ROLE OF INSECT NEUROPEPTIDES AND JUVENILE HORMONES IN SILK PROTEIN BIOSYNTHESIS

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Abstract

Brain or the neurosecretory system in insects being the wider part of the central nervous system does play important role in the neuroendocrine regulation of biological activities controlling growth, development and reproduction. The brain also regulates the synthesis and the release of juvenile hormones (JH) by the corpora allata (CA). A large number of neuropeptides are being identified in invertebrates and are found to play important roles in controlling brain functions of insects and their behaviour. Insect neuropeptides and juvenile hormones (JH) play important roles in all aspects of insect development and reproduction. Juvenile Hormone Acid Methyltransferase (JHAMT) is found to be the key regulatory enzyme in juvenile hormone biosynthesis. The ability of corpora allata to synthesize juvenile hormone is controlled by stimulatory and inhibitory signals. These signals are mediated through neuropeptides or its analogues are either stimulatory (Allatotropin, and its analogues), synthetic analogues (allatotropin analogue acetylated, ATAA and allatotropin analogue non acetylated, ATANA) or inhibitory (allatostatin, allatinhibin). It has been observed that the activities of the cellular ultra structure immediately after last larval moulting of the neuro secretory cells were very less compared to later part of the instar. Juvenile hormone and its analogues are found to cause various biochemical changes in the larval tissues. It also increases larval body weight, coccon weight and silk shell weight which is the result of secretion of more silk protein and lengthening of last instar. Similar observations are also shown by non-mulberry silkworm *Philosamia ricini* with JH-III. Juvenile hormones are found to inhibit silk gland function, prevent their degeneration and indirectly causing an increase in silk production. The practical application of these neuropeptides is also now undertaken by different groups for pest management as biocontrol agents. However, the molecular action and functional properties of these natural peptides are still a challenge to insect endocrinologists. In this paper, the role of neuropeptides and juvenile hormones with respect to silk protein biosynthesis in non-mulberry silkworms are being reviewed.

Key words: Brain, neuropeptides, juvenile hormone, silk protein

Introduction

The term ''neuropeptide'' was introduced by de Wied and co-worker to all mediator peptides of known chemical structures secreted by neurons (1). And that work marked the beginning of a new period in neuroscience. Over the past decade numerous peptides have been identified within the vertebrate and invertebrate nervous systems, and are present in the nervous as well as the non-neural endocrine systems. The mystery of some of the complex functions and mechanisms of action of neuropeptides are only beginning to be unravelled. Several mammalian neuropeptides may occur in a more or less similar form in insect nervous system. The lists of identified neuropeptides from the nervous system of invertebrates are increasing at alarming rate compared to that discovered before the year 1983, where the primary structure of only 7 neuropeptides was then known. Today over 40 neuropeptides are known in invertebrates. Yet very little information is known about the physiological functions of these neuropeptides in insects (2, 3). Before the concept of neuropeptides was made clear, Bargmann introduced the phrase ''peptidergic neuron'' to imply cholinergic, adrenergic or aminergic neurons (4). The confusion was created mainly due to the term being also used to the source of origin of the mediator peptides. Now the terminology has been aptly applied only to those neurons that synthesize peptides for release as signal substances at their terminal ends either into the blood stream (also known as *peptide neurohormones*) or into the neighbouring cells via ''peptidergic synapses'', ''synaptoid contacts'' or ''paracrine mechanisms''. Euler and Pernow introduced the term "neurotropic" for the characterization of neurons and elucidation of the structures of neuropeptides (5). Now from various studies it has become clear that neuropeptides play important roles in controlling different brain functions of an insect and its behavior.

On the basis of their similarity in the primary structures, neuropeptide are grouped in three families: (i) Intragenic families, (ii) Intraspecific families and (iii) Trans-specific families. 'Intragenic families' are those peptides that are released from the same precursor by limited proteolysis. Examples are the Bovine proenkephalin containing 6 copies of [Met]enkephalin and 1 copy of [Leu]enkephalin; and the FMRFa precursor of Aplysia containing 26 copies of FMRFa and 3 copies of the FLRFa (6). 'Intraspecific families' are those similar peptides that are synthesized in the same organism but released from different precursors. Examples are the 'relatives' of the pancreatic polypeptide (PP) NPY [a neuropeptide (NP) with N- and C-terminal tyrosine (Y) with N- and C-terminal tyrosine (Y) residues from porcine brain]; and PYY [a peptide (P) with N- and C-terminal tyrosine (Y) residues from porcine duedenum]. 'Trans-specific families' are those similar peptides synthesized in more or less related animals. Examples are the AKHs (Adipose-Kinetic Hormones) of the Arthropods and the nonapeptides of the neurohypophysis.

The two main classes of hormones synthesised in insects are: (i) the true hormones produced by the epithelial glands and belonging to the ecdysteroids or juvenile hormones and (ii) the neuropeptide hormones produced by the neurosecretory cells. The different hormones belonging to these classes regulate physiological, developmental and behaviour in insects (7). The juvenile hormones and ecdysteroids are produced in the epithelial hormonal glands, whereas, the peptides are synthesized in the neurosecretory cells that are abundant in the brain but are also found throughout the nervous system. The paired corpora cardiaca (CC), the main neurohemal organs are the source of neurosecretory products from the brain and the endogenous neuropeptides, while the perisympathetic organs release neurosecretory material from the ventral nerve cord. True hormones are defined by certain criteria as storage in and release from the neurohemal sites and transported via the hemolymph. But in many cases, these criteria have not been proved and hence they are often known as neuropeptide rather than neurohormone.

The last decade has been marked with success in the elucidation of the primary structure of insect neuropeptides mainly due to the availability of improved methods of isolation by High Performance Liquid Chromatography (HPLC), protein chemical detection methods, automated amino acid sequencers and mass spectrophotometers. The first neurosecretory hormone to be discovered in the animal kingdom, whether vertebrate or invertebrate was the insect brain hormone, described as an endocrine factor that induces pupation of the Gypsy moth, *Lymantria dispar*. The neurosecretory hormone was known as the brain hormone and is now commonly known as the prothoracicotropic hormone (PTTH) (8). Chemically the PTTH is a peptide and much has been commented on its mode of action. The PTTH is known to stimulate a pair of thoracic endocrine glands (the prothoracic glands) to synthesize and release ecdysone, a steroid that is vital for the overall development of the insect. Thus, PTTH plays an important role in the endocrine network by controlling insect development (8).

The first evidence that hormones control the intermediary metabolism in insects was given for carbohydrate mobilization in cockroaches (9) and for lipid metabolism in locusts (10, 11). Although the peptide structures of many insect species are known, most of the physiological data were on the adipokinetic peptides of locusts and hypertrehalosemic peptides of cockroaches. But little work has been reported on the neuropeptides and juvenile hormones of the silkworms, *Antheraea assama* Westwood and *Philosamia ricini* Biosduval.

Biosynthesis and mode of action of neuropeptides

The corpora cardiaca (CC) of insects are the main neurohemal organs of the endocrine system that store and release neurohormones that are synthesized by neurosecretory cells of the brain. In addition some insects as locusts, contain intrinsic neurosecretory cells which are clustered together. The intrinsic neurosecretory cells contain a large number of electron-dense secretory granules thah are the source of the adipokinetic hormone (AKH). During flight in locusts, a fraction of the stored AKH is released from CC into the hemolymph which may be controlled by octopamine and adenosine 3',5'-cyclic monophosphate (cAMP) (12). The released peptide in the hemolymph are then transported to their target cells, bind to specific membrane-bound receptors where they exert their biological function.

Biosynthesis of juvenile hormones

The juvenile hormones (JH) play unique roles in almost all aspects of insect development and reproduction that includes embryogenesis, larval moulting, metamorphosis, caste determination in the social life of insects, vitellogenin synthesis and ovarian development, phase determination in locusts and aphids, larval and adult diapause regulation, colour, polymorphism and various aspects of metabolism associated with these functions (13).

The chemical structure of a JH was first determined by Roller *et al*. (14) by using MS analysis, NMR and microderivatization techniques. The hormone (JH-I, C_{18}JH) showed that it is an unusual sesquiterpenoid with epoxide group near one end and a methyl ester on the other end. The hormone is one of a series of naturally occuring juvenile hormones, all having similar chemical structures. Juvenile hormone II (C_{17}JH) was later identified by Meyer *et al*. (15) and JH-III (C_{16}JH) by Judy *et al*. (16). Juvenile hormone III is the principal form of JH found in the Orthopteroidea, Coleoptera, Hemiptera and Hymenoptera. The Lepidoptera is unique in possessing a mixture of JH-I and JH-II. However, the corpora allata (CA) of the tobacco hornworm moth, *Manduca sexta* secretes JH-III in addition to JH-II (13) and JH-I. The male of certain Lepidoptera secretes JH acids (JHA-I, JHA-II, JHA-III, iso-JHA-II) from the CA that are later methylated in the accessory gland (17, 18). Methyl farnesoate (MF), an acyclic sesquiterpenoid ester that is closely related to JH-III in structure is also tentatively added to the group of JH acids. The MF was detected in high levels from the embryos of cockroach, *Nauphoeta cinerea* produced *in vitro* by CA.

Mechanism of action of juvenile hormone

The term juvenile hormone is derived from the fact that it blocks the developmental stages of nymphs into imagoes or the development of pupae into adult insects. JH controls the switches between alternative pathways of development at various points in the life cycle of insects (7). In support of these roles of JHs, many researchers have assumed that different concentrations of the hormone are responsible for specifying the different pathways. Most of the work on the hormonal control of moulting and metamorphosis gave results that are consistent with the hypothesis that metamorphosis in holometabolous insects is caused by gradual lowering of JH titer. Holometabolous insects continue as larvae only at high concentrations of JH, but pupation occurs when JH gradually declines to an intermediate or low level, and adult insects are formed in absence of JH.

The synthesis of JH-III, the homolog lacking branched side chains are now known to follow the path similar to the initial steps in cholestrol synthesis. The precursors for the carbon skeleton are

two-carbon (C-2) units that are products of metabolism of glucose, leucine, isoleucine and threonine and the precursor of acetate (20). The 3 units of C-2 in the form of acetyl CoA undergo enzymatic condensation to yield C-6 intermediate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The HMG-CoA is then reduced to mevalonate by HMG-CoA reductase which utilise NADPH as an electron donor. The next step in JH biosynthesis is the conversion of mevalonate to 3-isopentenyl pyrophosphate (IPP) that isomerizes to 3,3'-dimethylallyl pyrophosphate (DMAPP). The last step is the condensation of two units of IPP and one unit of DMAPP to form the basic farnesyl pyrophosphate unit. Farnesyl pyrophosphate is then converted to farnesol. Farnesol is oxidized to farnesal by a dehydrogenase that requires NAD, which is then oxidized to farnesoic acid by another dehydrogenase that also requires NAD. The terminal steps in biosynthesis are methyl ester formation at C-1 and epoxidation at the C-10/C-11 position.

The sequence of the terminal two steps in JH biosynthesis is reversed in the Lepidoptera (i.e., farnesoic acid to epoxyfarnesoic acid to JH-III) when compared to the order in other situations (**farnesoic acid → methyl farnesoate → JH-III**).

The branched precursors of JH0, JH-I and JH-II originate from propionyl-CoA. The branched chain of homoisoprenoid units are formed from condensation of 1 propionyl-CoA and 2 acetyl-CoA units and results in the synthesis of a homomevalonate intermediate, whose composition varies in the different forms of JH. The JH0 is composed of 3 homomevalonate units, JH-I of 2 homomevalonate and 1 mevalonate units, and JH-II consists of 1 homomevalonate and 2 mevalonate units (19).

Control of juvenile hormone synthesis in corpora allata

The ability of the corpora allata (CA) to synthesize JH is controlled by stimulatory and inhibitory signals that reach the glands through the hemolymph or via nervous connections (19). In many insect species a vital control mechanism in the brain neurosecretory cells is involved to stimulate each CA and exert paracrine influence on gland cells (21). The neuropeptides that stimulate (i.e., allatotropin), and inhibit (i.e., allatostatin) the JH production have recently been isolated from the brains of several insect species. The finding that crude brain extracts and partially purified peptides obtained from several species of insects stimulate the rate of JH production by CA *in vitro* provides strong evidence for the existence of allatotropins (22-26). Brain extracts from larvae of *Manduca sexta* appears to contain a factor that stimulates JH synthesis (27). Kataoka *et al*. have isolated and identified a tridecapeptide from the heads of adult *M. sexta* that activates JH synthesis in adult insects but shows no effect on CA of larvae or pupae (28). The synthetic allatotropin did not activate the CA in beetle, *Tenebrio molitor;* locust, *Schistocerca gregaria* and in cockroach, *Periplaneta americana* but stimulated the CA of the noctoid moth, *Heliothis virescens* suggesting the order in specificity. Allatotropin, a neurohormone secreted by the median neurosecretory cells (MNC) of the brain, stimulates the CA to secrete JH in silkworm, *Philosamia ricini* Biosduval (29). The chemically synthesized allatotropin used on the silkworm is the analogue of the allatotropin isolated and identified by Kataoka *et al.* in *Manduca sexta* (28). The cocoon weight, cocoon shell weight and silk production of *Philosamia ricini* was increased by treatment with JH (3.36 ± 1.01), hydroprene (4.39 ± 0.69) and methoprene (2.54 ± 0.59) compared to controls. The allatotropin analogue non-acetylated (ATANA) stimulates the rate of JH biosynthesis. The propionic acid after catabolism is converted to propionyl-CoA and its ethyl branches are used either for JH-I or JH-II synthesis in Lepidoptera insects. It was also reported that the two allatotropin analogues (ATAA and ATANA) increases the rate of biosynthesis of all three JH homologues, however JH-II showed the highest stimulation (30). Insect development is controlled by JH which are produced only in the corpora allata (CA), a small gland lying behind the brain. These hormones (JH-I, JH-II and JH-III) have been isolated not only from haemolymph and whole body extracts but also from incubation of CA *in vitro*. In insects, the activity of CA is regulated by the brain mediated through neuropeptides. The degree of activation of CA in the presence of allatotropin analogue (synthetic) under long term incubation enhanced the JH biosynthesis from 3 to 6 times compared to the control under the same experimental conditions. In insects, metamorphosis is initiated by ecdysteroids when the concentration of JH in the haemolymph is very low or undetectable. A major cause of the decrease of the JH titre is the inactivation of the CA, the gland that secrete JH (31). The biosynthesis of JH is regulated through neurohormonal factors/peptides secreted from the brain. More recently numerous peptides that effect CA have been described (32). Environmental and physiological factors influence neurosecretory cells in the brain and affect the activity of CA through peptidergic materials either transported directly to the CA by axons or released into the blood (33). These peptides are either stimulatory or inhibitory in nature-viz., allatostatin and allatinhibin. Much neural control has been demonstrated in some insects and is mainly inhibitory in nature (34). The mechanism of regulation of CA appears to differ between various species of insects. Unni *et al*. studied the role of such peptides that regulates the JH biosynthesis in *Manduca sexta* under short and long term incubations (30). The group reported on the action of allatotropic analogue, synthesized after modification at N- and C-terminals and substituted norleucine for methionine at the 7th and 8th position which corresponds to the active fragment (amino acids –5-13) of the natural Mas-AT (28). Allatotropin potency was evaluated by measuring the *in vitro* rates of JH biosynthesis in CA of *Manduca sexta*. The two synthetic analogues (ATAA-allatotropin analogues acetylated and ATANA-allatotropin analogues non-acetylated) were tested and found to be almost equally active *in vitro*. The substitution of methionine at 7th and 8th positions by norleucine has some effect on the biological activity of the peptide. In general, the two analogues increase the rates of biosynthesis of all the three homologues, but JH-II shows the highest stimulation. The long term incubation with the adult female CA of *Manduca sexta* in the presence of ATANA activates the CA to synthesize more JH. Thus, about 4 times at 3-6 hrs interval and about 6 times at 6-9 hrs and 9-12 hrs under the same incubation conditions. The degrees of activation of CA (control) without ATANA are also found to be 1 time and 2 times more for 6-9 hrs and 9-12 hrs

respectively. The same pattern of CA activity was reported by Gadot *et al.* (35). The CA of sexually mature female locusts, *Locusta migratoria* incubated for 24 to 48 hours shows a marked increase in JH activity than the initial low level. The higher JH assay may be due to the endogenous JH-III activity of mature CA or the presence of some inhibitors *in vivo*, which seems to be released during long term incubation (35). The natural allatotropin had no effect on the activity of CA of adult females of beetle, *Tenebrio molitor*; grasshopper, *Schistocerca nitans*, and cockroach, *Periplaneta americana*. However, *Manduca sexta* allatotropin stimulates JH biosynthesis strongly in CA of the adult female noctuid moth, *Heliothis virescens*. The JH biosynthesis is inhibited by two peptides, allatostatin and allatinhibin (36, 37). The isolation and establishment of the primary structure of allatostatin from the brain of adult virgin female *Diploptera punctata* was established by Woodhead *et al*., and also studied the activity of the natural and synthetic allatostatin with CA *in vitro* for the reversibility and species specificity of synthetic allatostatin and found to be reversible (37). JH rates were restored to pretreatment levels after the CA were transferred to normal medium. In contrast to the hormone listed above, the *Manduca sexta* allatinhibin derived from the brain of Vth instar and wondering stage inhibit the CA in a stable manner (38). The release of allatinhibin occurs only with post feeding period of the last larval stage. Allatinhibin prevents CA from responding to stimulatory factors with increased JH production. *In vitro*, allatinhibin has no immediate effect on CA and prolonged exposure is necessary to inhibit CA responsiveness (39). The peptide is secreted by day 4 of Vth instar brain kept overnight in Graces medium. The isolation and partial characterization of the peptide has been achieved very recently (40). The studies of matrix assisted laser desorption mass spectroscopy confirms its molecular weight of 894 Dalton (Prof G Bhaskaran, Texas A&M University, USA, 1995 personal communication). According to Bhaskaran *et al*., the CA of *Manduca sexta* are inhibited during the last larval instar by allatinhibin. The functional property of the peptide is different from that of allatostatin in that the inhibition is reversible and can be reversed by removal of the hormone or peptide. An *in vitro* approach to study the brain of silkworms have been successfully tried .The brain of the fifth instar second day larvae of silkworm is the capable form to survive *in vitro* for about 14to 16 hours at 37°C in the dark. The brain media were subjected to various molecular separation techniques for isolation of neuropeptides.. The molecular weight of this protein/peptides were found to be in between 23kDa-40 kDa **.(41)**

Effect of juvenile hormones on enzyme activity of silk gland

The effect of JH-II on the activities of trehalase and phosphorylase and on the level of disaccharide trehalose in the larvae of muga silkworm, *Antheraea assama* Westwood was investigated by Choudhury and Unni (42) and found that topical administration of JH to freshly ecdysed fifth instar larvae during larval and spinning period in the silk gland tends to decrease trehalase activity (from 4.61 to 25.98 units) and increase the phosphorylase activity (from 3.40 to 82.80 units), and in the haemolymph the enzyme activities were insignificant. However, JH seemed to increase the trehalose level in the silk gland (from 29.60 to 89.23 mg/gm) and decreases in the haemolymph. Studies also reveal that application of JH and its analogues cause various biochemical changes in the tissues. The application of JH also serves to increase larval body weight, cocoon weight and silk shell weight. This is possibly due to the indirect stimulatory effect of JH on the silk gland cells to secrete more silk protein as well as the direct effect of the hormone in prolonging the last instar leading to more silk production (43). Similar observation was reported by the same group on the effect of JH-III on the enzymes trehalase and phosphorylase, and on the metabolites trehalose and glycogen in the silk gland and haemolymph of the non-mulberry silkworm, *Philosamia ricini* during larval and spinning period (43). The topical application of JH increased the trehalase activity in the silk gland on the 2nd day of the Vth instar and in the early stages of spinning. In the initial stages of JH application phosphorylase activity increase in the silk gland but was low in the haemolymph. Low trehalose level was observed in the silk gland (during pre-spinning and spinning period) and haemolymph of treated larvae. Glycogen level in the silk gland of treated silkworm was found to be lower at early and late Vth instar which increases with the onset of spinning, whereas in haemolymph the level declines on JH treatment and increased in subsequent stages.

Effect of hormones on silk glands

In most Lepidoptera and Tricoptera, the posterior gland region secretes fibroin and one to several small proteins (44). In preparation for hatching, Lepidopteran silk glands become secretory. And during each stage of moulting the insects increase their secretory potential by growing in size and ploidy. This is characterized by fluctuation in function in each instar according to a larval pattern which is manifested by initiation of RNA transcription, by a high rate of proteosynthesis during feeding and by absence of these activities with the advancement of the next moult. In the last larval instar the silk glands develop according to a metamorphic pattern that differs from the larval one by enhanced function and programming of silk glands for histolysis. Development of silk glands is altered with hormonal treatment of the larvae. JH inhibits silk gland function, prevents their degeneration and indirectly cause an increase in silk production. Low doses of ecdysteroids stimulate silk gland development to increase the function, while high doses cause regression and degeneration. The JH anologues and anti-JH compounds are utilized in sericulture to control the yield and quality of silk. The changes from larval to the metamorphic developmental pattern is caused by a drop in JH titre. Trace amounts of JH in the last larval instars affect silk glands via regulation of feeding and moulting time. The function of the silk glands depends on nutrient supply which is stimulated by a brain neurohormone. Slight elevation of ecdysteroid titre that is associated with the termination of feeding and initiation of cocoon spinning may be implicated in the culmination of proteolysis and in the initiation of functional regression in the silk glands. The moult-inducing surge of ecdysteroids induces regression in the silk glands. Juvenile hormone, Juvenile hormone analogue (Methoprene), Juvenile

hormone activating peptide (ATANA) applied to freshly ecdysed Vth instar larvae of *Anthereae assama* causes better larval growth, larval body weight, cocoon weight and silk weight (45).

Future Scope

The silkworms may be exploited to produce important biomolecules and proteins with the help of advanced techniques of Molecular biology and Genetic engineering. With the help of standard molecular techniques genetically engineered 'most useful' genes can be manipulated to yield commercially important biomolecules, in addition to introduction of genes from the best insects of high quality in terms of silk productivity in to silkworms. We have demonstrated the linkage between the neurosecretory cells, neurosecretory materials, peptides and proteins with respect to juvenile hormone biosynthesis. Further its effect on silk biosynthesis within the silk gland was demonstrated with the help of synthetic juvenile hormones and its analogues in particular silkworms. The application studies of these factors on silkworms in laboratory and field trials caused a significant response on the silkworms in terms of better larval growth, silk gland weight and yield of silk fibre from 3-6%. The progress have already generated interest in North Eastern region, which can be utilized in sericulture for the better silk yield in terms of quality and quantity.

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