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Endocrine peptides and insect reproduction

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Summary

In insects, peptide hormones regulate many physiological and developmental processes such as growth, moulting, metamorphosis, reproduction, diapause, feeding and metabolism. This review focuses on those involved in reproduction, in particular vitellogenesis and oogenesis, spermatogenesis, ovulation and pheromone production. Most of these peptides regulate the production of the true gonadotrophic hormones (as in the case of allatostatins, allatotropins and ovary ecdysteroidogenic hormones) or the production of pheromones (as in the case of pheromone biosynthesis activating neuropeptides). Other peptides seem related to reproductive processes, but it is not clear whether they play a true regulatory role. Cloning approaches have greatly facilitated the solution of structural aspects of peptide research; the major challenge now is to approach the functional aspects.

Key words: Peptide, neuropeptide, hormone, insect reproduction

Introduction

Endocrinology of insect reproduction has traditionally focused on the two most thoroughly studied groups of insect hormones: juvenile hormones and ecdysteroids (Bellés, 2005; Wang et al., 2004). However, evidence is accumulating that endocrine peptides also play fundamental roles in reproductive processes. The present review deals with the involvement of endocrine peptides in processes related to reproduction, namely vitellogenesis, oogenesis, spermatogenesis, ovulation and pheromone production. A comprehensive review on this subject was published more than twenty years ago by Girardie (1983). More recent reviews deal with general physiology of insect peptides (Gäde et al., 1997) or applied aspects for pest control (Gäde and Goldsworthy, 2003).

Since there is a vast amount of information available, this review must be selective. This means that studies based on the effects of extracts from the brain or other tissue will not be considered, even though such studies might illuminate reproductive processes like accessory gland function or parturition, for example, for which peptides involved have not been identified yet. Moreover, the review focuses on endocrine peptides. We did not deal with compounds, like sex peptides, that are transferred from one sex to the other one and modify reproductive processes. Finally, the present contribution is not intended to be an exhaustive catalogue of peptide structures related to reproduction. The review by Gäde (1997) covers these structural aspects.

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Vitellogenesis and Oogenesis

Much information on the endocrine dependency of vitellogenesis has been reported for the most important insect groups. Comprehensive reviews have been published, for example, by Hagedorn (1985), Wyatt and Davey (1996), Wang et al. (2004) and Bellés (2005). Data available suggest that in less specialized groups (cockroaches, for example) vitellogenesis is governed mainly by juvenile hormone (JH) produced by the corpora allata (CA), whereas ecdysteroids are involved in the activation of choriogenesis (Bellés et al., 1993). In contrast, in more specialized insect groups (like mosquitoes), vitellogenesis is regulated mainly by ecdysteroids produced by the ovaries, whereas JH acts on several tissues, including fat body and ovaries, enabling them to perform their adult-specific functions (Fig. 1). However, in each of these extreme cases there are exceptions, and between them there are intermediate situations, sometimes modulated by contextual biological strategies, either reproductive, like in Lepidoptera, or social, like in Hymenoptera (Bellés, 2005). In addition to JH and ecdysteroids, a number of peptides are also involved in vitellogenesis. Most of these peptides have an indirect effect, regulating the production of JH or ecdysteroids in the vitellogenic cycle. Therefore, in species where vitellogenesis is JH-dependent, there are peptides that stimulate (allatotropins) or inhibit (allatostatins) the activity of the CA, thus influencing the production of JH and, in turn, that of vitellogenin. In species where vitellogenesis is directed by ecdysteroids produced by the ovary, the regulatory factors may be ootropic or oostatic (Fig. 1).

Allatotropins

The first allatotropin was identified by Kataoka et al. (1989) in the lepidopteran *Manduca sexta*, with the structure GFKNVEMMTARGF-NH_2. The same allatotropin has been found in the lepidopterans *Spodoptera frugiperda* (Oeh et al., 2000) and *Agrius convolvuli* (Kataoka unpublished, see Truesdell et al., 2000). Kataoka et al. (1989) reported that the *M. sexta* allatotropin stimulated the adult, but not the larval, lepidopteran CA. However, more recent experiments using ^{14}C-propionate instead of the usual radiolabelled [methyl]-methionine, have shown that *M. sexta* allatotropin stimulates JH biosynthesis in larval CA of the lepidopterans *Lacanobia oleracea* (Audsley et al., 2000) and *Samia cynthia ricini* (Li et al., 2002). In addition, it stimulates JH biosynthesis in the hymenopteran *Apis mellifera* (Rachinsky and Feldlaufer, 2000) and in the dipteran *Phormia regina* (Tu et al., 2001).

The gene coding for this allatotropin has been cloned and sequenced in the lepidopterans *M. sexta* (Taylor et al., 1996), *Pseudaletia unipuncta* (Truesdell et al., 2000), *Bombyx mori* (Park et al., 2002) and *S. frugiperda* (Abdel-latief et al., 2003). In the mosquito *Aedes aegypti*, a related peptide (APFRNSEMMTARGF-NH_2) has been isolated, and the corresponding cDNA has been cloned and sequenced (Veenstra and Costes, 1999). This peptide stimulates adult mosquito CA in a dose-dependent and age-specific manner (Li et al., 2003).

In *M. sexta*, *in situ* hybridization studies have revealed allatotropin-expressing cells in the brain, frontal ganglion and terminal ganglion (Taylor et al., 1996). In *P. unipuncta*, allatotropin gene expression

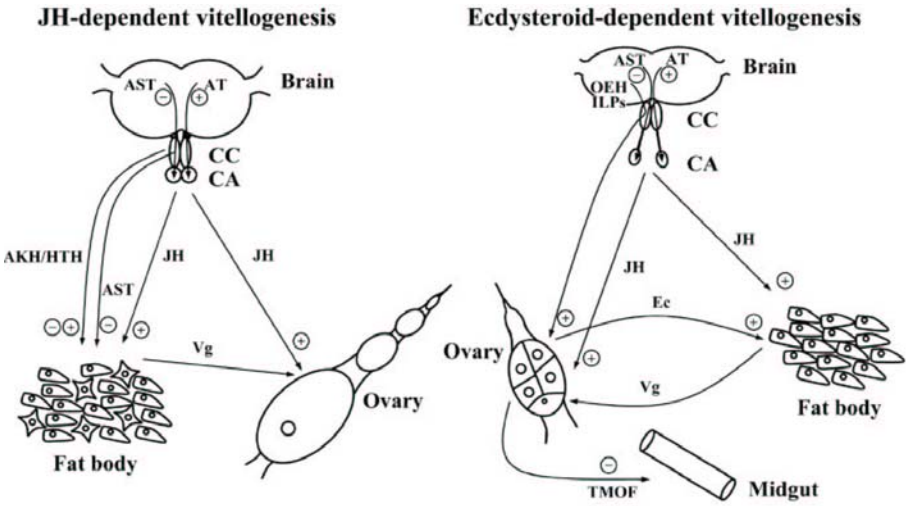

Fig. 1. Hormones influencing vitellogenesis in JH- and ecdysteroid-dependent insects. AKH: adipokinetic hormone; AST: allatostatin; AT: allatotropin; CA: corpora allata; CC: corpora cardiaca; Ec: ecdysteroids; HTH: hypertrehalosemic hormone; ILPs: insulin-like peptides; JH: juvenile hormone; OEH: ovarian ecdysteroidogenic hormone; TMOF: tripsin-modulating oostatic factor; Vg: vitellogenin. Signs + and − indicate influence on vitellogenesis.

starts in late pupae and continues during adult life. The pattern of expression parallels that of JH biosynthesis (Truesdell et al., 2000), which supports the hypothesis that allatotropin regulates JH production and vitellogenesis in the adult stage. As in *M. sexta*, *in situ* hybridization revealed that allatotropin is ubiquitously expressed in the nervous system of *P. unipuncta* (Truesdell et al., 2000). This suggests that this peptide may have other functions in addition to regulating JH synthesis. In this regard, it is worth noting that the allatotropin-like peptide GFKNVALSTARGF-NH_2 has been identified as a myotropin in the locust *Locusta migratoria* and in the Colorado potato beetle, *Leptinotarsa decemlineata* (Paemen et al., 1991; Spittaels et al., 1996a).

Allatostatins

YXFGL-NH_2 allatostatins

The first members of this group, characterized by a pentapeptide amidated C-terminal sequence YXFGL-NH_2, were reported in the cockroach *Diploptera punctata* (Woodhead et al., 1989). Later, similar peptides were identified in other cockroaches and crickets, for which the corresponding genes have been cloned and sequenced (Bellés et al., 1999; Meyering-Vos et al., 2001). Molecular studies in cockroaches and crickets have revealed that the YXFGL-NH_2 allatostatin gene codes for a pre-proprotein containing 13 or 14 similar allatostatin units, which are post-translationally cleaved and processed (Bellés et al., 1999; Meyering-Vos et al., 2001). All the resulting allatostatins inhibit the synthesis of JH by the CA of cockroach and cricket species to a greater or lesser extent, not only in adults but also in larvae and embryos (see Stay, 2000). Peptide levels measured in *D. punctata* adult female brain extracts inversely correlate with JH production in the CA (Yu et al., 1993). In the German cockroach, *Blattella germanica*, however, such tight correlation is not observed (Vilaplana et al., 1999), which is consistent with the pleiotropic role proposed for these peptides (see below). A number of YXFGL-NH_2 allatostatins have been identified in other insects, namely, locusts, bees, flies, mosquitoes, moths, stick-insects, and even in crustaceans, but they do not inhibit JH biosynthesis in these groups (see Bendena et al., 1999, Stay, 2000; Li et al., 2004). Indeed, immunolocalization studies in cockroach and crickets have shown that YXFGL-NH_2 allatostatins occur in several brain neurosecretory cells whose axons project to the CA (Maestro et al., 1998; Neuhäuser et al., 1994), whereas these glands are not innervated with allatostatin immunoreactive neurons in dipterans (Duve and Thorpe, 1994) or lepidopterans (Duve et al., 1997). Immunolocalization studies have also revealed that allatostatins are ubiquitously distributed, not only in parts of the central nervous system other than the axis brain-CA, but also in nerves peripheral to visceral muscles, in midgut endocrine cells, in hemocytes and in ovaries (see Stay, 2000; Witek and Hoffmann, 2001; Woodhead et al., 2003). Such wide distribution in a variety of tissues suggests that YXFGL-NH_2 allatostatins are multifunctional.

Regarding the mechanisms of action, Sutherland and Feyereisen (1996) proposed that inhibition of JH biosynthesis by YXFGL-NH_2 allatostatins occurs at early steps in the biosynthetic pathway, i.e., the mitochondrial citrate–malate shuttle or the ATP-citrate lyase, enzymes regulating the levels of acetyl-CoA in the cytoplasm. Finally, Birgül et al. (1999) isolated a G-coupled receptor from *Drosophila melanogaster* heads exhibiting structural similarities to mammalian somatostatin/ galanin/opioid receptor, whose bioactive ligand was a YXFGL-NH_2 allatostatin. Afterwards, other YXFGL-NH_2 allatostatin receptors have been identi-fied in *D. melanogaster* (Lenz et al., 2000a, 2000b), *Periplaneta americana* (Auerswald et al., 2001) and *B. mori* (Secher et al., 2001).

W^2W^9-NH_2 allatostatins

A second group of allatostatins is represented by four nonapeptides (GWQDLNGGW-NH_2, GWRDLNGGW-NH_2, AWRDLSGGW-NH_2 and AWERFHGSW-NH_2), identified in the cricket *Gryllus bimaculatus*, which share the C-terminal sequence $W(X)_6W$-NH_2 (W^2W^9-NH_2 allatostatins). These peptides show high sequence similarity to a formerly isolated myoinhibitory peptide from *L. migratoria* (see below) (Schoofs et al., 1991). All of them inhibit JH biosynthesis by the CA of virgin females of *G. bimaculatus* incubated *in vitro*, although the ED_{50} was one order of magnitude higher than of YXFGL-NH_2 allatostatins (Lorenz et al., 1995). Peptides of to the same family identified from brain extracts of the stick insect *Carausius morosus* do not inhibit JH production in this species (Lorenz et al., 2000). Five homologues (AWQSLQSSW-NH_2, AWKSMNVAW-NH_2, EAQGWNKFRGAW-NH_2, EPTWNNLKGMW-NH_2 and DQWQKLHGGW-NH_2) have been reported in *D. melanogaster*, as deduced from the corresponding cDNA (Williamson et al., 2001a). Also in this species, a G protein-coupled receptor which specifically responds to

W^2W^9-NH_2 allatostatins has been reported (Johnson et al., 2003). Nevertheless, the function of these peptides in fruit flies is unknown.

Manduca-*type allatostatins*

The third group of allatostatins is represented by the 15-amino acid, non-amidated peptide pQVRFRQCYFNPISCF, discovered in *M. sexta* (Kramer et al., 1991). It inhibits JH synthesis by the CA in adult lepidopteran females, but does not affect JH production in dictyopterans, orthopterans and coleopterans (Kramer et al., 1991). The same allatostatin has been deduced from the corresponding cDNA cloned in *P. unipuncta* (Jansons et al., 1996). In *P. unipuncta*, gene expression is low in sixth instar larvae, prepupae and early pupae, but increases in late pupae and in adults of both sexes on days 1 and 3. In 5-day-old adults, expression is quite constant in females but declines in males. This pattern of expression does not correlate with JH biosynthesis in *P. unipuncta*, which increases during the first five days of adult life, suggesting that the peptide has additional functions to JH inhibition.

More recently, the cDNA of a homolog of *M. sexta* allatostatin has been cloned in *D. melanogaster*. The fruit fly peptide is only one amino acid residue different (F to Y in position 4) from *M. sexta* allatostatin (Williamson et al., 2001b), and shows myoinhibitory activity in the fruit fly (Price et al., 2002). A peptide of the same family has been deduced from the genome of the mosquito *Anopheles gambiae* (Riehle et al., 2002), showing a single change (I to V in position 2) with respect to the fruit fly sequence. This peptide inhibits JH biosynthesis in the CA of *A. aegypti* (Li et al., 2004).

Two G protein-coupled receptors were isolated in *D. melanogaster*, the physiological ligands of which were purified from larval and adult peptide extracts and turned out to be two peptides belonging to the *Manduca*-type allatostatin family (Kreienkamp et al., 2002).

Peptides stimulating ecdysteroid synthesis in the ovary

Ovary ecdysteroidogenic hormone (OEH)

In mosquitoes vitellogenesis depends on the ecdysteroids produced by the ovary (Hagedorn, 1985; Wang et al., 2004). In 1967 Lea described the occurrence of a neurohormone in the mosquito *A. aegypti* that when released from the brain in response to a blood meal, stimulated ecdysteroid production in the ovaries. It was thus referred to as "ovary ecdysteroidogenic hormone" (OEH). Some 30 years later, Brown et al. (1998) identified this neurohormone as an 86-residue peptide (Fig. 2). The recombinant version of the peptide stimulates ecdysteroid synthesis in ovaries incubated *in vitro*. In addition, immunolocalization studies revealed that OEH is produced in two or three pairs of medial neurosecretory cells in the female brain, whose axons extend to the corpus cardiacum. The sequence of OEH shows a moderate similarity with the neuroparsin A of *L. migratoria* (Fig. 2), which is commented on below.

Bombyxins and insulin-like peptides (ILPs)

The first insect peptide belonging to the insulin superfamily, bombyxin, was purified from a head extract from the silkworm *B. mori* through its ecdysteroidogenic action on prothoracic glands of the moth *S. cynthia ricini* (Nagasawa et al., 1984). Thereafter, insect insulin-like peptides (ILPs) (Fig. 3) have been identified in several insect species, like the orthopteran *L. migratoria* (Lagueux et al., 1990; Hetru et al., 1991), the lepidopterans *S. cynthia ricini* (Nagata et al., 1999) and *A. convolvuli* (Iwami et al., 1996), and the dipterans *D. melanogaster* (Vanden Broeck, 2001) and *A. gambiae* (Riehle et al., 2002; Krieger et al., 2004).

In *L. migratoria*, a single gene has been identified (Lagueux et al., 1990), but in lepidopteran and dipteran species multiple ILP genes, mostly grouped in clusters, have been reported (Kondo et al., 1996; Vanden Broeck, 2001; Riehle et al., 2002; Krieger et al., 2004). Homologues of vertebrate insulin receptor have been identified in *D. melanogaster* (Ruan et al., 1995), *A. aegypti* (Graf et al., 1997) and *A. gambiae* (Riehle et al., 2002). As in vertebrates, they consist of a heterotetramer $\alpha_2\beta_2$ with tyrosine kinase activity, which acts through the phosphoinositide 3-kinase pathway (Fernandez et al., 1995).

Besides the role of ILPs in physiological processes like metabolic control, nutritional signaling, growth and ageing (Claeys et al., 2002), they also contribute to reproduction. For example, bombyxin II binds to ovarian cells in three species of Lepidoptera (Fullbright et al., 1997). In addition, insulin receptor is expressed in follicle and nurse cells of *A. aegypti* (Helbling and Graf, 1998), and vertebrate insulin activates ecdysteroidogenesis and protein synthesis in ovaries isolated from unfed mosquitoes (Graf et al., 1997). Similarly, vertebrate insulin and bombyxin II stimulate ovarian ecdysteroidogenesis in the blowfly *P. regina* (Maniere et al., 2004). In *D. melanogaster*, ovarian cells require an intact insulin

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                   *        20         *        40         *        60
Scg-NPP1 : MKPAAALAAATLLIAVILFHRAE-ANPISRSCE--GANCVVDLTRCEYGEVTDFFGRKVC
Lom-NPP  : MKATAALVAATLLLAVTLFHRAE-RNPISRSCE--GANCVVDLTRCEYGDVTDFFGRKVC
Scg-NPP3 : MKPAAALAAATLLIAVILFHRAE-ANPISRSCE--GANCVVDLTRCEYGEVTDFFGRKVC
Scg-NPP4 : MKPAAALAAATLLIAVILFHRAE-ANPISRSCE--GANCVVDLTRCEYGEVTDFFGRKVC
Scg-NPP2 : MKPAAALAAATLLIAVILFHRAE-ANPISRSCE--GANCVVDLTRCEYGEVTDFFGRKVC
Aea-OEH1 : MSAGPVLLLSVLLSGYIYWSLAQPTNVLEIRCKLYSGPAVQNTGECVHGAELNPCGKLSC

                   *        80         *       100         *       120
Scg-NPP1 : AKGPGDKCGGPYE---LHGKCGDGMDCRCGVCSGCSMQSLECFFFEGAAPNSC~~~~~~~
Lom-NPP  : AKGPGDKCGGPYE---LHGKCGVGMDCRCGLCSGCSLHNLQCFFFEGGLPSSC~~~~~~~
Scg-NPP3 : AKGPGDDCNV-------YASCGIGMDCRNKKCSGCSVKTLECY-FE-SFPMSEEE~~~~~
Scg-NPP4 : AKGPGERCSD-------YESCGDGLRCAQKLCVGCSLATLQCFSIV-SLPLSEEE~~~~~
Scg-NPP2 : AKGPSEHCND-------FIKCGDGLRCNCGRCTGCSMHTLQCYSDF-STPTTCP~~~~~~
Aea-OEH1 : LKGVGDKCGESTAGIIMSGKCASGLMCCGGQCVGCKNGICDHRLCPPRLTMNHHPFGLGL

                   *       140
Scg-NPP1 : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Lom-NPP  : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Scg-NPP3 : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Scg-NPP4 : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Scg-NPP2 : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Aea-OEH1 : MAGSPQQQQPVGVFPSLYKMFDYYSSESA
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Fig. 2. Alignment of neuroparsins (NPP) of *Schistocerca gregaria* (Scg) and *Locusta migratoria* (Lom) with the ovary ecdysteroidogenic hormone (OEH) of *Aedes aegypti* (Aea) (sequences from Janssen et al., 2001, and Claeys et al., 2003).

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A chains

Bombyxin-II     GIVDECCLRPCSVDVLLSYC
Human insulin   GIVEQCCTSICSLYELENYCN

B chains

Bombyxin-II     pQQPQAVHTYCGRHLARTLADLCWEAGVD
Human insulin       FVNQHLCGSHLVEALYLVCGERGFFYTPKT
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Fig. 3. Sequence comparison between bombyxin-II and human insulin, with A and B chains (sequences from Ishizaki and Suzuki, 1988).

pathway to fully upregulate their rate of proliferation in response to a protein-rich diet and to enter vitellogenesis (Drummond-Barbosa and Spradling, 2001). Also in *D. melanogaster*, adult female mutants for insulin receptor are sterile and produce less JH, whereas treatment with the JH analogue methoprene restores vitellogenesis (Tatar et al., 2001).

Other ootropic and vitellogenesis-stimulating peptides

Ovary maturating parsins

An ootropin that stimulates oocyte growth and precocious appearance of vitellogenin in the hemolymph has been isolated in the locust *L. migratoria* (Girardie et al., 1991). The neurohormone, called "ovary maturating parsin" (OMP), is localized in the

neuroendocrine cells of the pars intercerebralis and has been identified as a 65-residue peptide that occurs in two variants differing only in amino acid 26, which can be A or S. The sequence is:

YYEAPPDGRHLLLQPAPAAPAVAPAA/SPASWP HQQRRQALDEFAAAAAAAADAQFQDEEEDGG RRV

Moreover, Girardie et al. (1998) have reported homologue OMPs in *Schistocerca gregaria*. The OMP peptides of *S. gregaria* have been identified in four variants: two long variants corresponding to the OMP of *L. migratoria*, differing only by a tripeptide insertion (PAA) at position 21; and two short variants with the 13-residue N-terminal region deleted, and differing again by the PAA tripeptide insertion. The OMPs of *S. gregaria* induce precocious appearance of ecdysteroids and vitellogenin in the hemolymph, and stimulate oocyte growth (Girardie et al., 1998). The mechanism of action of OMPs is not well understood, but indirect evidence suggests that they could act as ecdysiotropins.

Short neuropeptide F-related peptides

Another type of ootropins is represented by two short neuropeptide F-related peptides, ARGPQLRLRF-NH_2 (Led-NPF-1; Spittaels et al., 1996b) and YSQVARPRF-NH_2 (Scg-NPF; Schoofs et al., 2001), isolated in the beetle *L. decemlineata*, and in the desert locust *S. gregaria*, respectively. Adult virgin females of *L. migratoria* treated with Led-NPF-1 mature terminal oocytes faster, whereas high doses induce vitellogenesis even in sub-basal oocytes. A second similar peptide from *L. decemlineata*: APSLRLRF-NH_2 (Led-NPF-2), was less active in the same locust assays (Cerstiaens et al., 1999). In *S. gregaria*, Scg-NPF treatment increases vitellogenin levels and accelerates ovarian maturation (Schoofs et al., 2001). The mechanism of action of these peptides is unknown.

In *D. melanogaster*, Mertens et al. (2002) have cloned and sequenced the cDNA of the G protein-coupled orphan receptor CG7395, which is closely related to type 2 mammalian NPY receptors. The putative ligands for CG7395 could be four peptides (AQRSPSLRLRF-NH_2, SPSLRLRF-NH_2, PQRLRW-NH_2 and PMRLRW-NH_2), encoded by a *D. melanogaster* gene, which belong to the group of short neuropeptide F-related peptides (Vanden Broeck, 2001). The CG7395 receptor is expressed in adult ovaries, but also in brain, gut, Malpighian tubules and fat body tissues of *D. melanogaster* larvae (Mertens et al., 2002).

Peptides inhibiting oogenesis and vitellogenesis

Tripsin-modulating oostatic factor (TMOF)

A factor inhibiting egg development in mosquitoes was identified from the ovaries of *A. aegypti* as the decapeptide: YDPAPPPPPP (Borowsky et al., 1990). It inhibits proteolytic enzyme biosynthesis in the midgut, which explains the indirect oostatic activity, and was thus referred as "trypsin-modulating oostatic factor" (TMOF). Similar activities were later found in ovarian extracts of the fleshfly *Neobellieria bullata*; purification of the active factor led to identification of the hexapeptide NPTNLH as a TMOF analogue. Treatment with NPTNLH inhibited trypsin-like synthesis by the midgut of liver-fed fleshfly female, resulting in a reduction of circulating vitellogenin and oocyte growth (Bylemans et al., 1994).

Colloostatin

A second factor with oostatic activity found in ovarian extracts of *N. bullata* has been identified as the peptide SIVPLGLPVPIGPIVVGPR. Given the similarity with certain collagens and its oostatic activity, it has been referred to as "colloostatin" (Bylemans et al., 1995). Colloostatin treatment inhibits yolk uptake by previtellogenic oocytes and reduces the levels of circulating vitellogenin in *N. bullata*. The mechanism of action involved in these activities is unknown, but it must be different from that of TMOF because colloostatin does not inhibit trypsin biosynthesis in gut tissues.

Allatostatins and vitellogenesis and oogenesis

As stated above, allatostatins of the YXFGL-NH_2 family are pleiotropic neuropeptides. Relevant to this review are the inhibitory effects of these allatostatins upon vitellogenin release observed in fat bodies of the cockroach *B. germanica* adult female incubated *in vitro* (Martín et al., 1996). The inhibitory effects were counteracted by mevalonolactone, thus suggesting that they were mediated by the inhibition of an early step of the mevalonate pathway, which had consequently inhibited the formation of dolichol, thus impairing the glycosylation and export of vitellogenin from the fat body (Fig. 4). Allatostatins of the W^2W^9-NH_2 family exhibit inhibitory properties upon ovarian ecdysteroid biosynthesis in the cricket *G. bimaculatus* (Lorenz et al., 1997).

Adipokinetic and hypertrehalosemic hormones and vitellogenesis

Peptides of the adipokinetic/red pigment-concentrating hormone (AKH/RPCH) family, which in insects

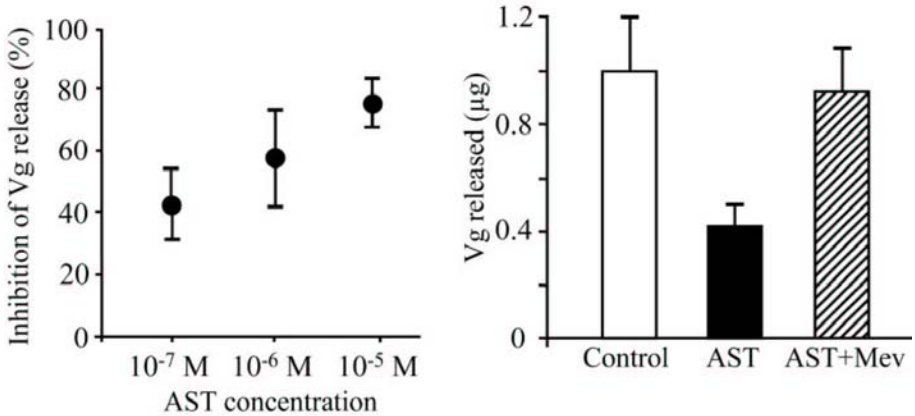

Fig. 4. Inhibitory effects of allatostatin (AST), DRLYSFGL-NH_2, on vitellogenin (Vg) release by the fat body of *Blattella germanica* females incubated *in vitro* for 7 h (left). The inhibitory effects induced by a concentration of 10^{-5} M of AST through 5 h of incubation were counteracted by 10^{-3} M of mevalonolactone (Mev) (right) (data from Martín et al., 1996).

regulate mobilization of energy storage, have been studied in the context of vitellogenesis. In the locust *L. migratoria*, AKH-I (pQLNFTPNWGT-NH_2) treatment lowers fat body total protein contents and vitellogenin synthesis (Moshitzky and Applebaum, 1990; Glinka et al., 1994). In the cricket *G. bimaculatus*, the peptide Grybi-AKH (pQVNFSTGW-NH_2) delays oocyte maturation and lowers the number of mature oocytes. These effects may be due to the interference with energy mobilization (Lorenz, 2003).

Experiments to study the effect of hypertrehalosemic hormone (HTH: pQVNFSPGWGW-NH_2), the peptide belonging to this family found in cockroaches, have given contradictory results. In *Blaberus discoidalis*, long-term HTH treatment *in vivo* was found to enhance JH-dependent synthesis of fat body export proteins (Keeley et al., 1991). In *B. germanica*, vitellogenesis *in vitro* induced by JH in fat body from cardioallatectomized females was partially inhibited by a 7 h treatment with HTH (Comas et al., 2001). This contradiction may be explained by the different experimental approaches used in these two experiments.

Neuroparsins

Two neuroparsins, A and B, were first isolated from the corpora cardiaca of *L. migratoria* (Girardie et al., 1987). Purified native compounds injected to adult females inhibited oocyte growth, whereas the injection of neuroparsin antibody had the opposite effects (Girardie et al., 1987). Neuroparsin A has been identified as an 83-residue peptide (Fig. 2) produced in the neurosecretory cells of the pars intercerebralis from a 107-residue precursor polypeptide (Lagueux et al., 1992). Neuroparsin B has 78 amino acids and results from N-terminus cleavage of neuroparsin A in the axon terminals. Furthermore, homologues of neuroparsins A and B have been isolated and identified in *S. gregaria*, and both are strikingly similar to *L. migratoria* compounds in terms of structure and function (Girardie et al., 1998). Recently, four *S. gregaria* brain cDNAs encoding distinct precursors for neuroparsin peptides have been reported (Scg-NPP-1 to 4, Janssen et al., 2001; Claeys et al., 2003). Whereas Scg-NPP-1 and 2 were only detected in brain, Scg- NPP-3 and 4 have been also detected in various other tissues, such as ventral nerve cord, fat body, testis and male accessory glands. Interestingly, whereas the gene expression pattern of Scg-NPP-1 is fairly constant during adult life, that of the other Scg-NPPs proved to be strongly regulated during the reproductive cycle in both males and females (Janssen et al, 2001; Claeys et al., 2003). However, the mechanism of action of neuroparsins remains to be determined.

Similarity between neuroparsin sequences is remarkable, and they also show modest similarity with respect to the *A. aegypti* OEH (see above) (Fig. 2), which could reflect an ancient common origin.

Spermatogenesis

The stimulatory role of ecdysteroids in early spermatogenesis involving mitoses and meioses has been demonstrated in many species of Orthoptera, Hemiptera, Lepidoptera and Diptera (Hagedorn, 1985). Further corroboration has been provided by Jacob (1992), who reported that fragments of testes of the rhinoceros beetle, *Oryctes rhinoceros*, incubated *in vitro* require ecdysteroids and the testis sheath to complete the mitotic and meiotic processes. The stimulatory function of ecdysteroids in early spermato-

genesis is consistent with the ecdysteroid production in the testes reported in moths of the genera *Heliothis, Lymantria*, *Ostrinia*, *Mamestra*, *Leucania* and *Spodoptera* (Loeb et al., 2001).

In relation to ecdysteroid biosynthesis in the testes, Wagner et al. (1997) have identified a 21-residue peptide (ISDFDEYEPLNDADNNEVLDF) in brain extracts of the gypsy moth, *Lymantria dispar*, that have ecdysiotropic properties. This "testis ecdysiotropin" did not induce ecdysteroid synthesis in prothoracic glands, which suggests that it is testis specific.

Besides testis ecdysiotropin, other peptidic factors have been implicated in spermatogenesis regulation. As early as 1953, Williams reported a macromolecular factor in the hemolymph of *B. mori* that stimulated spermatogenesis in spermatocysts incubated *in vitro*. In 1971 Kambysellis and Williams showed that 20-hydroxyecdysone induced the entry of this factor to the testis (see Hagedorn, 1985). However, no macromolecular factor directly promoting spermatogenesis has been sequenced. Indirect evidence that a protein or peptide is involved in insect spermatogenesis is that a peptidyl peptidase homologue of the mammalian angiotensin-converting enzyme (ACE) found in *D. melanogaster* (Hurst et al., 2003) is required for spermatid differentiation. In this dipteran, ACE mRNA is found mainly in large primary spermatocytes, whereas it is not detected in cyst cells (Hurst et al., 2003).

Ovulation

Since the purification of proctolin, the first insect peptide identified by Starrat and Brown in 1975, the use of cockroach hindgut or locust oviduct bioassays has facilitated the identification of a large number of peptides with myotropic properties. In cockroaches and locusts, the following peptides have been described as stimulators of oviduct contractions: proctolin, pyrokinins/myotropins, tachykinins, *L. migratoria* male accessory gland myotropins (Lom-AG-MT), *L. decemlineata* oviduct myotropin (Led-OVM), *M. sexta* Malpighian tubules myotropin (Mas-MG-MTs) or crustacean cardiactive peptide (CCAP) (Gäde et al., 1997; Schoofs et al., 1997; Predel et al., 2001). Conversely, *L. migratoria* myoinhibitory peptide (Lom-MIP) and other W^2W^9-NH_2 peptides, *S. gregaria* FLRFamide and schistostatins (YXFGL-NH_2 allatostatins from *S. gregaria*) are myoinhibitory in oviduct assays (Gäde et al., 1997; Schoofs et al., 1997). It is uncertain, however, whether any of these peptides is a physiological myomodulator of the oviduct or a regulator of ovulation.

In the cockroach *D. punctata*, a recent study suggests that YXFGL-NH_2 allatostatins are involved in ovulation (Woodhead et al., 2003), since they are present in the ooplasm of mature oocytes, and their levels in the oviduct increase between the completion of chorion formation and ovulation. Woodhead et al. (2003) postulate that allatostatins are released from the oocytes while travelling through the oviducts and, given that these peptides have no effect on oviduct muscle, they may facilitate the secretion of lubricating material during ovulation.

Pheromone Production

The three main types of hormones are involved in the regulation of pheromone production. JH has been described as the pheromone-regulating hormone in dictyopterans and coleopterans, whereas in dipterans that role generally seems to be played by ecdysteroids (Tillman et al., 1999). In lepidopterans the main regulatory factor is a peptide called pheromone biosynthesis activating neuropeptide (PBAN) (Rafaeli, 2002; Altstein, 2004).

The first PBAN was identified as a 33-residue C-terminally amidated peptide from brain-subesophageal ganglion extracts from the moth *Helicoverpa zea* (Raina et al., 1989). Homologous peptides with a similar sequence were subsequently characterized in other species of Lepidoptera (Fig. 5). The characteristic C-terminal pentapeptide of PBAN (FXPRL-NH_2) is shared by the PBAN/myotropin/pyrokinin family (Fig. 5). Peptides of this family have been described as pheromonotropic factors in lepidopteran species (PBANs, Rafaeli, 2002), myotropic peptides in locusts and cockroaches (myotropins and pyrokinins, Schoofs et al 1997; Predel et al., 2001), diapause-inducing hormones and cuticular melanization-epidermal pigmentation inducing peptides in the silkworm *B. mori* (Bom-DHs, Imai et al., 1991; Sato et al., 1992; Bom-MRCH, Matsumoto et al., 1990), and puparation accelerating factors in the flesh fly *N. bullata* (Neb-PK-2, Verleyen et al., 2004) (Fig. 5).

The PBAN gene has been characterized in a number of lepidopterans, the sequences showing high conservation (Iglesias et al., 2002; Rafaeli, 2002). The gene includes four peptide domains in addition to PBAN, most of them sharing with PBAN the C-terminal sequence FXPRL-NH_2. One is homologous to the diapause hormone, and the other three, called α-, β- and γ-neuropeptides, have an unknown function (Iglesias et al., 2002; Rafaeli, 2002).

Several authors (Jurenka, 1996; Iglesias et al., 2002) have proposed that PBAN is predominantly synthesized in the subesophageal ganglion and transported to the corpora cardiaca, from which it is released

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                                  *         20          *
A.ipsilon    : LADDTPATPADQE--MYRPDPEQIDS-RTKYFSPRL-NH2
M.brassicae  : LADDMPATPADQE--MYRPDPEQIDS-RTKYFSPRL-NH2
S.littoralis : LADDMPATPADQE--LYRPDPDQIDS-RTKYFSPRL-NH2
H.zea        : LSDDMPATPADQE--MYRQDPEQIDS-RTKYFSPRL-NH2
H.virescens  : LADDMPATPADQE--MYRQDPEQIDSRRTKYFSPRL-NH2
L.dispar     : LADDMPATMADQE--VYRPEPEQIDS-RNKYFSPRL-NH2
B.mori       : LSEDMPATPADQE--MYQPDPEEMES-RTRYFSPRL-NH2
S.cynthia    : LTEDMPATPTDQE--MFDQDPEQIDT-RTRYFSPRL-NH2
M.sexta      : ISEDMPATPSDQEYPMYHPDPEQIDT-RTRYFSPRL-NH2

LPK: pQTSFTPRL-NH2
Bom-DH: TDMKDESDRGAHSERGALWFGPRL-NH2
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Fig. 5. Sequence of the pheromone biosynthesis activating neuropeptide (PBAN) in *Agrotis ipsilon*, *Mamestra brassicae*, *Spodoptera littoralis*, *Helicoverpa zea*, *Heliothis virescens*, *Lymantria dispar*, *Bombyx mori*, *Samia cynthia ricini* and *Manduca sexta*. Also included are LPK (leucopyrokinin from *Leucophaea maderae*) and Bom-DH (diapause hormone from *B. mori*) which also have the motif FXPRL-NH_2 in the carboxy-terminus (references of the sequences in Rafaeli, 2002).

to the hemolymph where it acts on the pheromone gland. Other authors (Teal et al., 1989; Christensen et al., 1991) postulate that, at least in some instances, PBAN may be transported through the ventral nerve cord to the terminal abdominal ganglia, from where it may exert its pheromonotropic action in the pheromone gland.

In the pheromone gland, the PBAN would bind to a specific membrane receptor and activate the entrance of calcium ions into the cell, which would in turn stimulate pheromone production by mediation of adenosine 3,5-cyclic monophosphate (cAMP) as a second messenger (Jurenka, 1996; Rafaeli et al., 1997). A putative PBAN membrane-receptor protein, which is inducible by JH, has recently been purified in pheromone glands of *Helicoverpa armigera* (Rafaeli et al., 2003). In addition, the cDNA of the G protein-coupled PBAN receptor of *H. zea* (Choi et al., 2003) and *B. mori* (Hull et al., 2004) have been recently cloned and sequenced.

Concerning the enzymes affected, in some species (*Argyrotaenia velutinana*, *H. zea*, *Mamestra brassicae*), PBAN seems to increase acetyl-CoA carboxylase activity or the availability of substrate for fatty acid synthesis, whereas in others (*Spodoptera littoralis*, *Thaumetopoea pityocampa*, *B. mori*, *M. sexta*), it controls the reduction of fatty acyl groups to aldehydes or alcohols (see Tillman et al., 1999, and references therein).

Conclusions

In most cases, the endocrine peptides related to vitellogenesis appear to have an indirect action. Experiments *in vitro* and *in vivo* have shown that allatotropins and allatostatins modify rates of JH biosynthesis in a number of insect groups. In most cases, however, there is no correlation between allatotropin or allatostatin levels and JH production. In addition, other activities have been described for the peptides and localization studies have identified them in organs and tissues other than the CA. Taken together, the data suggest that regulation of JH production is not the only, nor even the most relevant, function of allatotropins and allatostatins. Indeed, the pleiotropic activity and the relatively broad distribution of these allatomodulatory peptides in the nervous system points to a basic neuromodulatory role (Stay, 2000; Elekonich and Horodyski, 2003).

In species dependent on ecdysteroids for vitellogenesis, like mosquitoes, the OEH appears to modulate vitellogenesis, given that it mediates the production of the vitellogenic hormone. The case is reminiscent of vertebrate models, in which the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) stimulate gonad development and steroidogenesis. However, in contrast to OEH, FSH and LH are glycoproteins composed by α- and β-chains. Indeed, no FSH or LH homologues are known in insects, and although two receptors named DLGR-1 and DLGR-2 found in *D. melanogaster* seem homolo-

gous to FSH and LH receptors (Hauser et al., 1997; Eriksen et al., 2000), the modest sequence similarity (about 20%), in comparison with the mammalian receptors in the ligand binding N-terminal region, suggests that fruit fly ligands have diverged with respect to LH and FSH.

ILPs are also pleiotropic peptides involved in many functions, some of them being related to reproduction. The role of ILPs in the transduction of nutritional signals to metabolic processes may contribute to vitellogenesis, especially in anautogenous species. Their ecdysiotropic action also links ILPs to reproduction in ecdysteroid-dependent insects. A relevant feature of ILPs is the signal transduction mechanism, involving binding to a heterotetrameric membrane tyrosine kinase receptor, which is conserved in different animal phyla.

The contribution of other peptides such as TMOF and AKH/RPCH to vitellogenesis appears to be indirect. The physiological function of OMP, neuropeptide F-related peptides, colloostatin and neuroparsin is a mystery. The activities observed in relation to vitellogenesis could be unspecific, in most cases related to pharmacological ecdysteroidogenic effects. The structural similarity of some of these peptides with known ecdysteroidogenic hormones (like the similarity between neuroparsins and OEH) might explain the postulated unspecific effects.

The ecdysiotropic action of testis ecdysiotropin on testes but not on the prothoracic gland indicates that this peptide is tissue-specific. This and evidence pointing to ecdysteroids as regulators of spermatocyte and spermiocyte formation suggest that testis ecdysiotropin mediates modulation of these early spermatogenesis steps.

The copious collection of data on peptides with myotropic activity on the oviducts does not help us to understand the endocrine regulation of ovulation. Data on tissue-specificity, and on the expression of these peptides in relation to ovulation and oviposition might help to elucidate whether the myomodulatory action on the oviduct is specific.

Although PBAN belongs to a family of peptides that elicit a diversity of effects (pheromonotropic, myotropic and diapause- and pigmentation-inducing), modulation of pheromone production is one of its best established roles. The challenge here is to unveil the pheromonotropic mechanisms of action at molecular scale. It would also be interesting to elucidate the function of the other peptides co-expressed with PBAN (α-, β- and γ- neuropeptides), the physiological significance of the co-expression of PBAN and diapause hormone, and the mechanisms of allocation of the pheromonotropic and chromatotropic activities of PBAN.

Perspectives

Notable progress has been made in identifying peptides using a pre-fixed biological assay to monitor the biological activity during purification. Then, the synthetic version of the peptides are tested with the same assay to confirm the biological activity. Often, the biological part of the study is based on opportunistic approaches (like the availability of a standard biological assay that is easy, fast and reliable), rather than on strategies to answer physiological questions. This has produced biased results and ambiguities, especially from a functional point of view, that should be clarified with deeper physiology-focused studies.

Now, immersed as we are in the genomic era, the solution of structural aspects of peptide research seems easier. Availability of the entire genome of model insects allows us to assess the diversity of regulatory peptides occurring in a single species (Vanden Broeck, 2001; Riehle et al., 2002). Moreover, sequences from genomes of model species can be used as templates to obtain the corresponding homologues in other species. Therefore, gene cloning is often the fastest way to identify the structure of the peptide or protein. In this context, biochemical approaches would play a corroborative role. This means that, although the gene provides the sequence of the peptide under study, this does not substitute the biochemical studies that elucidate the structure of the physiologically functional compound and determine the post-translational modifications. These biochemical studies will include peptidomics approaches and the analysis of the corresponding peptide receptor.

Peptidomic approaches use nanoscale liquid chromatography combined with mass spectrometry and data base searching to identify the whole peptidome, i.e., all the peptides, with their post-translational modifications, expressed in a cell or tissue (Clynen et al., 2003). This powerful tool is expected to allow the analysis of the peptidome corresponding to a specific tissue (for example, neurohemal releasing sites) in different physiological states, and to provide clues to the physiological events in which the peptides are involved.

Genome databases have facilitated the identification of a number of insect peptide receptors (Johnson et al., 2003). Expression studies at level of the genes coding for the peptides and for the corresponding receptors, can illuminate the analysis of peptide functions. Finally, and functionally more relevant, mole-

ular characterization of the target genes can allow the use of the new techniques of protein silencing, like RNAi, which may overcome the limitations imposed by the difficulty of using transgenesis in non-drosophilid insects (Plasterk, 2002). RNAi may help to unveil the functions of those peptides that in this review appear circumstantially linked to reproduction but whose physiological role remains enigmatic.

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