peptides 2001; 22:241-254.
4. Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. Gen Res 2001; 11:1126-1142.
5. Liu F, Baggerman G, D'Hertog W et al. In silico identification of new secretory peptide genes in Drosophila melanogaster. Mol Cell Proteomics 2006; 5:510-522.
6. Liu F, Baggerman G, Schoofs L et al. Uncovering conserved patterns in bioactive peptides in Metazoa. Peptides 2006; 27:3137-3153.
7. Clynen E, De Loof A, Schoofs L. The use of peptidomics in endocrine research. Minireview. Gen Comp Endocrinol 2003; 132:1-9.
8. Baggerman G, Verleyen P, Clynen E et al. Peptidomics J Chromatogr B Analyt Technol Biomed Life Sci 2004; 803:3-16.
9. Schulz-Knappe P, Zucht HD, Heine C et al. Peptidomics: the comprehensive analysis of peptides in complex biological mixtures. Comb Chem High T Scr 2001; 4:207-217.
10. Baggerman G, Cerstiaens A, De Loof A et al. Peptidomics of the larval Drosophila melanogaster central nervous system. J Biol Chem 2002; 277:40368-40374.
11. Baggerman G, Boonen K, Verleyen P et al. Peptidomic analysis of the larval Drosophila melanogaster central nervous system by two-dimensional capillary liquid chromatography quadrupole time-of-flight mass spectrometry. J Mass Spectrom 2005; 40:250-260.
12. Schoofs L, Baggerman G. Peptidomics in Drosophila melanogaster. Briefings in functional genomics and proteomics 2003; 2:114-120.
13. Zupanc GKH. Peptidergic transmission: from morphological correlates to functional implications. Micron 1996; 27:35-91.
14. Bockaert J, Pin JP. Molecular tinkering of G protein-coupled receptors: an evolutionary success. Embo J 1999; 18:1723-1729.
15. Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocrine Reviews 2000; 21:90-113.
16. Schlyer S, Horuk R. I want a new drug: G-protein-coupled receptors in drug development. Drug Discovery Today 2006; 11:481-493.
17. Drews J. Drug discovery: a historical perspective. Science 2000; 287:1960-1964.
18. Mertens I, Vandingenen A, Meeusen T et al. Postgenomic characterization of G-protein-coupled receptors. Pharmacogenomics 2004; 5:657-672.
19. Thomsen W, Frazer J, Unett D. Functional assays for screening GPCR targets. Current Opinion in Biotechnology 2005; 16:655-665.
20. Leifert WR, Aloia AL, Bucco O et al. G-protein-coupled receptors in drug discovery: nanosizing using cell-free technologies and molecular biology approaches. J Biomol Screen 2005; 10:765-779.
21. Nässel DR, Homberg U. Neuropeptides in interneurons of the insect brain. Cell Tissue Res 2006; 326:1-24.
22. Santos JS, Voemel M, Homberg U et al. Three-dimensional charting of peptidergic neurons in the larval ventral nerve cord of the fruit fly, Drosophila melanogaster. J Neurogenet 2006; 20:214-215.

23. Predel R, Wegener C, Russell WK et al. Peptidomics of CNS-associated neurohemal systems of adult Drosophila melanogaster: a mass spectrometric survey of peptides from individual flies. J Comp Neurol 2004; 474:379-392.
24. Wegener C, Reinl T, Jansch L et al. Direct mass spectrometric peptide profiling and fragmentation of larval peptide hormone release sites in Drosophila melanogaster reveals tagma-specific peptide expression and differential processing. J Neurochem 2006; 96:1362-1374.
25. Neupert S, Johard HAD, Nässel DR et al. Mass spectrometric analysis of peptide content in single circadian pacemaker neurons (large LNVS) in Drosophila melanogaster. J Neurogenet 2006;20:186-187.
26. Neupert S, Predel R. Analysis of neuropeptide expression in single hugin-neurons using MALDI-TOF MS. J Neurogenet 2006;20:187-187.
27. St Johnston D. The art and design of genetic screens: Drosophila melanogaster. Nat Rev Genet 2002; 3:176-188.
28. Adams MD, Sekelsky JJ. From sequence to phenotype: reverse genetics in Drosophila melanogaster. Nat Rev Genet 2002; 3:189-198.
29. Rubin GM, Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science 1982; 218:348-353.
30. Spradling AC, Rubin GM. Transposition of cloned P elements into Drosophila germ line chromosomes. Science 1982; 218:341-347.
31. Ryder E, Russell S. Transposable elements as tools for genomics and genetics in Drosophila. Brief Funct Genomic Proteomic 2003; 2:57-71.
32. Echols H, Gingery R, Moore L. Integrative recombination function of bacteriophage lambda: evidence for a site-specific recombination enzyme. J Mol Biol 1968; 34:251-260.
33. Gong WJ, Golic KG. Ends-out, or replacement, gene targeting in Drosophila. Proc Natl Acad Sci USA 2003; 100:2556-2561.
34. Rong YS, Golic KG. Gene targeting by homologous recombination in Drosophila. Science 2000; 288:2013-2018.
35. Fire A, Xu S, Montgomery MK et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998; 391:806-811.
36. Kennerdell JR, Carthew RW. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell 1998; 95:1017-1026.
37. Ashburner M, Golic KG, Hawley RS. Drosophila: a laboratory handbook. 2005; second edition.
38. Renn SC, Park JH, Rosbash M et al. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell 1999; 99:791-802.
39. Park Y, Filippov V, Gill SS et al. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development 2002; 129:493-503.
40. Lee G, Park JH. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster. Genetics 2004; 167:311-323.
41. Dulcis D, Levine RB, Ewer J. Role of the neuropeptide CCAP in Drosophila cardiac function. J Neurobiol 2005; 64:259-274.
42. Park JH, Schroeder AJ, Helfrich-Forster C et al. Targeted ablation of CCAP neuropeptide-containing neurons of Drosophila causes specific defects in execution and circadian timing of ecdysis behavior. Development 2003; 130:2645-2656.
43. Baker JD, McNabb SL, Truman JW. The hormonal coordination of behavior and physiology at adult ecdysis in Drosophila melanogaster. J Exp Biol 1999; 202:3037-3048.
44. Yoshii T, Heshiki Y, Ibuki-Ishibashi T et al. Temperature cycles drive Drosophila circadian oscillation in constant light that otherwise induces behavioural arrhythmicity. Eur J Neurosci 2005; 22:1176-1184.
45. Wen T, Parrish CA, Xu D et al. Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. Proc Natl Acad Sci USA 2005; 102:2141-2146.
46. Wu Q, Wen T, Lee G et al. Developmental control of foraging and social behavior by the Drosophila neuropeptide Y-like system. Neuron 2003; 39:147-161.
47. Meng X, Wahlstrom G, Immonen T et al. The Drosophila hugin gene codes for myostimulatory and ecdysis-modifying neuropeptides. Mech Dev 2002; 117:5-13.
48. Taylor CA, Winther AM, Siviter RJ et al. Identification of a proctolin preprohormone gene (Proct) of Drosophila melanogaster: expression and predicted prohormone processing. J Neurobiol 2004; 58:379-391.
49. Winther AM, Acebes A, Ferrus A. Tachykinin-related peptides modulate odor perception and locomotor activity in Drosophila. Mol Cell Neurosci 2006; 31:399-406.
50. Gotz KG. Visual guidance in Drosophila. Basic Life Sci 1980; 16:391-407.

51. Lee KS, You KH, Choo JK et al. Drosophila short neuropeptide F regulates food intake and body size. J Biol Chem 2004; 279:50781-50789.
52. Hammond SM, Bernstein E, Beach D et al. An RNA-directed nuclease mediates posttranscriptional gene silencing in Drosophila cells. Nature 2000; 404:293-296.
53. Bernstein E, Caudy AA, Hammond SM et al. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001; 409:363-366.
54. Breer H, Sattelle DB. Molecular properties and functions of insect acetylcholine receptors. J Insect Physiol 1987; 33:771-790.
55. Rauh JJ, Lummis SC, Sattelle DB. Pharmacological and biochemical properties of insect GABA receptors. Trends Pharmacol Sci 1990; 11:325-329.
56. Raymond-Delpech V, Matsuda K, Sattelle BM et al. Ion channels: molecular targets of neuroactive insecticides. Invert Neurosci 2005; 5:119-133.
57. Kubiak TM, Larsen MJ, Burton KJ et al. Cloning and functional expression of the first Drosophila melanogaster sulfakinin receptor DSK-R1. Biochem Biophys Res Commun 2002; 291:313-320.
58. Radford JC, Davies SA, Dow JAT. Systematic G-protein-coupled receptor analysis in Drosophila melanogaster identifies a leucokinin receptor with novel roles. J Biol Chem 2002; 277:38810-38817.
59. Johnson EC, Bohn LM, Barak L et al. Identification of Drosophila neuropeptide receptors by G protein-coupled receptors-bèta-arrestin2 interactions. J Biol Chem 2003; 278:52172-52178.
60. Birse RT, Johnson EC, Taghert PH et al. Widely distributed Drosophila G-protein-coupled receptor (CG7887) is activated by endogenous tachykinin-related peptides. J Neurobiol 2006; 66:33-46.
61. Rosenkilde C, Cazzamali G, Williamson M et al. Molecular cloning, functional expression and gene silencing of two Drosophila receptors for the Drosophila neuropeptide pyrokinin-2. Biochem Biophys Res Commun 2003; 309:485-494.
62. Jorgensen LM, Hauser F, Cazzamali G et al. Molecular identification of the first SIFamide receptor. Biochem Biophys Res Commun 2006; 340:696-701.
63. Garczynski SF, Brown MR, Shen P et al. Characterization of a functional neuropeptide F receptor from Drosophila melanogaster. Peptides 2002; 23:773-780.
64. Feng GP, Reale V, Chatwin H et al. Functional characterization of a neuropeptide F-like receptor from Drosophila melanogaster. Eur J Neurosci 2003; 18:227-238.
65. Mertens I, Meeusen T, Huybrechts R et al. Characterization of the short neuropeptide F receptor from Drosophila melanogaster. Biochem Biophys Res Commun 2002; 297:1140-1148.
66. Meeusen T, Mertens I, Clynen E et al. Identification in Drosophila melanogaster of the invertebrate G protein-coupled FMRFamide receptor. Proc Natl Acad Sci USA 2002; 99:15363-15368.
67. Iversen A, Cazzamali G, Williamson M et al. Molecular cloning and functional expression of a Drosophila receptor for the neuropeptides capa-1 and -2. Biochem Biophys Res Commun 2002; 299:628-633.
68. Park Y, Kim YJ, Adams ME. Identification of G protein-coupled receptors for Drosophila PRXamide peptides, CCAP, corazonin and AKH supports a theory of ligand-receptor co-evolution. Proc Natl Acad Sci USA 2002; 99:11423-11428.
69. Iversen A, Cazzamali G, Williamson M et al. Molecular identification of the first insect ecdysis triggering hormone receptors. Biochem Biophys Res Commun 2002; 299:924-931.
70. Johnson EC, Garczynski SF, Park D et al. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in Drosophila melanogaster. Proc Natl Acad Sci USA 2003; 100:6198-6203.
71. Egerod K, Reynisson E, Hauser F et al. Molecular cloning and functional expression of the first two specific insect myosuppressin receptors. Proc Natl Acad Sci USA 2003; 100:9808-9813.
72. Cazzamali G, Torp M, Hauser F et al. The Drosophila gene CG9918 codes for a pyrokinin-1 receptor. Biochem Biophys Res Commun 2005; 335:14-19.
73. Birgul N, Weise C, Kreienkamp HJ et al. Reverse physiology in Drosophila: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. Embo Journal 1999; 18:5892-5900.
74. Larsen MJ, Burton KJ, Zantello MR et al. Type A allatostatins from Drosophila melanogaster and Diplotera puncata activate two Drosophila allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. Biochem Biophys Res Commun 2001; 286:895-901.
75. Kreienkamp HJ, Larusson HJ, Witte I et al. Functional annotation of two orphan G-protein-coupled receptors, Drostar1 and -2, from Drosophila melanogaster and their ligands by reverse pharmacology. Journal of Biological Chemistry 2002; 277:39937-39943.
76. Staubli F, Jorgensen TJD, Cazzamali G et al. Molecular identification of the insect adipokinetic hormone receptors. Proc Natl Acad Sci USA 2002; 99:3446-3451.
77. Cazzamali G, Saxild NPE, Grimmelikhuijzen CJP. Molecular cloning and functional expression of a Drosophila corazonin receptor. Biochem Biophys Res Commun 2002; 298:31-36.

78. Cazzamali G, Hauser F, Kobberup S et al. Molecular identification of a Drosophila G protein-coupled receptor specific for crustacean cardioactive peptide. Biochem Biophys Res Commun 2003; 303:146-152.
79. Mendive FM, Van Loy T, Claeysen S et al. Drosophila molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2. Febs Letters 2005; 579:2171-2176.
80. Luo CW, Dewey EM, Sudo S et al. Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. Proc Natl Acad Sci USA 2005; 102:2820-2825.
81. Sudo S, Kuwabara Y, Park JI et al. Heterodimeric fly glycoprotein hormone-alpha 2 (GPA2) and glycoprotein hormone-beta 5 (GPB5) activate fly leucine-rich repeat-containing G protein-coupled receptor-1 (DLGR1) and stimulation of human thyrotropin receptors by chimeric fly GPA2 and human GPB5. Endocrinology 2005; 146:3596-3604.
82. Johnson EC, Bohn LM, Taghert PH. Drosophila CG8422 encodes a functional diuretic hormone receptor. J Exp Biol 2004; 207:743-748.
83. Mertens I, Vandingenen A, Johnson EC et al. PDF receptor signalling in Drosophila contributes to both circadian and geotaxic behaviors. Neuron 2005.
84. Lear BC, Merrill CE, Lin JM et al. A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. Neuron 2005; 48:221-227.
85. Hyun S, Lee Y, Hong ST et al. Drosophila GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron 2005; 48:267-278.
86. Johnson EC, Shafer OT, Trigg JS et al. A novel diuretic hormone receptor in Drosophila: evidence for conservation of CGRP signaling. J Exp Biol 2005; 208:1239-1246.
87. Cvejic S, Zhu Z, Felice SJ et al. The endogenous ligand Stunted of the GPCR Methuselah extends lifespan in Drosophila. Nat Cell Biol 2004; Epub.
88. Rorth P. A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc Natl Acad Sci USA 1996; 93:12418-12422.
89. Staudt N, Molitor A, Somogyi K et al. Gain-of-function screen for genes that affect Drosophila muscle pattern formation. PLoS Genet 2005; 1:e55.
90. Roseman RR, Johnson EA, Rodesch CK et al. A P element containing suppressor of hairy-wing binding regions has novel properties for mutagenesis in Drosophila melanogaster. Genetics 1995; 141:1061-1074.
91. Bellen HJ, Levis RW, Liao G et al. The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 2004; 167:761-781.
92. Lukacsovich T, Asztalos Z, Awano W et al. Dual-tagging gene trap of novel genes in Drosophila melanogaster. Genetics 2001; 157:727-742.
93. Bier E, Vaessin H, Shepherd S et al. Searching for pattern and mutation in the Drosophila genome with a P-lacZ vector. Genes Dev 1989; 3:1273-1287.
94. Torok T, Tick G, Alvarado M et al. P-lacW insertional mutagenesis on the second chromosome of Drosophila melanogaster: isolation of lethals with different overgrowth phenotypes. Genetics 1993; 135:71-80.
95. Beinert N, Werner M, Dowe G et al. Systematic gene targeting on the X chromosome of Drosophila melanogaster. Chromosoma 2004; 113:271-275.
96. Mlodzik M, Hiromi Y. Enhancer trap method in Drosophila: its application to neurobiology. 1992; 397-414.
97. Thibault ST, Singer MA, Miyazaki WY et al. A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat Genet 2004; 36:283-287.
98. Huet F, Lu JT, Myrick KV et al. A deletion-generator compound element allows deletion saturation analysis for genomewide phenotypic annotation. Proc Natl Acad Sci USA 2002; 99:9948-9953.
99. Golic KG, Golic MM. Engineering the Drosophila genome: chromosome rearrangements by design. Genetics 1996; 144:1693-1711.
100. Deak P, Omar MM, Saunders RD et al. P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: correlation of physical and cytogenetic maps in chromosomal region 86E-87F. Genetics 1997; 147:1697-1722.
101. Bellotto M, Bopp D, Senti KA et al. Maternal-effect loci involved in Drosophila oogenesis and embryogenesis: P element-induced mutations on the third chromosome. Int J Dev Biol 2002; 46:149-157.
102. Oh SW, Kingsley T, Shin HH et al. A P-element insertion screen identified mutations in 455 novel essential genes in Drosophila. Genetics 2003; 163:195-201.
103. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993; 118:401-415.
104. Toba G, Ohsako T, Miyata N et al. The gene search system. A method for efficient detection and rapid molecular identification of genes in Drosophila melanogaster. Genetics 1999; 151:725-737.