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Peptidomics and processing of regulatory peptides in the fruit fly *Drosophila*

Dennis Pauls[a], *Jiangtian Chen*[a], *Wencke Reiher*[a], *Jens T. Vanselow*[b], *Andreas Schlosser*[b], *Jörg Kahnt*[c], *Christian Wegener*[a,*]

[a] *Neurobiology and Genetics, Theodor-Boveri-Institute, Biocenter, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany*
[b] *Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany*
[c] *Max-Planck-Institute of Terrestrial Microbiology, Karl-von-Frisch-Strasse 10, D-35043 Marburg, Germany*

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ABSTRACT

More than a decade has passed since the release of the *Drosophila melanogaster* genome and the first predictions of fruit fly regulatory peptides (neuropeptides and peptide hormones). Since then, mass spectrometry-based methods have fuelled the chemical characterisation of regulatory peptides, from 7 *Drosophila* peptides in the pre-genomic area to more than 60 today. We review the development of fruit fly peptidomics, and present a comprehensive list of the regulatory peptides that have been chemically characterised until today. We also summarise the knowledge on peptide processing in *Drosophila*, which has strongly profited from a combination of MS-based techniques and the genetic tools available for the fruit fly. This combination has a very high potential to study the functional biology of peptide signalling on all levels, especially with the ongoing developments in quantitative MS in *Drosophila*.

1. Introduction

Regulatory peptides have important functions: neuropeptides act as neuromodulators within the nervous system, and peptide hormones are released from neurohemal sites or endocrine cells into the circulation to regulate body functions. As the most diverse group of molecules involved in cell-to-cell communication, regulatory peptides are involved in the regulation of many physiological processes ranging from the control of diuresis to the orchestration of complex behaviours. As peptides secreted by the regulated secretory pathway, they are packaged into dense-core vesicles within which they are processed from larger precursor molecules (prepropeptides) by a specific set of enzymes.

Due to its genetic amenability, *Drosophila* is today a favourite organism to study the physiological functions of regulatory peptides, with strongly increasing numbers of publications in the recent years especially on the role of

* *Corresponding author*. Tel.: +49 931 31 85380; fax: +49 931 31 84452.
E-mail addresses: dennis.pauls@uni-wuerzburg.de (D. Pauls), jiangtian.chen@uni-wuerzburg.de (J. Chen), wencke.reiher@uni-wuerzburg.de (W. Reiher), jens.vanselow@virchow.uni-wuerzburg.de (J.T. Vanselow), andreas.schlosser@virchow.uni-wuerzburg.de (A. Schlosser), kahnt@mpi-marburg.mpg.de (J. Kahnt), christian.wegener@uni-wuerzburg.de (C. Wegener).
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neuropeptides in the control of feeding and metabolism, ageing, learning and memory, circadian clock and ecdysis behaviour (see [1]). Besides the advanced genetic tools available for the fruit fly, the advent and still ongoing improvement of mass spectrometric methods combined with the miniaturisation of HPLC techniques represent an important corner stone of what is currently a highly exciting field of fruit fly research. As predicted more than 10 years ago [2], these techniques allowed to chemically characterise the regulatory peptides in an efficient and straight-forward manner, thus providing the fundamental knowledge of "which peptides are present" (or in more elegant terms "the peptidome") upon which functional studies are based. The *Drosophila* peptidome was predicted *in silico* [3–5] already soon after the release of the *Drosophila* genome [6] and was later substantially expanded and refined [7]. In retrospect, these predictions have in most but not all cases been correct (see below), showing that peptide processing and posttranslational modification is not fully predictable. Thus, peptidomics was and still is the only way to identify which regulatory peptides are existing and thus could be employed as communication signals by the fruit fly.

In this review, we will briefly summarise the peptidomic studies performed in *Drosophila* with focus on regulatory peptides (for information on the different immune-related peptides see [8–10]). We compile a list of the hitherto chemically identified fruit fly regulatory peptides, and review the genetic and peptidomic studies of peptide processing in the fruit fly. At the end, we give an outlook on emerging quantitative peptidomic methods in *Drosophila* that will allow to address regulatory peptide dynamics under different physiological regimes or after transgenic or mutational manipulations.

2. Peptidomics in *Drosophila*

While *Drosophila* as a genetic model organism has clear experimental advantages also in the study of peptide processing, its small size had for a long time been a substantial challenge for the biochemical analysis of neuropeptides. Up to the year 2000, only 7 neuropeptides had been biochemically characterised in *Drosophila* [11–14], while at the same time several dozens of peptides each had been sequenced by Edman degradation from larger insect species such as locusts and cockroaches [15–17]. Yet, with the sequencing of the fruit fly genome [6] and the advent of powerful peptidomic techniques such as LC/MS–MS [18] and direct peptide profiling [19] it eventually became possible to chemically characterise the *Drosophila* peptidome. Supplementary Table 1 lists the sequences and tissue distribution of all peptides that have so far been chemically characterised in *Drosophila*.

2.1. *Peptide characterisation by LC–MS*

The history of *Drosophila* peptidomics starts with the pioneering work of Baggerman and colleagues in *Drosophila* larvae [20], using capHPLC/ESI-Q-TOF MS/MS to identify 28 neuropeptides from an extract of central nervous systems (CNS) prepared from 50 larvae (see Supplementary Table 1). This could later be improved by switching from a one- to a two-dimensional capHPLC-separation, yielding 38 peptides from an extract of 50 larval CNS [21] (see Supplementary Table 1). Using different combinations of LC/MS–MS methods to analyse the peptidome from adult brain extracts, Yew et al. [22] could characterise 42 neuropeptides by MALDI-FTMS and fully sequence 26 peptides by ESI-QTOF MS/MS (see Supplementary Table 1). Intriguingly, some of the peptides found in the brain were later on also identified in extracts of the midgut, the part of the fruit fly digestive tract that contains enteroendocrine cells. In fact, all 23 enteroendocrine peptides identified by offline capHPLC combined with MALDI-TOF MS/MS [23] can be classified as brain–gut peptides (see Supplementary table 1). Besides these studies with a peptidomic scope, LC/MS alone [24] or in combination with reverse pharmacology [25] or radioimmuno-assay [26] was also employed to specifically identify and characterise single peptides.

2.2. *Peptide characterisation by direct peptide profiling*

Along with LC–MS, direct peptide profiling has been successfully used to identify fruit fly peptides. In this method, fly tissues such as a neurohemal organ or part of the brain is dissected and transferred to a MALDI target plate and left to dry. After addition of an appropriate matrix solution, peptides are extracted and can be directly analysed by MALDI-MS (see [19]). While not working for all tissues, a major advantage of direct profiling is the circumvention of the unavoidable peptide loss during LC and adsorption to plastic ware. In addition and in contrast to conventional extraction methods leading to cell disruption, the on-plate extraction in direct profiling apparently leads to a highly selective extraction of regulatory peptides but not intracellular peptides. Direct peptide profiling thus allows peptide identification from single tissues of single flies. Measuring neurohemal organ and brain tissue, this technique allowed the first characterisation of the neuropeptidome of adult flies and revealed 32 different neuropeptides [27] (see Supplementary Table 1). The same approach was later used for larval neurohemal organs and the endocrine Inka/epitracheal cells, detecting 23 different peptides [28] (see Supplementary Table 1). The ability of direct peptide profiling to reveal the finer tissue distribution of peptides led to the discovery of a differential processing of the CAPA neuropeptides between neurohemal organs of the brain and ventral ganglion [27,28] as described below. Direct peptide profiling also allowed the chemical identification of not less than 27 neuropeptides within the antennal lobes of adult fruit flies [29]. Like LC–MS, direct peptide profiling was also employed to specifically characterise and identify single peptides [30–33] (see Supplementary Table 1).

2.3. *Peptide characterisation in single cells or enriched neuron populations*

While the high sensitivity of mass spectrometers enables direct peptide profiling even from single cells (e.g. [34–37]), it is more an art than a technology to isolate single fly neurons suited for MS analysis, as even small neuropeptide contaminations from membrane remains of passing neurites may result in detectable mass peaks and conventional enzyme digestion for cell separation can lead to a loss of peptide

signals [38]. On the other hand, peptidergic fly neurons can be selectively labelled by green fluorescent protein (GFP) using the GAL4-UAS binary expression system [39], as many specific peptide GAL4-lines are available. Using GAL4-UAS-driven GFP expression, Neupert and colleagues [40] could manually isolate PDF- or HUGIN-PK-expressing neurons and analyse their peptide contents. Taking another approach, Yew and colleagues [22] used the specific peptidergic c929-GAL4 line or a Dopa-decarboxylase-GAL4 (Ddc-GAL4) line to express mCD8-GFP in around 200 peptidergic or dopaminergic/serotonergic neurons. After enzymatic treatment, suspended cells were enriched by immunocapture. This led to the successful identification of neuropeptides that are contained within the c929-GAL4 neurons or co-localised with dopamine/serotonin. While this cell enrichment is susceptible against contaminations, immunocytochemistry verified the results for those peptides tested.

2.4. *Disagreements with peptide predictions and limitations of peptidomics in Drosophila*

Since the first peptidome predictions [3–5], prepropeptide prediction and especially the prediction of peptide processing has been improved and new bioinformatic tools and pipelines have been developed (e.g. [7,41,42]). This improvement is largely based on the growing genomic and peptidomic data, which allows to bioinformatically compare a large set of actually made peptides with the respective preproproteins derived from the genome. Nevertheless, still today to predict a processed peptide does not necessarily infer it is really made. In *Drosophila*, e.g. HUG-γ has been predicted, and shown to activate the respective pyrokinin receptor to a significant degree but with a considerably higher EC_{50} than the chemically identifiable peptide paracopy from the same prepropeptide [43–45]. The native peptide or its corresponding mass was, however, not found in any of the peptidomic studies in contrast to the other peptide paracopy (HUG-PK) contained on the propeptide. This mismatch may – at least for HUG-γ – be caused by amino acid differences in propeptide areas outside the basic cleavage sequence [46]. To give another example: although sNPF-1 occurs in the predicted long form with 11 amino acids [3], the shorter unpredicted form $sNPF_{4–11}$ appears to be more abundant and is identical to sNPF-$2_{12–19}$ which apparently does not occur in the predicted long form (see Supplementary Table 1). This short form is however fully compatible with the rules postulated earlier on by Veenstra [47]. Moreover, masses corresponding to the predicted sNPF-3 and -4 [3,41] could not be detected until Yew and colleagues [22] showed that these peptides are unexpectedly cleaved and retain a basic Lys residue at their N-terminus. An interesting example are also the predicted *amnesiac*-derived PACAP-like peptides [48]. While *amnesiac* has convincingly been shown to play an important role in learning and memory and other processes (e.g. [49–51]), none of the predicted peptides has so far been found in the brain. Clearly, the failure to detect a peptide by mass spectrometry does not prove the non-existence of a peptide. Nor does the biochemical detection of a peptide prove its function as a neuronal signalling molecule or a peptide hormone, since also immune-induced peptides are produced by the CNS and midgut. Furthermore, some peptides may actually act intra- rather than extracellularly (see Ferro et al., this special issue). These examples highlight, however, the importance and indispensability of a biochemical characterisation as still the only way to demonstrate the presence of bioactive peptides.

A caveat of the peptidomic approach is that peptides larger than 6 kDa have so far been very difficult to analyse by MS. Thus, eclosion hormone, prothoracicotropic hormone (PTTH), insulin-like peptides (DILPs) and other larger fruit fly peptides are still chemically uncharacterised. Recent advances in mass spectrometry, i.e. top-down proteomics on instruments with high resolving power in combination with peptide fragmentation techniques such as ETD [52–54] are starting to change this situation.

3. Peptide processing in *Drosophila*

Once peptidomic techniques yielded a (certainly still not complete) overview of the *Drosophila* peptidome, it was time to employ these techniques to tackle the so far ignored biochemical level of peptide processing in the fruit fly. Neuropeptides and peptide hormones are produced from larger preproproteins within the regulated secretory pathway. Like other secreted or membrane-bound proteins, the preproproteins carry an N-terminal signal peptide which directs them to the lumen of the rough endoplasmic reticulum. After removal of the signal peptide, the resulting proprotein can be modified (e.g. glycosilated) and is then exported to the trans-Golgi-network and finally packaged within dense-core vesicles and transported to release or storage sites. Within the trans-Golgi and the dense-core vesicles, the bioactive peptides are processed from the proprotein by a set of specific enzymes, including furins, convertases, carboxypeptidases and amidating enzymes (Fig. 1, see [55] for more details). The different processing steps and the responsible enzymes have largely been worked out in the 1980s and 1990s in mammalian model species and cell cultures, often using preproinsulin as a substrate [56]. This focus has not at least been driven by the role of processing enzymes in obesity, cancer and other pathologies as well as viral infections (e.g. [57–60]).

So, what then is the significance of studying peptide processing and proprotein processing enzymes in *Drosophila*? A medically relevant future direction in the protein processing field is to unravel how processing enzymes, especially convertases, are regulated in disease state [61]. The regulation of peptide processing may also be an important yet unappreciated factor within the (neuro)endocrinological control of behaviour and physiology, since processing enzymes are in principal well positioned to influence peptide production in a general way affecting all tissues in an organism. This field is basically unexplored, yet recent evidence suggests that convertase and carboxypeptidase activity can be regulated by biogenic amines in cultured chromaffin cells [62]. Convertase gene expression has also been shown to be regulated by photoperiod in the Siberian hamster [63] and by the molecular circadian clock work in *Drosophila* [64]. The functional significance of these findings are unclear, but *Drosophila* lends itself as an excellent model to study the cellular and systemic mechanisms and functions of processing regulation: (a) *Drosophila*

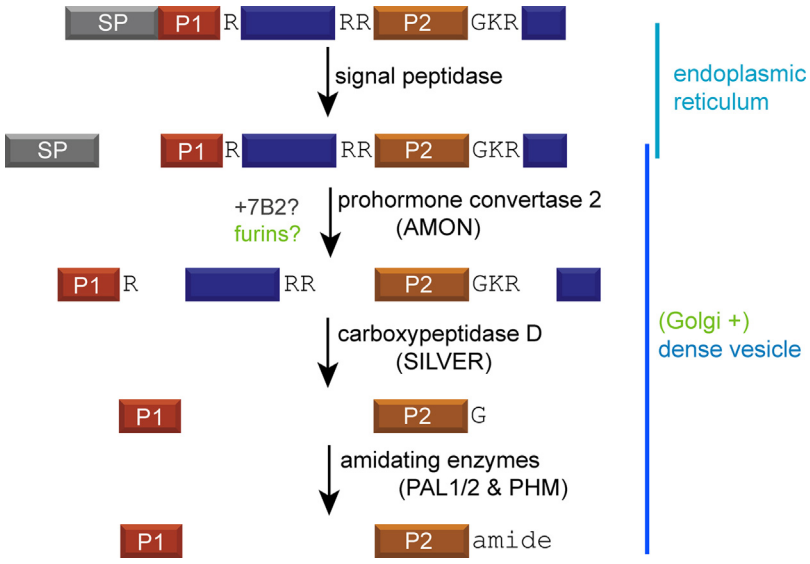

Fig. 1 – Current model of peptide processing in the regulated secretory pathway of *Drosophila*. During translation, the prepropeptide containing a signal peptide (SP, grey), two peptide-containing stretches (P1, P2 in red and orange) and spacer regions (in blue) is translocated into the endoplasmic reticulum and the signal peptide is removed. The resulting propeptide is then transported to the Golgi apparatus where peptide processing by furins (and perhaps some early PC activity) may already start. Dense-core vesicles containing propeptides and processing enzymes bud from the *trans*-Golgi. Within the dense-core vesicles, (further) processing takes place by the indicated enzymes. First, the prohormone convertase AMON cleaves the propeptide C-terminally of mono- or dibasic cleavage sites [47]. Then, the carboxypeptidase D SILVER removes the C-terminal cleavage sequence. In case of the presence of a C-terminal Gly residue after carboxypeptidase action (P2), Gly is cleaved at the C_α-atom by the amidating enzymes PAL1 or PAL2 plus PHM, resulting in a C-terminal amidation. The role of the chaperone 7B2 in activating/modulating AMON is not fully explored yet. Further peptide modification may occur (e.g. forming of an N-terminal pyroGlu, sulfatation) but is not indicated. The resulting bioactive peptides are then released by Ca^{2+}-mediated vesicle exocytosis. (For interpretation of the references to colour in text, the reader is referred to the web version of this article.)

has an unrivalled genetic toolbox; (b) the *Drosophila* genome only contains one proprotein convertase gene (*amon*) and only one relevant carboxypeptidase gene (*silver*), eliminating functional redundancy which complicates genetic interference with peptide processing in mammals, and (c) biochemical analyses in the small fly have become possible with the advent of highly sensitive and sophisticated mass spectrometers and the miniaturisation of chromatographic flow rates to the μl (capLC) or nl (nanoLC) range (see 2.1).

After release into the extracellular space, peptides are further processed and degraded by peptidases such as neprilysins. This has recently been reviewed for *Drosophila* and other insects [65] and is not discussed here.

3.1. *Genetics and structure of Drosophila processing enzymes*

The *Drosophila* genome contains several genes predicted to encode enzymes potentially involved in neuropeptide biosynthesis (Table 1, [5]): three subtilisin-like serine endoproteases, two carboxypeptidases, three amidating enzymes, and one prolyl-endoprotease (CG5355). The functions and genetics of dCPM and prolyl-endoprotease are uninvestigated, in contrast to the remaining genes which have been well characterised even before the *Drosophila* genome became accessible. In addition, there are six angiotensin-converting enzyme (ACE)-like enzymes (ACER, ANCE, ANCE2-5). Of the ACE-like enzymes, only ACER and ANCE appear to be active enzymes [66] and are involved in peptide degradation or modification after release rather than in prohormone processing (see [65]) These ACE-like enzymes are therefore not reviewed here.

Cathepsins provide an alternative propeptide processing pathway in mammals, where they can act as endopeptidases and aminopeptidases inside secretory vesicles [67,68]. These cysteine proteases participate in lysosomal protein degradation and other processes, yet it is unknown whether any of the *Drosophila* cathepsins is involved in the processing of regulatory peptides.

3.2. *Subtilisin-like endoproteases:furins*

The characterisation of prohormone processing genes started 1991 by the work of Roebroek and colleagues [69], who used a phage library of *Drosophila* genomic DNA to screen for human *furin* sequences. This approach revealed *dfur1* and *dfur2* [69,70]. *dfur1* is a 7.3 kb gene of 13 exons which, by alternative splicing, gives rise to three furin-like proteins dFURIN1 (892 aa), dFURIN1-CRR (1101 aa) and dFURIN1-X (1269 aa, [71]). All three dFURIN1 are identical up to amino acid residue 775, but differ in their C-terminal sequence. The common part of all three proteins includes a putative transmembrane domain, a pro domain followed by a subtilisin-like

Table 1 – Enzymes encoded in the *Drosophila* genome with relation to prepropeptide/preproprotein processing.

Protein symbol	Enzyme	Protein family	Gene
AMON (dPC2)	Prohormone convertase 2	Peptidase S8/proprotein convertase	*amontillado*
dFUR1	dFURIN1	Peptidase S8/furins	*furin 1*
dFUR2	dFURIN2	Peptidase S8/furins	*furin 2*
CG5355	Prolyl endopeptidase	Peptidase S9	*CG5355*
dCPD (SVR)	Carboxypeptidase D	Peptidase M14/carboxypeptidase	*silver*
dCPM	Carboxypeptidase M	Peptidase M14/carboxypeptidase	*CG4678*
PAL1	Peptidyl-α-hydroxyglycine-α-amidating lyase 1	Peptidyl-alpha-hydroxyglycine alpha-amidating lyase	*Peptidyl-α-hydroxyglycine-α-amidating lyase 1*
PAL 2	Peptidyl-α-hydroxyglycine-α-amidating lyase 2	Peptidyl-alpha-hydroxyglycine alpha-amidating lyase	*Peptidyl-α-hydroxyglycine-α-amidating lyase 2*
PHM	Peptidylglycine-α-hydroxylating monooxygenase	Copper type II ascorbate-dependent monooxygenase	*Peptidylglycine-α-hydroxylating monooxygenase*

catalytic domain and the middle domain, and is also found in dFURIN2 (1680 aa). dFURIN1-X has a unique domain of 377 amino acid residues encoded by exon 10, located after amino acid residue 775 and without special structural feature. dFURIN-CRR consists of a cysteine-rich region, divided into two subdomains, beyond amino acid residue 856. dFURIN1 and dFURIN2 lack an N-terminal signal peptide but contain an additional potential transmembrane domain at the C-terminal end. In contrast, dFURIN1-X and dFURIN1-CRR as well as the mammalian FURINs only contain one transmembrane domain in the N-terminal part. Similar to dFURIN1-CRR, dFURIN2 proteins consist of a large cysteine-rich region beyond the middle domain [70]. The consensus cleavage site of mammalian furins is R-X-K/R-R [72,73] which can at least *in vitro* also be cleaved by proprotein convertases [73]. All dFURINS display a cleavage specificity similar to that of mammalian FURINs [74,75], and both fly and human furins have been shown to also cleave at dibasic RR or KR and monobasic R sites when heterologously expressed in cell culture along with different mammalian proproteins [75]. In this assay, the cleavage specificity towards mono- and dibasic cleavage sites appeared to be similar to mammalian PC1 but differed to mammalian PC2 [75].

Since PCs show a redundancy of substrate cleavage specificity *in vitro* with specific substrates *in vivo*, the cleavage of precursors very likely depends on structural properties of the substrate and amino acids surrounding recognition sites and the differential subcellular distribution of subtilisin-like endoproteases within the secretory pathway [76] (see Fig. 1). This opens the possibility that dFURINS are involved in the processing of regulatory peptides in *Drosophila*, and dFURIN1 is co-expressed with dPC2 AMONTILLADO (AMON, see 3.3) in a few but not all peptidergic neurons: the FMRFamide-like peptide-expressing Tv neurons and other putatively peptidergic ap-let neurons [77,78]. The functional importance of dFURINS in the biosynthesis of regulatory peptides remains however unclear for *Drosophila*, especially since furins are commonly regarded as ubiquitous house-keeping genes involved in the production of secreted proteins [72]. This is supported by the finding that a dFurin1 homolog of the mosquito *Aedes aegypti* is involved in the processing of the major yolk protein vitellogenin [79]. Furthermore, FMRFamide-like immunoreactivity in the Tv neurons is completely abolished in *amon* mutants, suggesting that dFURIN1 is not involved in FMRFamide-like peptide processing [33]. Unlike in mammals, dFURINS appear also not to be required for the maturation of d7B2, a chaperone important for functional activation of dPC2 [80] (see 3.3). In contrast, the role of dFURINS in processing bone morphogenetic protein signals (Glass bottom boat, Screw) is well investigated [81–83].

3.3. *Subtilisin-like endoproteases:proprotein convertases*

The gene encoding *Drosophila* prohormone convertase 2 AMONTILLADO (AMON) is composed of 12 exons with around 16 kb of genomic sequence resulting in a 97 kDa AMON protein with 654 amino acid residues [84]. AMON shows a high 66% overall sequence identity to its human homolog PC2, with 75% identity within the catalytic domain [84,85]. In contrast, the sequence identity of the AMON catalytic domain to dFURINs is only about 50% [84]. AMON contains a signal sequence or pre-domain, a pro-domain followed by a subtilisin-like catalytic domain and the typical P-domain. The C-terminal part consists of a short extension beyond the P-domain and no transmembrane domain. Essential for enzymatic activity is the active site formed by a catalytic triad (Asp, His, Ser) and Asp at the catalytically important oxyanion hole [84]. During enzyme maturation, dPC2 autocatalytically activates itself, with the release of catalytically active enzyme fully depending on the presence of the small neuroendocrine protein 7B2 [61,80]. Unlike for the dFURINs, the cleavage specificity of dPC2 AMON has not been investigated in detail. Inferred from the reduction of peptide levels in *amon* mutants (see 3.6) it seems that dPC2 shows a broader cleavage specificity for both mono (R)- and dibasic (RR, KR, RK, KK) sites. This would be concurrent with the situation in vertebrates [61].

The localisation of *amon*-expressing cells has been studied by *in situ* hybridisation [23,84,86], immunostainings [78,87] and promotor-driven GAL4 expression [33,87]. AMON is

widely expressed in what appears to be largely or exclusively peptidergic cells. *amon* mRNA expression peaks at late embryogenesis [84], and appears to be low during the larval and adult stage. This may explain the large differences in labelled cell numbers between stages and between methods, and prevents reliable cell counts. It is however clear that AMON is localised to the enteroendocrine cells in both larvae and adults [23,87], in the endocrine AKH cells [86] as well as the secretory peptidergic neurons innervating the neurohemal organs (corpora cardiaca, perisympathetic neurons) and expressing CAPA peptides, HUGIN-PK, FMRFa-like peptides, myosuppressin, sNPFs and corazonin [33,78,87].

The importance of functional AMON in the production of bioactive neuropeptides was genetically demonstrated by an *amon* deficiency or nonsense or missense point mutations that lead to a lack or impairment of the catalytic centre [85]. These mutants die after hatching from the egg or latest during larval ecdyses [84,85]. A similar phenotype was also visible when ectopically overexpressing serpin4.1 in large sets of peptidergic neurons, suggesting that this protein is an inhibitor of AMON [88]. An inhibitory action of this serpin on subtilisin-like endoproteases (human furin) has been demonstrated *in vitro* [88,89].

3.4. *Carboxypeptidases*

The *silver* gene in *Drosophila* was first cloned and characterised by Settle and coworkers [90]. *silver* encodes a metallocarboxypeptidase D (dCPD) with three carboxypeptidase-like domains, a transmembrane domain and a cytosolic tail region. The domain composition of dCPD clearly identifies this protein to be a homolog of avian CPD and mammalian CPE [91]. dCPD occurs in long and short protein forms. A detailed approach analyzing *Drosophila* genomic and EST database sequences as well as RT-PCR and sequencing led to the finding that the *silver* gene contains eight exons [91]. The first exon is alternatively spliced into three forms: 1A, 1B and 1C. Exons 1A and 1B are found in both long and short forms including N-terminal regions encoding signal peptides. In contrast, 1C mRNA does not encode for an N-terminal signal peptide and contains a truncated part of the first carboxypeptidase-like domain. The long forms of CPD contain all three CP-like domains, short bridge regions b1 and b2 between the CP-like domains, and a C-terminal transmembrane domain followed by a cytosolic tail. While all CP-like domains of dCPD fold into a carboxypeptidase-like structure [91,92], the third domain shares only a low sequence similarity with the first two active domains and lacks several key residues essential for substrate binding and enzymatic activity [91]. It is therefore predicted to be enzymatically inactive [91]. Domain 1A lacks a metal-binding His residue (replaced by Gln) which strongly reduces enzymatic activity based on reduced affinity to the co-factor Zn^{2+}. This catalytical inactivity of 1A is confirmed by the inability of transgenically expressed domain 1A to rescue the lethality of a defective *silver* gene [93]. The 1A domain is thus rather involved in Zn^{2+}-independent substrate binding. Domains 1B and 2 show a comparable enzymatic activity but have a different pH optimum. Domain 1B is maximally active at pH 7–8, and domain 2 between pH 5 and 6.5. These different pH optima seem to be essential for full enzymatic efficiency as CPD functions in the trans-Golgi network, the secretory pathway and the reuptake pathway, i.e. different compartments with different pH values. Both domains trim C-terminal Arg and Lys residues after FURIN/PC cleavage, with domain 1B preferring Arg and domain 2 Lys [93]. All active domains of *Drosophila* CPD can be inhibited by different chemicals like e.g. *para*-chloromercuriphenylsulfonate (with domain 1 less sensitive than domain 2) while they are activated by e.g. Co^{2+} (with domain 2 less sensitive than domain 1B) [91].

The importance of functional dCPD in the production of bioactive neuropeptides was genetically demonstrated by embryonic lethality in a number of *silver* mutants [90] and flies with a P-element insertion that disrupts the *silver* gene [94].

The function of the second carboxypeptidase encoded in the *Drosophila* genome (dCPM) is uninvestigated. As its gene expression appears to be upregulated in the nervous system of both larval and adult flies [95], it is possible that dCPM also contributes to neuropeptide processing.

3.5. *Amidating enzymes*

Most *Drosophila* peptides are C-terminally amidated (see Supplementary Table 1), a modification that typically is essential for bioactivity. Each of the two steps of peptide amidation in the fruitfly is catalysed by an own enzyme encoded on a separate gene [96]. This is in contrast to the bifunctional peptidylglycine-α-amidating mono-oxygenase (PAM) of vertebrates (see [97]). First, glycine-extended intermediate peptides obtained after PC and subsequent CPD action are hydroxylated by a copper-containing peptidylglycine-α-hydroxylating mono-oxygenase (PHM; [77,96,97]). Then, a peptidyl-α-hydroxyglycine-α-amidating lyase (PAL) catalyses the cleavage of the hydroxylated intermediate to yield the final α-amidated peptide [96,97]. The dPHM gene of around 1.4 kb contains eight exons and seven introns and encodes a protein of 365 amino acids. dPHM consists of a hydrophobic N-terminus and a signal peptide. It shares a high sequence similarity to the PHM domain of vertebrate PAMs including eight cysteine residues in homologous positions and two histidine-rich sequence clusters likely to be required for copper binding. dPHM and the PHM domain of vertebrate PAM differ in the presence of alternative splicing sites, which appear to be absent in the *Drosophila* gene. Although dPHM is separately encoded, its exon/intron structure is fairly similar to that of the dPHM domain of vertebrate PAM, indicating a common ancestral gene.

Two genes code for dPAL proteins [98]. Both dPAL1 and dPAL2 share four conserved cysteine residues with the PAL domain of vertebrate PAM, forming disulfide bonds, and contain two potential N-glycosylation sites. In addition, dPAL1 is characterised by putative transmembrane domains close to both its N- and C-terminal end. dPAL2 contains a typical signal sequence motif, whereas it seems to be absent in dPAL1 [98]. dPAL2 is thus secreted, while dPAL1 remains membrane-bound, as suggested by a differential localisation within the regulated secretory pathway and by release studies in heterologous cell culture systems [98].

The pH-optimum of dPHM that requires copper and ascorbate is in an acidic pH range about 5.0 similar to the activity of vertebrate PHM [96]. In contrast, the enzymatic activity of

dPALs is increasing with higher pH and is at max around pH 7.0 unlike for vertebrate PAL activity [98]. This appears to coincide with a generally higher pH in *Drosophila* peptidergic vesicles compared to vertebrate vesicles [99]. Both dPHM and dPAL act in the secretory granules. The different pH optima for these enzymes are thus somewhat surprising. During amidation, PHM catalyses the production of a peptidyl-α-hydroxyglycine intermediate, which is then processed further to the amidated peptide plus glyoxylate [97]. Thus, the sequential action of PHM and PAL may correspond with or even be regulated by a decreasing acidic pH during secretory granule maturation.

The importance of functional dPHM in the production bioactive neuropeptides was genetically demonstrated by a P-element insertion that disrupts the dPHM gene and causes embryonic lethality in homozygous stocks [77,96]. This lethality appears to be caused by a lack of important motifs including one of the two dihistidine motifs for copper binding [77]. With a mosaic approach, Taghert and colleagues also showed that peptide amidation is required for neuropeptide-regulated behaviour [100].

3.6. Combining genetics and peptidomics to characterise the role of AMON in peptide processing

Given the occurrence of only one canonical PC (dPC2 AMON) and the phenotypes indicative of impaired peptide signalling observed in *amon* mutants, it is not far-fetched to assume that AMON is the major PC involved in the processing of fruit fly peptides. This hypothesis was first tested for adipokinetic hormone (AKH) which is produced by endocrine cells intrinsic to a neurohemal organ known as corpora cardiaca (CC) [86]. $amon^{C241Y}$ mutant flies die early during larval development. Lethality can however be rescued by ubiquitous ectopic expression of a *hs-amon* heatshock construct [84,85]. In $amon^{C241Y}$ mutant L3 larvae that received one heatshock per day, AKH and its C-terminally extended processing intermediate AKH-GK could be detected by direct MALDI-TOF mass spectrometric profiling of CC of individual larvae similar to genetic controls [86]. Suspension of the heatshocks for three days however resulted in a complete loss of the detectability of AKH and AKH intermediates. This phenotype could be rescued by giving a heatshock a day prior to mass spectrometric analysis after suspending the heatshock. This provided direct evidence that AMON is involved in the processing of native fruit fly peptides. Surprisingly, repeating this experiment for the CC of adult flies revealed that AKH and its intermediates are still detectable in $amon^{C241Y}$ mutants even after several days without heatshock. While the underlying reasons for this are not well understood, the difference in AKH detectability may be related to the rapid growth and hence constant need of new AKH synthesis in larvae, while the non-growing adults living in small glass tubes with permanent access to food may not need high rates of AKH production as AKH release is not triggered by extensive flight or starvation. Nevertheless, adding a heavy, stable isotope-labelled synthetic AKH to the MALDI matrix in direct peptide profiling allowed to semiquantify native AKH levels in $amon^{C241Y}$ mutants by calculating the intensity ratio between labelled and native AKH. This approach showed that adult $amon^{C241Y}$ mutants several days without heatshock had 16 times lower AKH levels than controls. Also AKH-GK was strongly reduced [86].

The semiquantitative direct peptide profiling approach (with heavy stable-isotope labelled synthetic AKH, HUGIN-PK and myosuppressin added to the tissue along with the matrix) allowed to analyse the levels of other neuropeptide hormones stored in the CC [33]. Like AKH, all the neuropeptide hormones detectable in the CC of controls were undetectable in $amon^{C241Y}$ mutant larvae 3 days after last heatshock. In adult CC, these neuropeptide hormones could be detected in a substantial fraction of $amon^{C241Y}$ mutants several days after last heatshock, albeit at strongly reduced levels [33]. In contrast to neurohemal organs such as the CC, more complex tissues such as the brain or gut cannot be analysed by direct peptide profiling. Pooled CNS and midgut extracts (the midgut houses all enteroendocrine cells in the fly) were thus analysed by LC/MS–MS to investigate the role of AMON in processing enteroendocrine and interneuronal peptides. In $amon^{C241Y}$ larvae and adults several days after last heatshock, the detection rate for most enteroendocrine peptides dropped to zero or was very much reduced compared to controls. In adults, however, 3 out of 24 enteroendocrine peptides were detected at the same rate in $amon^{C241Y}$ mutant as in controls [23]. In the CNS, the peptide detection rate did not differ significantly for most peptides between $amon^{C241Y}$ mutant and controls (Pauls, Reiher, Wegener, unpublished data). The results from semiquantitative peptide profiling in the CC suggest, however, that the CNS peptides detectable in $amon^{C241Y}$ mutants may occur – albeit detectable – at much lower concentration than in controls.

These results demonstrated on the biochemical level that AMON is a key enzyme in the processing of fruit fly peptides. It is however not entirely clear why peptides are still – albeit at low level – detectable in adult *amon*-deficient flies. Several reasons may account for this finding. Probably some other subtilisin-like endoproteases (e.g. the furins) secure a baseline production of peptides even in the absence of AMON. This would be somewhat similar to the situation in mammals, where redundancy between different PCs has been demonstrated (e.g. [101]). It is also possible that the $amon^{C241Y}$ mutation represents a hypomorph, resulting in a PC with strongly reduced yet residual processing activity. This is, however, less likely since similar results have been obtained by direct peptide profiling of adult CCs in $amon^{Q178st}$ mutants (Wegener, unpublished) that are expected to produce a truncated PC2 without the catalytic sites [85]. Nevertheless, several cases are known in *Drosophila* where despite a stop codon or a nonsense mutation genes have been fully translated albeit at a lower rate [102,103]. The least exciting hypothesis is that of a background expression of the hs-rescue transgene even at low temperature, which is known from hs-*phm* constructs [77]. More research will be needed to address these hypotheses.

3.7. Combining genetics and peptidomics to characterise the role of SILVER in peptide processing

Since homozygous loss of SILVER = dCPD function is lethal, and rescue lines have so far not been produced, the general relevance of dCPD for peptide processing has not been tested

by peptidomics. Processing intermediates resulting from PC cleavage and still carrying the C-terminal mono- or dibasic cleavage signal have not been found in peptidomic studies of insects, with exception of AKH using MALDI-TOF MS (e.g. [22,27,28,104–106]). This suggests that the C-terminal removal of the basic cleavage signal usually occurs fast and is not a rate limiting step during peptide processing. In *Drosophila* and other insects, AKH is usually not detected as $[M+H]^+$ adduct, but as $[M+Na]^+$ or $[M+K]^+$ adducts in MALDI-TOF MS. In contrast, the AKH processing intermediates containing a C-terminal basic cleavage signal are easily protonated and occur predominantly as $[M+H]^+$ adducts. This chemical peculiarity of AKH likely is the reason why the presumably low-abundance processing intermediates can be detected for AKH, but not for other peptides. In *Drosophila*, both AKH processing intermediates (AKH-GK and AKH-GKR) are detectable by MALDI-TOF MS [33,93], and this fortuitous finding was used to test for cleavage specificity of the different dCPD domains. Different *silver* constructs corresponding to different endogenous CPD forms produced by alternative splicing were specifically expressed in *silver*-expressing cells in a mutant background or in AKH cells in a wildtype background. Then, the level of AKH, AKH-GK, and AKH-GKR was quantified by semiquantitative direct peptide profiling. All transgenic CPD lines showed a large and significant reduction of AKH-GK compared to controls [93]. The results also revealed that CP domain 1 of dCPD more readily removes R, while CP domain 2 more readily removes K, though both domains can act redundantly. This led to the suggestion that CP domain 1 acts earlier and CP domain 2 acts later (at lower pH) in the maturation of secretory vesicles [93].

These results provided the so far only biochemical evidence that dCPD is involved in processing endogenous *Drosophila* peptides. Using a similar genetic rescue as performed for dPC2 (see above) would be one way to test whether dCPD is the sole enzyme responsible for C-terminal trimming during peptide processing.

3.8. *Combining genetics and peptidomics to characterise the role of amidating enzymes in peptide processing*

While there is as yet no direct peptidomic data available on the effect of mutations in the amidating enzymes PHM and PAL, peptide amidation is strongly compromised in flies with cell-specific RNAi-mediated downregulation of the P-type ATPase copper transporter ATP7 [107]. PHM is one of the few enzymes that use copper as a cofactor, with an optimal copper concentration for isolated *Drosophila* PHM between 0.5 and 2 μM [96]. During the ascorbate-dependent monoxygenase reaction, the two spatially separated coppers alternate between a Cu(I) and Cu(II) state (see [97]). ATP7 transporters are regulators of intracellular copper homeostasis and typically located in the membrane of the trans-Golgi network [108]. In the fly, ATP7 is expressed in many if not all peptidergic neurons as suggested by ATP7-promotor-driven marker expression and peptide co-immunostaining [107]. In mammals, ATP7A delivers Cu(I) to PHM and other cuproenzymes in the lumen of the secretory pathway [109]. When the single *Drosophila* ATP7 was down-regulated by cell-specific RNAi using the binary GAL4-UAS expression system in flies kept on a copper-reduced diet, this resulted in the occurrence of non-amidated glycine-extended neuropeptides in direct peptide profiling of neurohemal and nervous tissue [107]. It has to be stressed that such glycine-extended processing intermediates have never been observed in wildtype flies, suggesting that the amidation process is not a rate-limiting step in peptide processing. Since most *Drosophila* peptides are C-terminally amidated and the amidation is essential for receptor activation, sufficient copper supply may be as essential for the fly as it is for humans [110].

3.9. *Differential processing of neuropeptides in the fruit fly nervous system*

It has become textbook knowledge that vertebrate prepropeptides like pro-opiomelanocortin (POMC) or proglucagon undergo tissue-specific processing, leading to differential peptide expression between peptidergic cells expressing the same prepropeptide gene (e.g. [111]). In contrast, the CAPA prepropeptide is so far the only insect prepropeptide that has been shown to be differentially cleaved in a cell-specific manner in various species incl. *Drosophila* [27,28,112–114] (see also Neupert et al., 2014 – this special issue). The molecular reason for this is unclear, while alternative mRNA splicing of the *capa* mRNA can be excluded for the fruit fly [115]. CAPA processing is strongly impaired in all *capa*-expressing cells in *amon* mutants [33]. Future research has to show whether the differential CAPA processing is due to differences in the processing enzyme complement (AMON plus further enzymes such as furins [115]), or whether AMON specificity is modulated by the vesicle milieu or may be modulated by other factors such as 7B2 [116].

4. Outlook

As highlighted in this review, we have gained a solid knowledge on the peptidomics of *Drosophila*, and sophisticated genetic and peptidomic techniques are established. What is needed now is to take the next step and go from more or less descriptional peptidomics to functional peptidomics, as the fruit fly offers unmatched opportunities to combine advanced genetic tools with quantitative peptidomic techniques to study peptide biology at all levels. The studies on peptide processing reviewed above are a first step into that direction. Another set of experiments combined ectopic overexpression/*in vivo* RNAi of a transcription factor (DIMMED) and ectopic expression of a heterologous prepropeptide with LC–MALDI-TOF or LC–ESI-MS/MS with an ion trap to study DIMMED functions [117,118]. After ectopic expression of DIMMED (similar to vertebrate MIST-1) and a designed peptide precursor in non-peptidergic photoreceptors, Hamanaka and colleagues could show the presence of processed peptide in extracts from whole heads (including the photoreceptors) by LC–MS, while the peptide is not processed when only the peptide precursor alone is expressed [117]. This elegantly demonstrated that DIMMED conveys a peptidergic secretory phenotype onto otherwise non-peptidergic cells. This combination of sophisticated genetics and LC–MS was then later

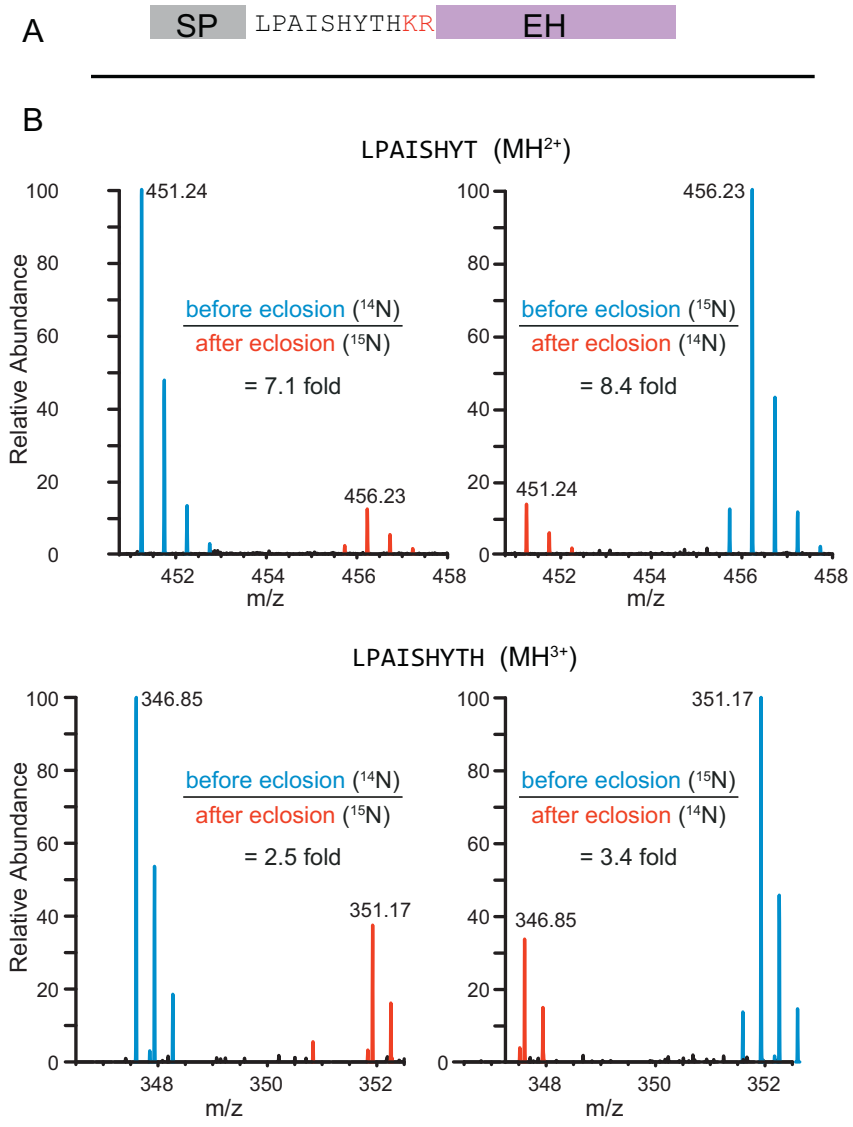

Fig. 2 – "Proof-of-principle" of quantitative peptidomics in *Drosophila*, using a spacer peptide generated during eclosion-hormone (EH) processing. EH is a key neuropeptide orchestrating the motor behaviour that propels flies out of their puparium during eclosion. A comparison of immunofluorescent staining intensities just before or after eclosion has shown that EH is massively released during eclosion [126]. Flies were raised on either ^{14}N- or ^{15}N-labelled yeast, then brains were dissected from pharate females that were either very close to eclosion or freshly eclosed with unexpanded wings. Ten brains and thoracico-abdominal ganglia from each group were pooled and peptides were extracted with 90% MeOH containing 0.5% TFA. The pooled peptides were analysed by nanoLC–MS/MS on an Orbitrap mass spectrometer (EASY-nLC 1000 coupled to LTQ Orbitrap Velos, Thermo). For relative precursor peptide quantitation, a peptide ratio ($^{14}N/^{15}N$) was calculated for each identified peptide using Mascot Distiller (Matrix Science) software. Since EH (8 kDa) is very difficult to detect by MS, a smaller spacer peptide (expected to be generated in equimolar amounts to EH during processing) was quantified. (A) Shows the structure of the EH prepropeptide. The signal peptide (SP, in grey) is removed during the translational process. Then a subtilisin-like endoprotease (most likely dPC2) cleaves C-terminal of the dibasic cleavage sequence KR (in red), which is then removed by a carboxypeptidase (most likely CPD). This generates EH (in mauve) and the sequence LPAISHYTH, and – after further carboxypeptidase action – LPAISHYT, all peptides being released upon regulated exocytosis. (B) Quantification of LPAISHYTH and LPAISHYT before and after eclosion. To exclude unspecific effects of ^{15}N, reciprocal experiments were carried out. The results show a 7–8 fold (LPAISHYT) and a around 3 fold (LPAISHYTH) decrease of peptide levels, confirming the EH stainings and indicating a massive release of EH during eclosion. (For interpretation of the references to colour in text, the reader is referred to the web version of this article.)

used to screen for target genes downstream of DIMMED [118].

The rather easy metabolic labelling of whole flies with heavy isotopes by feeding yeast raised on ^{15}N medium

[119,120] provides an excellent opportunity to conduct quantitative peptidomic studies in the fruit fly, with achievable peptide labelling efficiencies of >98% ([119] Chen, Vanselow, Schlosser, Wegener, unpublished). When feeding ^{15}N-labelled yeast to an experimental group ("heavy"), and normal unlabelled yeast to controls ("light"), tissues of both groups can be pooled, extracted and analysed by LC–MS. This excludes all artefacts of unequal peptide loss, extraction efficiency, etc. between experimental and control groups. Fig. 2 gives an example of peptide quantification using ^{15}N. As ^{15}N-labelled yeast appears to have mild effects on the growth of flies, labelling should always follow a reciprocal design (Fig. 2). In combination with qPCR, quantitative LC–MS has an excellent potential to study the activation of peptide signalling under specific physiological or behavioural conditions such as stress, hunger or different behaviours.

Still a major quest is to develop techniques to identify and ideally also quantify peptides by LC–MS in the hemolymph of fruitflies. Already a very difficult task in large insects with a large hemolymph volume such as moths [121], all attempts to reliably identify peptides in the fruit fly hemolymph have failed so far. This failure is caused by the small amount of hemolymph (in the lower μl range in larvae, <1 μl per adult fly) and the low concentration of circulating peptide hormones (expected to be in the nM range), and not the least by the high peptide/protein complexity of hemolymph [122] which prevents simple peptide identification by mass only [121]. In contrast, immune-induced peptides occur in the mM range and are easily mass spectrometrically identified in the hemolymph [8,10,123]. Development of a MS-based method to analyse peptide hormones would be extremely helpful to establish *Drosophila* also as a true endocrinological model, e.g. for the circadian control of peptide hormone secretion.

Peptidomic approaches have so far been limited to the identification and quantitation of smaller peptides or to the indirect analysis of non-bioactive peptides originating from prepropeptide processing as a proxy for larger peptide hormones (see Fig. 2). With recent developments in mass spectrometry, i.e. high sensitive, high resolution mass analyzers (e.g. Orbitrap), combination of different fragmentation techniques [124] and advanced analysis software [125], the analysis also of large peptide hormonesas well as low abundant peptide modifications is now becoming technically feasible.

So, it's exciting times for *Drosophila* peptidomics!

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.02.007.

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