CHARACTERIZATION OF THE EXPRESSION, FUNCTION AND SIGNALING OF THE OVARY ECDYSTEROIDOGENIC HORMONE IN THE FEMALE YELLOW FEVER MOSQUITO, *Aedes aegypti*

by

ANIMESH DHARA

(Under the Direction of MARK R. BROWN)

ABSTRACT

The neuropeptide, ovary ecdysteriodogenic hormone (OEH), is released from medial neurosecretory cells (MNCs) in the brain of female *Aedes aegypti*, in response to blood feeding. Transcript and protein expression profile of OEH in different body parts at different life stages and in female tissues during egg maturation cycle indicated that brain OEH is main source of OEH present in other tissues. Besides head tissue, OEH transcript was found only in ovary at any point during egg maturation cycle. The basis of OEH isolation from head extracts was its stimulation to yolk deposition in blood-fed decapitated females *in vivo* and ecdysteroid production by ovaries *in vitro*. A truncated version of OEH peptide was shown to be bioactive in a previous study. In this study, we compared the bioactivity of both full length and truncated peptides and did not see any significant difference indicating the truncated version could be the mature peptide, processed from prepropeptide. Insulin-like peptides (ILPs), in particular ILP3, are also produced by cells in the same region of the female brain and have the same demonstrated bioactivity. This study in fact showed the colocalization of OEH and ILP3 in MNCs. The functional *in vivo* bioassays indicated redundant functions of OEH with ILP3 in
stimulating midgut digestion, nutrient storage, yolk protein synthesis and yolk uptake by the oocytes. In *in vitro* tissue culture condition OEH could not stimulate those functions indicating OEH required some other signals or resources to exhibit its function, shown *in vivo*. The study further showed, as that of ILP3, the ecdysteroidogenic function of OEH could be modulated through amino acid sensing, target of rapamycin (TOR) pathway. The signaling study revealed that despite many overlapping functions, unlike ILP3, OEH neither bound with nor activated the mosquito insulin receptor (MIR). However, OEH activated the MIR downstream protein kinase, Akt indicating that there might be some cross-talk with ILP3 signaling. As expected, OEH activated the TOR downstream signaling proteins, ribosomal S6 kinase and 4EBP1. Finally, this research demonstrated that OEH mediated activation of signaling proteins differs in a tissue specific way.

**INDEX WORDS:** Ovary ecdysteroidogenic hormone (OEH), insulin-like peptides (ILPs), *Aedes aegypti*, egg maturation, mosquito insulin receptor (MIR), target of rapamycin (TOR),
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MOSQUITO, AEDES AEGYPTI

by

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DEDICATION

To all my teachers, family members and friends whose unconditional support paved my way to this destination.
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CHAPTER 01
INTRODUCTION AND LITERATURE REVIEW

Mosquitoes are the invertebrate vectors of many pathogens that cause deadly diseases like malaria, filaria, dengue, west nile fever, and encephalitis. These diseases result in millions of deaths and sicknesses every year. One way to reduce mosquito-borne diseases is to control mosquito populations in the wild. Several insecticides (including DDT) have been used in the past to control mosquito populations, many of which either have adverse effects (Bouwman et al., 2011; Eskenazi et al., 2009) on other biological entities due to improper use or have no effect because mosquitoes have developed resistance over time (Kerah-Hinzoumbe et al., 2008; Ndiath et al., 2012). For these reasons, scientists seek better ways to control mosquito populations.

Other methods, like biological control (Das and Amalraj, 1997) and releasing transgenic sterile mosquitoes in the wild (Alphey et al., 2010) are emerging as effective approaches to reduce mosquito populations.

The reason why mosquitoes transmit those deadly pathogens is females of some mosquito species are anautogenous i.e. they require a blood meal to mature their eggs. The whole process, starting from blood ingestion to the laying of eggs, is tightly regulated by different endocrine pathways. So, understanding the mechanisms how the endocrine signals coordinate different aspects of egg maturation process is of paramount importance, to develop new approaches for
disrupting mosquito reproduction. The yellow fever mosquito, *Ae. aegypti* is an important vector of arboviruses and also an important model to study mosquito reproduction.

1.1. Egg maturation in *Aedes aegypti*

*Ae. aegypti* females require a blood meal for each batch of eggs deposited. Egg maturation occurs in three distinct phases in this mosquito (Raikhel et al., 2002). The first phase is called “previtellogenic,” which starts with the eclosion (emergence of adult from pupae) or after oviposition and ends when females ingest the next blood meal. The second phase is called “vitellogenic” in which synthesis of major yolk protein, vitellogenin (Vg), occurs in the fat body, and yolk proteins are taken up by the oocytes in the primary follicle. The last phase is called “postvitellogenic,” which starts as soon as yolk protein uptake is complete. During the postvitellogenic phase, a thick chorion membrane is formed surrounding the yolk filled oocytes, and this phase continues until the females finishes laying eggs from that blood meal. After emergence, females require some time (approximately 72 h post emergence) to mature the ovary and other tissues to undergo a complete egg maturation cycle.

a. Mosquito ovary structure and development

Ovary development in mosquito starts during embryogenesis. When the mesoderm differentiates, 6-8 primordial germ cells are enclosed by a fine sheath to form a pair of gonadal rudiments, but the differentiation and development do not take place until post embryonic phase. By the 4th larval instar, ovaries in the abdomen become prominent and differentiate into various cell types. By the early pupal stage, ovaries start to form ovarioles, the functional units of the ovary, which are enclosed in a sheath and connected to a calyx lumen by the follicular stalk. The
ovarioles are called meroistic because they consist of oocytes as well as nurse cells. The ovarioles are also called polytrophic because a group of nurse cells is enclosed with an oocyte in each ovarian follicle. Each ovariole contains a germarium, secondary follicle, and primary follicle. Primary follicle consists of one oocyte and some nurse cells are enclosed by a monolayer of epithelial cells, called follicular epithelium. The nurse cells provide nutrition to the oocytes and synthesize maternal ribosomes and mRNA to be deposited in the eggs. At eclosion, the number of cells in the follicular epithelium may be fewer than 20. During 24-72 h post eclosion, primary follicles continue to develop until each has 200-250 epithelial cells. This growth is under the influence of juvenile hormone (JH III) (Raikhel and Lea, 1991) and 20-hydroxyecdysone (20-HE, active form of ecdysone in insects) (Beckemeyer and Lea, 1980). Development of the accessory ducts of the female reproductive systems occurs during this time. Each of the 60-75 ovarioles is posteriorly connected to a lateral oviduct. At the seventh abdominal segment, the lateral oviducts join together and form a common oviduct. The cell types change from columnar in lateral oviduct to cuboidal in common oviduct. The common oviduct leads to atrium of gynecophoric canal which is connected to the caecum and the common spermathecal duct. The ovarioles are covered by a thin membrane, ovarian sheath with an outer muscle layer formed by isolated cell bodies interconnected with muscle fibers. The muscle cells in ovarian sheath are continuous with those of the muscle layer of the lateral oviduct.

b. Previtellogenic phase

In this phase ovary and fat body in the female prepare for the reproduction. Follicular cells surrounding the oocyte grow in number and undergo ultrastructural changes to provide passage of yolk proteins into oocytes (Raikhel and Lea, 1985). At the late previtellogenic stage, the oocyte membrane projects microvilli to contact the epithelial cells which are cubic. The
oocyte occupies one pole of the follicle (Soumare and Ndiaye, 2005). Nurse cells at the other end synthesize mostly the ribosomal RNAs (28S, 18S and 4-5S) during the first 48 h after emergence (Frelinger and Roth, 1971). The nurse cells within a follicle may develop differently, some may develop polytene chromosome even before a blood meal is taken (Fiil, 1976).

During the previtellogenic phase, fat body also undergoes changes. The number of ribosomes and ploidy increases in the fat body cells (Dittman et al., 1989; Raikhel and Lea, 1990) to enable synthesis of yolk protein precursors (YPPs). A study by Zhu et al. showed that an orphan receptor, βFTZ-F1, is responsible for the development of fat body competence and responsiveness to 20 HE titer during the previtellogenic phase (Zhu et al., 2003). By about 3 days post eclosion, previtellogenic development of ovary and fat body enters into a state of arrest until a female ingests a blood meal. During this previtellogenic phase, host seeking behavior is promoted by an unknown mechanism. A study shows that blood feeding stimulates the release of Aedes head peptide-I (Aea-HP-I) which inhibits this host seeking behavior (Brown et al., 1994). The titer of Aea-HP-I reaches peak around 36 h PBM and comes back to very low level by 72 h PBM, when females usually finish laying eggs and resume host seeking for the next previtellogenic cycle.

c. Vitellogenic phase

Blood feeding lifts the previtellogenic developmental arrest in Ae. aegypti by stimulating ovary to produce ecdysteroid hormones. Ingestion of a blood meal stimulates the release of neuropeptides into the hemolymph from medial neurosecretory cells (MNCs) in the female brain. These neuropeptides stimulate follicle cells in the ovary to produce and release the ecdysteroid hormone, ecdysone, into the hemolymph, where it is taken up by the peripheral tissues and
converted into its active form, 20-HE. In the fat body, 20-HE binds to the heterodimeric nuclear hormone receptor complex formed by the ecdysone receptor (EcR) and ultraspiracle (Usp) for activating downstream target genes (Wang et al., 1998). Following binding, the ligand-receptor complex enters the nucleus where it binds to the ecdysteroid responsive element (EcRE) sequences in the upstream region of specific genes which are activated or inhibited. Most of the ecdysteroid regulated genes are transcription factors such as E74, E75 or GATA factors that activate the transcription of Vg gene in the fat body (Martin et al., 2001).

A repressor protein, AHR38 attaches to Usp and prevents formation of the EcR-Usp complex, thus inhibiting ecdysteroid signaling in the fat body during previtellogenic phase (Zhu et al., 2000). The increased titer of 20-HE during postvitellogenic phase lifts that inhibition by displacing AHR38 from Usp. Ecdysone or 20-HE alone is not enough to stimulate Vg synthesis in anautogenous females (Borovsky and van Handel, 1979; Fuchs and Kang, 1981). Fat body also requires the nutritional signal from blood-fed midgut to relieve the previtellogenic arrest. Increased amount of amino acids in the hemolymph obtained from the digested blood meal provides an essential signal to initiate vitellogenesis in the fat body (Attardo et al., 2005). Vitellogenin and other yolk protein are synthesized, processed in the fat body, and secreted into the hemolymph to be taken up by the oocytes through receptor mediated endocytosis via clathrin coated pits. The Vg uptake by oocytes increases between 6 and 24 h PBM, and uptake is highest between 24 and 30 h PBM. The uptake is completed by 36 h (Koller et al., 1989).

When the Vg uptake by oocyte is over, lysosomes in the fat body cell start degrading biosynthetic machinery responsible for producing YPPs, and the vitellogenic activity of the fat body trophocytes halts (Raikhel, 1986). It is thought that prolonged 20-HE exposure to fat body
negatively regulates Vg synthesis (Raikhel et al., 2002). At around 36 hours PBM, fat body completely resumes its other functions, e.g. storage and metabolism of nutrients to maintain the energy homeostasis until the next cycle of vitellogenesis starts (Raikhel and Dhadialla, 1992).

d. Post vitellogenic phase

The post vitellogenic phase starts around 30-36 h PBM when the uptake of yolk protein by the oocytes is complete. The length of the yolk mass reaches about 400 µm. In this phase, maternal vitelline plaques fuse to block the gap between the follicle cells and oocyte, and form the vitelline envelope. Next, an endochorion layer is deposited between 40 and 60 h PBM, an exochorion layer assembles surrounding the smooth endochorion (Raikhel and Lea, 1991). By 60-72 h PBM, the exochorion formation is completed (Anderson and Spielman, 1973; Mathew and Rai, 1975; Powell et al., 1988) and females start laying eggs. By 96 h PBM, egg laying is completed. This terminates the postvitellogenic cycle and begins the next previtellogenic cycle.

1.2. Neuroendocrine regulation of egg maturation

A classic study by Arden Lea, demonstrated that surgical ablation of MNCs from female brain of four Aedes mosquito species (Ae. aegypti, Ae. taeniorhynchus, Ae. triseriatus and Ae. sollicitans) impaired their egg maturation after receiving a blood meal (Lea, 1967). Among those four species, Ae. aegypti was totally unable to mature any eggs. Egg maturation was restored by transplantation of one pair of MNCs from a donor. So, it was evident that the MNCs were essential for egg maturation in mosquito. Previous studies showed that blood meal stimulates MNCs to the release a hormone called, egg development neurosecretory hormone (EDNH), from the axons in the neurohemal organ, corpus cardiacum (CC). This EDNH was shown to be responsible for egg maturation (Clements, 1956; Gillete, 1956; Menn et al., 1989). Work by
Meola and Lea indicated that some EDNH was already stored in the MNCs and reaches to corpus cardiacum (CC) by four days time after emergence (Meola and Lea, 1971; Meola et al., 1970). It was also demonstrated that EDNH present in head extract stimulated ovaries to release ecdysone in vitro (Hagedorn et al., 1979).

Several attempts were made to purify and characterize EDNH from female head extract but were not successful (Borovsky and Thomas, 1985; Fuchs et al., 1980; Gade et al., 1997; Masler et al., 1983; Wheelock and Hagedorn, 1985; Whisenton et al., 1987). The basic protocol for all these studies was to fractionate the female head extract, and test each fraction for gonadotropic activity (yolk deposition in blood-fed decapitated females) and ovary ecdysteroidogenic activity. Bioactive fractions were further purified by using conventional chromatography, gel filtration chromatography and reversed phase HPLC. Matsumoto et al purified three groups of fractions (range between 6.5 and 13 kDa) that had both gonadotropic and ecdysteroidogenic activity. Out of these three groups, one group had comparatively high bioactivity. Amino acid composition analysis revealed that the peptide having gonadotropic activity in that group was basic in nature.

The final breakthrough came a decade later, when Brown et al. (1998) separated three bioactive peptides from six million heads. Only one peptide was isolated in sufficient quantity for structural analysis. Mass spectrometry of the portion of the peptide sample revealed a protein of approximately 8.8 kDa. Partial amino acid sequences obtained from amino terminal sequencing were used to retrieve a full length cDNA for sequencing (Gen bank accession no. U69542). After obtaining the full length sequence, recombinant protein was expressed in bacteria.
to check the bioactivity. Since this peptide was the first structurally characterized and shown to directly stimulate ecdysteroid production by ovary in vitro, it was named “ovary ecdysteroidogenic hormone” or OEH rather than EDNH.

a. Ovary ecdysteroidogenic hormone

*Ae aegypti* OEH is the first gonadotropic neuropeptide sequenced and characterized in insects (Brown et al., 1998). It encodes a single polypeptide chain containing 149 amino acids which is thought to be a preprohormone (Fig 3.1A, chapter 3). The first 22 residue of the open reading frame are the signal peptide. During isolation from the head extracts, the mass obtained for the bioactive peptide was 8803 Da, which is same as the predicted molecular mass of the first 86 residues excluding the signal peptide (residues 23 to 108). There is an arginine at 108 position which is thought to be an endoproteinase site for post-translational cleavage. This truncated version of OEH was later expressed in bacterial expression system and found to have gonadotropic activity in vivo and ecdysteriodogenic activity in vitro on ovaries. The presence of 12 cysteines in the truncated OEH peptide suggests six internal disulfide bonds are possible, but based on the mass spectrometry data, the expected mass value is the same as that estimated for the sequence with four disulfide bonds. The bioactivity of the full length long OEH (149 amino acids) was not determined. Sequence analysis (Fig.1) revealed that OEH belongs to a family of neuropeptides, called “neuroparsin” (Brown et al 1998), which was first identified in locusts (See section 1.2.b)

b. Tissue distribution and functions of OEH in *Ae. aegypti*

OEH is thought to be released from the MNCs of the female brain in response to the blood meal, but it is not clear when and for how long OEH is released into the hemolymph after
blood ingestion. Other tissues in females may also be a source of OEH. The results from immunocytochemical studies (Brown and Cao, 2001) showed at least two OEH release sites: MNCs, their axons extending to CC along the anterior midgut and axons extending from the cells in the abdominal ganglion to perivisceral organs (PVO). No qualitative changes in the anti-OEH immunostaining of MNCs and associated axons were observed at different time points after a blood meal, indicating two possibilities, either OEH release was rapidly replenished or OEH was not at all released from CC. In contrast, OEH immunostaining diminished in the PVO by 12 h PBM and did not return until 48 h PBM. This suggests that OEH release is correlated with the activation of egg maturation. It is still unresolved, when and where OEH is released into the hemolymph after the ingestion of blood meal.

c. OEH in other mosquitoes and dipterans

Based on the sequence of *Ae. aegypti* OEH, similar sequences were identified and annotated in the genome database for *Anopheles gambiae* and *Culex quinquefasciatus*. *An. gambiae* OEH (XP_311039) denoted as AgamOEH and *Culex quinquefasciatus* OEH (XP_001870999) denoted as CqOEH consist of 137 and 144 amino acids, respectively (Halt et al., 2002, Arensburger, 2010). Like in the AaeOEH, the first 22 amino acids in both AgamOEH and CqOEH peptide comprise signal peptides (Fig.1). AgamOEH shares 37.7% amino acid sequence identity and 60.6% similarity with AeOEH whereas CqOEH shares 75.8% amino acid sequence identity and 76.6% similarity with AeOEH. Unlike in AeOEH, there is no putative endoproteinase site in AgamOEH and CqOEH.
d. Neuroparsins in locusts

Neuroparsin was first purified from migratory locust, *Locusta migratoria* (Girardie et al., 1989) and later identified in desert locust, *Schistocera gregaria* (Claeys et al., 2003; Girardie et al., 1998; Janssen et al., 2001). Neuroparsin A (NP-A) consists of 83 amino acids and neuroparsin B (NP-B) is a 78 amino acid truncated form of NP-A. Neuroparsins from both locusts share the conserved 12 cysteine residues with mosquito OEH (Badisco et al., 2007). Different neuroparsin isoforms (4 in *S. gregaria*, 3 in *L. migratoria*) (Fig. 1) are generated by alternative mRNA splicing in brain and fat body of the locust, but the proteins for those isoforms were never isolated from those tissues. Locust neuroparsins exhibit sequence similarity with vertebrate insulin-like growth factor binding proteins (IGFBPs) at the N-terminal domain, with the highest similarity to IGFBP7 (Claeys et al., 2003; Janssen et al., 2001).

In locust, a structurally unrelated parsin was also identified and called ovary maturing parsin (OMP) because it stimulated Vg synthesis, possibly by inducing ovarian ecdysteroid production (Badisco et al., 2007). Although OMP has a similar function as OEH, it is structurally different from OEH. Interestingly, no OMP orthologues have been identified in any other insects, including the mosquitoes.

e. Neuroparsin in dipterans

*Drosophila* is the only group in Diptera other than mosquito in which neuroparsins have been reported so far. Surprisingly, no neuroparsin is found in *D. melanogaster* but it is found in eight other related *Drosophila* species (Veenstra, 2010). *Drosophila* neuroparsins also show conservation of the 12 cysteine residues across the species. Veenstra (2010) showed that *D. auraria* which is phylogenetically between *D. ananassae* and *D. melanogaster*, has a full length
neuroparsin sequence and functional promoter, whereas *D. ananassae* has a truncated neuroparsin sequence, lacks the functional promoter, and has lost some conserved cysteines, indicating an evolutionary loss of the neuroparsin gene before the separation of *melanogaster* sub group (Fig.1).

**f. Neuroparsins in other arthropods**

Putative neuroparsin sequences have also been identified from ESTs and gene databases of honey bee (*Apis melifera*), silk moth (*B mori*), crustaceans (*Litopenaeus vannamei, Litopenaeus setiferous*, etc.) and chelicerates (*Limulus polyphemus, Amblyomma varigatum*, etc.) (Claeys et al., 2003). Sequence alignment showed that the certain amino acid residues are highly conserved between mosquito OEH, locust neuroparsins, and cockroach neuroparsin-like peptides despite their evolutionary distance.

**g. Tissue distribution and functions of neuroparsin**

In locust, neuroparsin A (NP-A) is produced in the A1- type neurosecretory cells of *pars intercerebralis*, and neuroparsin B is found in the CC. In locust, mRNA of some neuroparsin isoforms are only expressed in the brain, but others are expressed in fat body, testis, and male accessory glands (Claeys et al., 2005).

Female locust injected with neuroparsin at day 0 after elcosion and repeatedly every two days until day 7, were found to have shorter terminal oocytes, which was an opposite effect of juvenile hormone found in locust (Girardie et al., 1987). It was also shown that it had no effect on Juvenile hormone biosynthesis by corpora alata (Girardie et al., 1987). In the same study, locust nymphs were injected with neuroparsin antiserum at the end of 3\textsuperscript{rd} instar and again at the
beginning and end of the fourth and fifth instar nymph, resulting in a developmental arrest occurred resulted with the formation of a metathetic green pigmented nymph-adult intermediary phenotype. This result suggested a role for neuroparsin in development and metamorphosis.

In addition, *L. migratoria* neuroparsin was shown to have anti-diuretic activity (Fournier et al., 1987), neuritogenic (increase neurite out growth) activity (Vanhems et al., 1990), hypertrehalosomic and hyperlipemic functions, although it is less potent than adipokinetic hormone or AKH (Moreau et al., 1988). In the honey bee, queen brain-specific expression of neuroparsin may indicate a potential role in caste determination or queen longevity (Gronke and Partridge, 2009). Neuroparsins were also found in the extract of CC of two cockroach species, *Blaberus craniifer* and *Leucophaea maderae*, and they were shown to have roles in the regulation of water reabsorption in the posterior gut of cockroach (Boureme et al., 1989).

1.3. Ecdysone biosynthesis in insects: regulation of ecdysone biosynthesis in mosquito ovary

Ecdysone, initially identified as “molting hormone” required for metamorphosis, was later found to have important roles in the reproduction of insects including mosquitoes. During the larval stages of insects, prothoracicotropic hormone originating from brain neurosecretory cells and released from corpora allata (CA) stimulates the prothoracic glands (which disappear during the pupa-adult molt) to produce ecdysteroids. A study by Jenkins et al. showed, unlike other insects, the prothoracic glands in the fourth instar mosquito larva did not produce ecdysone rather some unidentified cells in thorax and abdomen released ecdysteroids *in vitro* at similar times and amounts present in hemolymph of the 4th instar larva *in vivo* (Jenkins et al., 1992).
The regulation of ecdysteroid biosynthesis is thought to occur in insects mainly by three mechanisms: substrate availability, autocrine feedback and peptide hormones (Brown et al., 2009). No published study is available to report the first mechanism. There is only one study which specifically demonstrated the autocrine feedback mechanism for ecdysteroid biosynthesis in insect prothoracic gland (Takaki and Sakurai, 2003). The third mechanism, peptide hormone stimulation of ovarian ecdysteroidogenesis has been well documented.

Cholesterol is the main substrate for ecdysteroid biosynthesis. Unlike vertebrate animals that synthesize cholesterol, mosquitoes and other insects cannot synthesize cholesterol and must acquire cholesterol from food through midgut digestion. Peripheral tissues like ovary and fat body receive cholesterol from the midgut via lipoproteins in the hemolymph (Soulages and Wells, 1994). Once in the specific tissues or cells, some transporter proteins, such as, diazepam-binding inhibitors, start1, sterol career protein-2 are thought to transport the ecdysteroid precursors to specific cellular compartments (Brown et al., 2009). Ecdysteroid biosynthesis reactions are catalyzed by a group of cytochrome p450 enzymes, encoded by a family of “Halloween genes”. In the very first step of ecdysteroid biosynthesis, cholesterol is delivered to endoplasmic reticulum (ER) where the enzyme, 7,8 dehydrogenase converts it to 7-dehydrocholesterol. Then, 7-dehydrocholesterol is transported to mitochondria where it undergoes a series of unknown reactions, termed the “black box” (Brown et al., 2009), to be converted into 3-oxo-Δ4 intermediate, which is then transformed into 5β diketol in the cytosol. 5β diketol is again transported back to the ER to get the first of the terminal hydroxylation reactions and then the hydroxylated intermediate again comes back to mitochondria. In this way, ecdysteroid intermediates are transported back and forth from ER to mitochondria. Terminal
hydroxylase enzyme homologues (CYP302A1, 22-hydroxylase; CYP315A1, 2-hydroxylase) of *Drosophila* were identified in the ovary of *Ae. aegypti* and their transcript expression increased following a blood meal (Sieglaff et al., 2005) indicating a correlation between blood meal ingestion and ecdysteroid production. The final product of ecdysteroid biosynthesis, ecdysone is released by the ovary and converted into the biologically active form, 20-HE by 20-hydroxylase (CYP314A1) in peripheral tissues. A *Drosophila* homologue of 20- hydroxylase was identified (Sieglaff et al., 2005) in the ovaries of *Ae. aegypti* but its transcript level did not increase following a blood meal. This result supported the findings of other studies that showed the ovary lacked 20-hydroxylase activity, and ecdysone was the major ecdysteroid secreted by the ovary in *Ae. aegypti* (Hagedorn et al., 1975; Smith and Mitchel, 1986).

1.4. Insulin-like peptides and their receptors

Insulin-like peptides (ILPs), known to be important for growth, development and metabolism, are highly conserved from insects to human. These peptides belong to the insulin super family and consist of linked B, C and A chains as a propeptide. During post-transcriptional processing the C chain gets cleaved, and the mature peptide forms with the B and A chain connected by interchain and intrachain disulfide bonds. Based on the homology of conserved amino acid residues in the A chain of *An. gambiae* and *D. melanogaster* ILPs, eight ILPs were identified from the *Ae. aegypti* EST and genomic database and characterized by cloning and sequencing (Riehle et al., 2006). Brown et al. demonstrated that ILP3 binding to the mosquito insulin receptor (MIR) in ovary membranes (Brown et al., 2008). Another member of the same peptide family, ILP4, activates MIR signaling but does not directly bind to the MIR, rather binds to a low molecular protein (~ 55 kDa), which is not yet identified (Wen et al., 2010).
Given that ILP3 binds to the MIR and the MIR is the only insulin/insulin-like growth factor receptor present in *Ae. aegypti*, it makes the receptor-ligand interaction of ILPs in mosquito as complex as in vertebrates. In vertebrates, insulin binds to the insulin receptor (IR) which is a tyrosine kinase receptor (RTK), and activates phosphatidylinositol-3 (PI3) kinase, whereas insulin-like growth factors (IGFs) bind both to insulin-like growth factor receptors (IGFR) and IR with different affinities. IGFR, an RTK, activates mitogen activated protein (MAP) kinase pathway (Fig.4.1B, Chapter 4).

In mammals, relaxin and other ILPs were shown to bind to the G-protein coupled receptors (GPCRs), which contain cystein and leucine-rich repeat domains at their extracellular amino terminus. Some of these GPCRs, LGR 7 and LGR 8, stimulate cAMP production through Gs-cAMP-dependent pathway (Hsu et al., 2002) and some of them inhibit cAMP production through Gi-cAMP dependent pathway (Liu et al., 2003). Many GPCRs were identified in *Ae. aegypti* and *An. gambiae* but not all their ligands are known yet. Transcripts of these putative relaxin GPCRs are present in the mosquito ovary suggesting a role in ovarian ecdysteroidogenesis by the production of cAMP (Shapiro, 1983).

**a. Functions of ILPs**

Both ILP3 and ILP4 have gonadotropic and ecdysteroidogenic activities but differ in potency, which can be attributed to the difference in their receptor binding property. Recently, it was also shown that ILP3 and ILP4 differentially regulate carbohydrate and lipid metabolism (Wen et al, 2010). ILP3 acts more like vertebrate insulin, which is hypoglycemic in that it increased the glycogen level in fat body and reduced the soluble carbohydrate, trehalose, in the hemolymph after a sugar meal. ILP3 was also found to be hypolipidemic, i.e. it increased the
storage lipid in the body tissue (Brown et al., 2008). ILP4 had no effect on glycogen or lipid stored in the body tissue following sugar feeding (Wen et al., 2010). A recent study (Gulia-Nuss et al., 2011) showed that ILP3 could restore transcript expression and activity of late serine proteases in the midgut of bloodfed, decapitated females and in the tissue in vitro. ILP3 also restored the Vg transcript and protein expression in the fat body of the blood-fed decapitated animals. A study by Roy et al showed that bovine insulin alone could not stimulate vg transcript expression in the fat body, but in the presence of 20 hydroxyecdysone, it exerted a stimulatory effect (Roy et al., 2007). In the same study, knockdown of an insulin signaling kinase, Akt inhibited upregulation of Vg transcription caused by the combined effect of ILP3 and 20 HE, suggesting a role for insulin receptor signaling via PI3K pathway in Vg synthesis.

b. Cross-talks between OEH and ILP

It is important to note that even though OEH and ILPs are structurally different, they both stimulate ecdysteroid synthesis by the ovary in vitro and yolk deposition in blood-fed decapitated mosquito. Although these regulatory functions overlap, ILPs or OEH may have their own predominant functions which are not identified yet. It will be interesting to investigate if OEH has any regulatory role in the nutrient metabolism, midgut digestion and or Vg synthesis of Ae. aegypti.

No receptor or binding protein for OEH/ neuroparsin has been identified to date. So, whether OEH stimulates ecdysteroidogenesis via an RTK or any GPCR is still an open question to answer. Recently, a recombinant locust neuroparsin, Scg-NP4, having sequence similarity to vertebrate IGFBP was shown to bind to the S. gregaria insulin-related peptide (Scg-IRP) in vitro using affinity column chromatography (Badisco et al., 2008). A molluskan IGFBP-like peptide, termed as “perlustrin,” was also shown to interact in vitro with vertebrate IGF and insulin (Weiss
et al., 2001). Given some sequence similarity with vertebrate IGFBP, it is possible that OEH may bind to any of the ILPs to activate the physiological processes in mosquito.

1.5. Objective of the study

Based on the literature described above, OEH is an important regulator of egg maturation in mosquito, but its other functions and signaling mechanism are still unknown. Further characterization of the tissue distribution, functions and the mechanism of action of OEH will give us a better understanding of mosquito reproduction and thus may provide a better way to control mosquito population in the wild. We will investigate some important questions in the following chapters.

In the second chapter, we will investigate if OEH is solely expressed in the heads or in other body parts (thorax, abdomen wall, ovary and hemolymph) as well, and how ingestion of the blood meal changes the expression pattern of OEH in different body tissues during a gonotrophic cycle. It will be interesting to know if OEH and ILPs are localized in the same or different MNCs of brain.

In the third chapter, we will examine if the bioactivity of short form of synthetic OEH is similar or different to that of long form of recombinant OEH. We will use gonadotropic (in vivo) and ecdysteroidogenic (in vitro) bioassays to compare the bioactivity of the two OEH forms. Using the same bioassays, we will also test if OEH and ILP3 both act in an additive or synergistic way when both the peptides are present. We will also seek the answer if OEH can restore trypsin expression and activity in midgut and Vg synthesis in fat body of the blood-fed decapitated females in vivo or in the midgut and fat body tissue culture in vitro. Previous studies (Brown et al., 1998; Wen et al., 2010) demonstrated that ILPs acted differently in regulating
nutrient storage in the sugar-fed females. We will also investigate if OEH regulates nutrient storage in the sugar-fed females.

In the fourth chapter, we will investigate which signaling pathways are activated by OEH with reference to ILP3. We know, ILP3 stimulates ovary ecdysteroid synthesis through insulin receptor signaling. So, we will investigate if OEH works through insulin receptor signaling or other pathways to induce ecdysteroid synthesis. ILP3 induced ovarian ecdysteroid production was shown to be modulated through amino acid sensing and target of rapamycin pathway. We will examine if OEH mediated ecdysteroid production can be modulated through the same pathway. cAMP was also shown to be an inducer of ecdysteroid production. So, we will examine if OEH can stimulate ecdysteroid production through the production of cAMP by the ovaries.

In the fifth chapter, we will examine if OEH binds to any ovary membrane protein by radio-receptor binding experiment. By immunocytochemistry and immunoblot, we will investigate whether OEH is taken into the ovary. If taken up by the ovary, in which cellular compartment (cytoplasm or nucleus) OEH is destined to after entering through the cell membrane.

The sixth chapter will summarize the results from the preceding chapter and highlight the important findings about OEH functions and signaling in comparison to ILP functions and signaling. The results from this study will provide a better understanding of anautogenous mosquito reproduction. This dissertation work will also guide the direction of future studies to answer unresolved questions.
1.6. References


Fig. 1: Alignment of the amino acid sequences of mosquito OEH and neuroparsin from *Drosophila* and locust species: (adapted and modified from Veenstra, 2010) Amino acid residues highlighted in yellow comprise of the predicted signal peptide for each individual OEH/neuroparsin protein. Conserved cysteine residues within or across the groups are labeled in red (numbered C1-C13). Other conserved residues are highlighted in black or gray.
2.1. Introduction

In insects, neurosecretory cells and midgut endocrine cells are the source of many different neuropeptides throughout all life stages (Gade et al., 1997). A classic endocrine study (Lea, 1967) showed when the medial neurosecretory cells (MNCs) were surgically removed from female mosquitoes, they were unable to mature eggs after receiving the blood meal, and egg maturation was restored by transplantation of one pair of MNC from a donor. These findings indicated that brain neurosecretory cells were the main source of the neuropeptides which regulate egg maturation. Later, another study (Van Handel and Lea, 1984) showed that decapitated Ae aegypti females given a blood enema could produce vitellogenin. Another study (Masler and Kelly, 1995) demonstrated that the extracts of thorax or abdomen had ecdysteroidogenic and gonadotropic activity although to a lesser extent than head extract. These studies indicated that the source of ecdysteroidogenic or gonadotropic factors could also exist in the body tissues other than head.

Knowledge of OEH distribution and expression is very important to identify specific roles of OEH in any physiological processes. Previous immunocytochemistry study with brain section showed anti-OEH staining in bilateral MNCs (Brown et al., 1998). Later, another
immunocytochemistry study with whole brain confirmed the previous observation and identified OEH immunoreactivity in the peptidergic cells in the optic lobes, cardiac valve, anterior midgut, and neuronal cells in the subesophageal ganglion and thoracic ganglia, abdominal ganglion and perivisceral organs (PVO) of larva and adult of *Ae. aegypti* and *An gambiae* (Brown and Cao, 2001). OEH belongs to the peptide family called “neuroparsin” which was first identified from the migratory locust (Girardie et al., 1989). In locusts, transcripts of some neuroparsin isoforms are expressed in other body tissues in addition to brain neurosecretory cells, but no protein expression was reported in those tissues (Claeys et al., 2005).

A study on *Ae aegypti* ILPs (Riehle et al., 2006) showed that transcripts of some ILPs (ILP 1, 3 and 8) are specifically expressed in head but others (ILP2, 4, 5 and 7) are also expressed in other tissues (Riehle et al., 2006). In the same study, a monoclonal antibody against silk worm ILP (bombyxin) stained bilateral clusters of MNCs and their connecting axons, extending up to the anterior midgut of both fourth instar larva and adult, but the locust insulin related peptide (LIRP) polyclonal antibody stained lateral clusters of neurosecretory cells and subesophageal ganglion, abdominal ganglia and gut endocrine cells indicating the neurosecretory cells might be specific for individual ILPs.

So, based on previous studies, it is very likely that the brain neurosecretory cells are the main source of OEH, but it is not known when and how much OEH is released in the hemolymph in adult. Because, OEH immunoreactive cells are also found in other body tissues, we also cannot rule out the possibility that these tissues are the source of OEH. In this chapter, we examined the expression and distribution of OEH transcript and protein at different life stages.
and in female tissues during a gonotrophic cycle. It is also not known whether same set of neurosecretory cells in the brain produce both ILPs and OEH. By using dual label immunocytochemistry and antibodies against ILP3 and OEH, we investigated that as well.

### 2.2. Materials and Methods

**a. Mosquito rearing**

*Aedes aegypti* (UGAL strain) was maintained at 27°C in a 16 h light/8 h dark photo period. Larvae (150-200 / tray with 500 ml of deionized water) were fed ground rat chow: lactalbumin: brewer yeast (1:1:1) at defined amount according to standard rearing protocol. Newly emerged were provided with the deionized water. On day 2 post eclosion, the adults were given 5% sugar water for only one day and then again provided with deionized water for the rest of the time. Blood feedings were done on an anesthetized rat for 10-15 min when required by the experiments or for the purpose of collecting eggs.

**b. Characterization of OEH protein expression during development and a gonotrophic cycle**

Samples of different life stages were collected in ice cold phosphate buffered saline (PBS): eggs (approx. 300 at 36 h post oviposition), heads, thoraces and abdomens (10 each) from fourth instar larva, pupa and 3 day old sugar-fed females and males. Also, tissue samples (head, abdomen, hemolymph, and ovary) were collected from sugar-fed and blood-fed females at different time points (2, 6, 12, 18, 24, 30, 36, 48 and 72 h) post blood meal (PBM). All samples were collected in 100 ul of ice cold 0.2 N acetic acid, ground with plastic pestles, rinsed with 300 ul of 0.2 N acetic acid, sonicated (3-4 medium pulse), centrifuged at 13,000 xg for 5 min at 4°C.
Supernatant was removed without disturbing the pellet and frozen at -80°C before lyophilization. Lyophilized samples were resuspended in Tris-Tricine sample buffer (Nusep Tris-tricine, 125 mM DTT). Samples and standards were heated at 65°C for 45 min, put into ice immediately and centrifuged at 13,000 xg for 2 min. After centrifugation, 4 body parts equivalent samples were loaded per lane of a 16.5% Tris-Tricine gel (Criterion, BioRad) and run at 90 V at 4°C until the dye front reached to the bottom of the gel. Following gel electrophoresis, the proteins were transferred to 0.1 μm nitrocellulose membrane for 1 h at 45 V at 4°C in transfer buffer (12 mM Tris-base, 96 mM glycine, 20% MeOH). After transfer, the nitrocellulose membrane was dried over night. On the next day, the membrane was washed with TBST (Tris-buffered saline pH 7.4 with 0.1% Tween-20) for 5 min and then blocked with ECL blocking agent (0.1 gm/ 10 ml) and treated overnight at 4°C with OEH primary antibody (rabbit anti AaeOEH 304C) (Brown and Cao, 2001) at a 1:10,000 dilution. After washing three times with TBST (each wash for 20 min) and the membrane was incubated with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:20,000 final concentration). The membrane was washed 3 times (each for 20 min) with TBST and developed with ECL advance chemiluminescence kit. Images were taken by GeneGnome (SynGene) imaging system.

c. Characterization of OEH transcript expression in different tissues

Tissue samples were collected in ice cold PBS from head, thorax, gut, ovary and abdomen wall of both sugar-fed and blood-fed females from different time points and kept at – 80 °C until further use. Total RNA was extracted from the collected tissues by using high pure RNA isolation kit (Roche, cat #11828665001) with on-column DNase digestion. Total RNA was quantified with a nanodrop spectrophotometer (Thermo scientific). Up to 1 μg of total RNA was used for cDNA synthesis. PCR was done using OEH specific primers (forward primer: 5’-
TGGAGATTCGCTGCAAGCTGTACT – 3’ (ORF position: 83-106) and reverse primer: 5’-ACATCTTGTAGAGCGACGGGAACA – 3’ (ORF position: 421-398). PCR conditions were as follows: 94°C for 3 min, 94°C for 30 sec, 65°C for 45 sec, and 72°C for 1 min for 35 cycles and 72°C for 5 min. Single primer and no template controls were used to rule out any non-specific amplification. PCR products were run in 1.2% agarose gel and photographed using a Gene-Gnome imaging system.

d. Whole brain dissection and immunocytochemistry

Whole brains were dissected from 3 to 5 day old females in PBS and placed in fixative (4% paraformaldehyde in PBS) in a 1.5 ml microtube cap. Fixation was done for 2 h at RT on a rocker. After fixation, tissues were permeabilized with 0.2% Triton X-100 in 4% paraformaldehyde in PBS for 15 min on rocker at room temperature (RT). Then, the tissues were washed 3 times (5 mins each on rocker) with PBS at RT. Next, the tissues were treated with a blocking solution (PBS-GS-T: PBS with 5% goat serum and 0.1% Tween-20) for 2 h on a rocker at 4°C. After blocking, the tissues were incubated in anti-OEH antibody (304C) at 1:1000 dilution (with blocking solution) for overnight at 4°C on a rocker. On the next day, the tissues were washed 3 times (30-40 min each) with PBS-GS-T on a rocker at 4°C and incubated with goat-anti rabbit antibody (Alexa Fluor™ 488 fragment of goat anti-rabbit IgG (H+L); Molecular Probes, A11070, lot no. 73B2-1) 1/2000, overnight, on a rocker at 4°C. On the next day, the tissues were washed with PBS-T (PBS with 0.1% Tween) 3 times (30-40 min each). The tissue was blocked again with same blocking solution for 2 h at 4°C on a rocker and then incubated with Aedes aegypti anti-ILP3 antibody (R-3850, affinity purified) in block solution at 1:100 dilution for overnight at 4°C. Next day following three washes with PBS-GS-T (PBS-goat
serum-Tween 20) for 30-40 min each, the tissues were incubated with goat-anti rabbit (Alexa Fluor™ 568 fragment of goat anti-rabbit IgG (H+L); Molecular Probes, A21069, lot no. 57028A) 1/2000 for overnight at 4°C. The tissue was washed again with PBS-T (PBS with 0.1% Tween) 3 times (30-40 min each) at 4°C. The tissue was mounted on slide with 1:1 (PBS: glycerol) and looked under the SP2 confocal microscope at dual wavelengths (488 nm & 568 nm).

2.3. Results

a. OEH peptide expression in females at different life stages

Previous immunocytochemistry studies (Brown and Cao, 2001; Brown et al., 1998) detected staining with OEH antiserum in the brain and other tissues of larvae and adults. Here, we conducted a comparative study on OEH protein expression in different body parts and life stages with a Western blot approach to examine whether the immunoreactivity detected previously by OEH antiserum recognizes a protein consistent with predicted mass of OEH. We used synthetic short form of OEH peptide (86 amino acids) as a positive control and a prestained protein marker to analyze the molecular weight of immunoreactive bands. The OEH antiserum detected two immunoreactive peptide bands (approximately 9,000 Da and 13,000 Da) in head samples of pupae and adults but in larva the upper band is not very distinct (Fig. 2.1). Our result also shows that OEH protein is not expressed in embryos (eggs) but is expressed in later life stages (larvae, pupae and adults). The intensity and distinction of OEH peptide bands in larval heads is not as good as in head samples of pupae or adult stages. A faint immunoreactive band of expected size (9,000 Da) was observed in female but not in male abdomen.
b. OEH peptide and transcript expression in different female tissues during a gonotrophic cycle

Because OEH is one of the major regulatory peptide hormones for egg maturation in females, we focused on examining OEH protein and transcript expression in different female tissues before and after blood feeding. OEH peptide is always present in the head, and blood feeding seems to have no effect on OEH protein expression in the head (Fig. 2.2) which corresponds to the uninterrupted transcript expression in the head during a gonotrophic cycle. In the ovary, OEH peptide was first detected at 18 h PBM and the peptide level increased up to 48 h PBM and then started decreasing. The level of OEH transcript in ovary is much lower than in the head tissue and transcript expression pattern in the ovary followed an inverse trend of its protein expression. Upon blood feeding, OEH transcript levels increased in ovary slightly from sugar-fed females and expressed at the same levels until 18 h PBM and then started falling by around 72 h PBM, it comes back to sugar-fed level. In hemolymph, OEH peptide was first detected at around 12 h PBM and the protein level gradually increased and reached to a maximum level at 36 h PBM (due to high content of protein at 36 h, the chemiluminesce signal burned out, and appeared as doublet, but it is a single protein band) and fall to a low level by 72 h PBM. In the abdominal wall, OEH was detected at around 36 h PBM and increased to a substantial level by 72 h PBM. OEH peptide was not detected in thorax at any time point of the gonotrophic cycle. Even though protein was detected in hemolymph or abdominal wall, no transcript was found in those tissues at any of the time points (not shown for the corresponding blots).
**c. Medial neurosecretory cells produce both OEH and ILP3**

In the mosquito protocerebrum, there are both medial and lateral clusters of neurosecretory cells (Fig. 2.3A). The axons from bilateral medial clusters, cross over and exit from the brain and extend to the primary neurohemal organ, corpus cardiacum (CC). Axons, coming from bilateral clusters of lateral neurosecretory cells directly join the CC without making any cross-over. A previous study showed (Fig. 2.3B) OEH immunoreactivity in the bilateral clusters of MNCs and their axons, crossed over and exited the brain and reached to the CC (Brown and Cao, 2001). In the present study, we did whole brain immunocytochemistry with ILP3 antibody and found a similar immunostaining pattern (Fig. 2.3C) as that of OEH. Because OEH and ILP3 antibody both stained MNCs, we wanted to examine whether the same cells in MNC cluster produce both OEH and ILP3 or certain cells are specific for an individual peptide. Our dual label immunocytochemistry of the whole mount brain showed that *Aedes aegypti* ILP3 antibody or *Bombyx mori* insulin-like peptide antibody (A1A3) staining was mostly concentrated in the medial clusters (Fig. 2.4. A2 & B1), whereas OEH immunoreactivity (Fig. 2.4. A1 & B2) was observed in some cells of medial cluster as well as adjacent cells lateral to the medial clusters. Merged images (Fig. 2.4. A3 & B3) showed that majority of the MNCs were stained for anti-OEH or anti-ILP3 immunoreactivity, but a few are specific for the individual peptide.

**2.4. Discussion**

In this study, we examined OEH expression in more detail than previously reported. First, we demonstrated the protein expression of OEH in the body parts of different life stages of both sexes. Then we examined OEH expression in tissues in both sugar-fed and blood-fed females. In the blood-fed females, we examined OEH expression at certain intervals up to 72 h PBM to
monitor if blood meal ingestion regulates OEH transcript and protein expression in different tissues and if that correlates with any known function of OEH in relation to the egg maturation process. By using double label immunocytochemistry method, we demonstrate for the first time that some cells in MNC cluster produce both ILP3 and OEH.

We did not find OEH protein in the *Aedes aegypti* embryo (eggs) indicating that OEH may not have any role in the differentiation of organs during embryogenesis. In later life stages, OEH is always present in the head of larva, pupa and both adult male and females (Fig. 2.1). The higher molecular weight immunoreactive band (~13 kDa) is not present in larva but present in pupa and adult stages. Higher molecular weight band may represent the unprocessed prepropeptide or else a protein complex in which OEH is bound to some other protein. The lower band (~ 9 kDa) is probably the processed mature peptide present in the tissue. Mass spectrometric analysis of these two OEH immunoreactive bands may validate our assumption. OEH expression in female head is much higher than in male indicating OEH may have more important function in females than in male counterparts. Moreover, expression of OEH in males suggests its different function(s) other than stimulating ovaries to produce ecdysteroid and mature eggs in females.

We found that OEH peptide and transcript were present in the head tissue all the time independent of blood ingestion by females (Fig. 2.2) suggesting that blood feeding is not required for OEH synthesis in the MNCs which is in agreement with the findings of a previous study (Brown and Cao, 2001) showing that there is no qualitative changes in the immunostaining of MNCs by OEH antisera at different time points after blood meal ingestion which also
indicated two possibilities that either OEH released from CC was rapidly replenished or OEH was not at all released from CC.

Interestingly, in addition to the head, we also detected OEH transcript in the ovaries of both sugar-fed and blood-fed females. Based on the current literature, the blood meal triggers the release of OEH from female brain and stimulates ovary to produce ecdysteroids, that initiate the egg maturation process. The presence of OEH transcript in the ovaries indicates that ovary may produce OEH but may use brain OEH as a trigger to synthesize its own peptide. In contrast to the brain, OEH protein was detected in ovaries at around 18 h PBM when its transcript level started decreasing. It is still not clear whether ovaries uptake OEH from the hemolymph or produce their own OEH, thus are not dependent on brain OEH. It is plausible that ovary may need brain OEH as a stimulus to produce its own OEH at the later part of the gonotrophic cycle.

We detected OEH on Western blots of hemolymph extracts at around 12 h PBM. This is very late for OEH to be in the hemolymph according to the time frame of egg maturation cycle. It is possible that OEH is released from the brain immediately after a blood meal but the level present in four body parts equivalent as loaded per lane on gel may be too low to be detected until 12 h PBM. Since we did not find any OEH transcript in the hemolymph, we think that the hemocytes present in hemolymph do not transcribe OEH mRNA. So, the OEH protein, we see in the hemolymph, comes from brain MNCs after being released by blood-feeding induced stimulation. The previous immnuocytochemistry study (Brown and Cao, 2001) showed OEH immunoreactivity in the some endocrine cells present in the gut of both larva and adult and in the PVO and abdominal ganglion of both male and female Aedes aegypti. They demonstrated that OEH staining disappears in PVO at around 12 h and again comes back at 48 h PBM. In contrast, we
did not see OEH peptide in the extract of female abdomen wall tissue until 36 h PBM (Fig. 2.2), neither did we observe any OEH transcript expression in gut nor in whole thorax and abdomen wall of both non-bloods fed and blood-fed females. This discrepancy in OEH distribution may be due to the relative amount of peptide present vs the detection efficiency of western blot and also the cross-reactivity of OEH antisera in the context of other peptidergic cells present in those tissues.

OEH is not the only peptide produced by MNCs in *Ae aegypti*. The MNCs in *Ae aegypti* showed immunoreactivity with antisera against neuropeptide F (Stanek et al., 2002), leucokinin-like peptides (Chen et al., 1994), FMRFamide-like peptides and bovine pancreatic polypeptide (Brown and Lea, 1988) and *Bombyx mori* ILP (Brown et al., 2008; Riehle et al., 2006). In the whole brain immunocytochemistry study by Brown et al. an antisera against silkmoth ILP (bombyxin), stained the MNCs in the brain and also cross-reacted with *Ae aegypti* synthetic ILP3 peptide on dot blot (Brown et al., 2008). Here I used ILP3 antisera (raised against synthetic *Ae aegypti* ILP3 peptide) for the first time to stain the whole mount female brain and detected similar staining pattern of MNCs and their connecting axons (Fig. 2.3) as detected by previously the bombyxin antibody. The ILP3 antibody staining of MNCs and associated axons is comparable to the staining of whole brain by OEH antisera (Brown and Cao, 2001). This similar distribution of OEH and ILP3 in MNCs and associated axons made us investigate whether the same cells in the MNC cluster produce both OEH and ILP3. Our dual label immunocytochemistry showed that within the bilateral MNC clusters, some cells located at the anteromedial axis stained with both OEH and ILP3 antibody whereas a few cells on the lateral to anteromedial axis were specifically stained with OEH (Fig. 2.4 A3) indicating some cells produce both peptides and some are specific to OEH. Although a good number of MNCs produce both OEH and ILP3,
it is still not known how the synthesis and release of these two different peptides are regulated within the same cell which needs to be further investigated.

2.5. References


The expression of OEH peptide at different life stages of *Ae. aegypti*. Proteins were extracted from eggs and different body parts (H = head, T = thorax, A = Abdomen) of fourth instar larvae, pupae, male and female adults (3 days old, sugar-fed) and electrophoresed on a 16.5% Tris-tricine polyacrylamide gel under reducing conditions. Blots were incubated with an anti AaOEH antibody (304C) for chemiluminescent detection. Molecular weights of standards (MW STD) and *Aedes* OEH standard are displayed to the left. This immunoblot is representative and the summary of immunoreactive peptide detection from at least three independent cohorts: +, peptide present in majority of samples or -, peptide absent in the majority of samples. An immunoreactive band of the approximate size (9.0 kDa) of AaeOEH peptide was detected in heads of larva, pupae and adults and faint band was detected in the abdomen of the females. Another larger (approx 13.0 kDa) immunoreactive peptide band was also detected in heads of pupae, males and females only.
Figure 2.2. OEH protein and transcript expression in female *Ae. aegypti* tissues during a gonotrophic cycle. Only the tissues showing OEH protein or transcript expression at at least one time point during a gonotrophic cycle are displayed here. For protein detection, 4 body parts equivalent tissue extracts were loaded per lane and OEH antisera detected a single immunoreactive band of expected size (~9 kDa). For RT-PCR, equal amounts of RNA were used for cDNA synthesis and equal amounts of cDNA were used as template to amplify an OEH specific product (339 bp) without introns. Each blot or transcript expression data is representative of at least three experiments from independent cohorts.
Figure 2.3. Whole brain immunocytochemistry in female *Ae. aegypti* with ILP3 and OEH antiserum. **A.** Diagram showing cross section (anterior to posterior) of female head displaying protocerebrum containing peptidergic neurosecretory cells clusters. **B.** OEH antisera stained (black arrowhead) the clusters of MNCs and their axons which formed a chiasma and arch in the brain (BR), exited in the nervi corporis cardiaci (NCC), and passed over the pharyngeal pump (PP) to the corpus cardiacum (CC) [reprinted from Cao and Brown, 2001]. **C.** Immunostaining of whole mount brain with ILP3 antisera demonstrated immunoreactivity in two clusters of MNCs (white arrow) in the dorso-posterior region of the brain. ILP3 immunoreactive axons extended (red arrow) from the MNCs, crossed over (yellow arrow) and exited the brain to the CC (white arrow), a possible release site for ILP3 into the hemolymph. Some axons branched laterally projected towards the optic lobes.
Figure 2.4. Dual immunostaining of *Ae. aegypti* female brain with OEH, ILP3 and silkworm ILP antibody. **A1**. OEH staining in the cluster of medial neurosecretory cells (MNCs) (blue arrow) and other adjacent cells (white arrow). **A2**. ILP3 staining (blue arrow) is located in MNCs. **A3**. Merged images show some MNCs (blue arrow, yellow color cells) produce both ILP3 and OEH and others only OEH (white arrow). **B1**. Silkworm ILP antibody (A3A1) staining in MNCs (blue arrow). **B2**. OEH staining in same MNCs (blue arrow), lateral cells (white arrow) and axon tract (yellow arrow) extended from the MNCs towards corpora cardiaca. **B3**. Merged images show that some cells and axon tract are specifically stained for OEH (white arrow) and some (blue arrow, yellow color cells) for both OEH and A3A1. The results show that some MNCs produce both OEH and ILPs which might reflect their functional redundancy.
3.1. Introduction

OEH was first isolated and identified from the head extract of *Aedes aegypti* adults (Brown et al., 1998) on the basis of its stimulation of yolk deposition in blood-fed decapitated females and *in vitro* ecdysteroid production by ovaries. During isolation, the bioactive fraction that eluted as a single peak through high performance liquid chromatography was subjected to mass spectrometry to determine the molecular mass of OEH peptide. The molecular mass obtained from mass-spectrometry was 8803 Da, which was equivalent to mass of the predicted 86 amino acid sequences of the open reading frame excluding the signal peptide and spanning from 23 to 108 residues (Fig. 3.1 A). There is an arginine (R) residue at 108th position which is thought to be an endoproteinase site where the cleavage might have occurred during the processing of propeptide to mature peptide. Brown et al suggested that this truncated peptide might have originated from degradation during peptide extraction and purification (Brown et al., 1998). In the same study this truncated form of OEH (short OEH, 86 amino acids) was expressed in a bacterial vector and the purified peptide showed high specific activity in both *in vitro* and *in vivo* bioassays. The mass spectrometry of the purified peptide showed similar molecular mass to that of native OEH which indicated that OEH peptide was bioactive without its 41 residues (109th to 149th amino acid) at the C-terminal end. So, there is still an open question whether the full length peptide (127 amino acids, excluding the signal peptide, predicted MW: 13.3 kDa) is
bioactive or not. If this long OEH is bioactive, how it differs from shorter version form in terms of bioactivity.

In this study, we compared the bioactivity of the long and short form of OEH by *in vivo* and *in vitro* bioassays with the ovary. In all the experiments, we used *Ae. aegypti* insulin-like peptide-3 (ILP3) for comparison and reference because of its similar gonadotropic and Ecdysteroidogenic function (Brown et al., 2008).

A recent study (Gulia-Nuss et al., 2011) showed that ILP3 could restore late-phase serine protease activity in midguts of blood-fed decapitate females. In this study, we examined if OEH has similar roles as that ILP3 in blood meal digestion by regulating late serine protease activity. As OEH stimulates yolk deposition in the ovaries of blood-fed decapitated females, it is worth investigating whether or not OEH increases the vitellogenin (Vg) synthesis in the fat body of blood-fed decapitated mosquitoes. It is also not known whether the OEH alone can stimulate all these physiological processes (midgut digestion, Vg synthesis in fat body) *in vitro* in a non blood-fed background or needs the nutritional signal (blood meal) to stimulate these processes. ILP3 but not ILP4 was shown to have roles in nutrient storage in body tissue in the decapitated sugar-fed females. Here, we have investigated role of OEH in nutrient storage using ILP3 and ILP4 alongside as controls.

### 3.2 Materials and Method

#### a. Mosquito rearing

Mosquitoes were reared as per the standard protocol (See chapter 2.2.a).
b. Synthetic short OEH (sOEH)

The 86-residue (residue 23 to 108 from amino terminal) OEH-peptide was synthesized by the solid phase method (Merrifield, 1963) using standard Fmoc-chemistry (Atherton et al., 1978) on a Perkin Elmer ABI433 A peptide synthesizer (PE Applied Biosystems). The crude peptide was purified by HPLC. During purification by HPLC column (Jupiter 5 µM, 300A, C18, 100 mm), based on absorbance and the predicted retention time of synthetic peptide, fractions were collected, diluted and checked for gonadotropic and ecdysteroidogenic bioactivity. Bioactive fractions are pooled, lyophilized and stored.

c. Recombinant long OEH (rlOEH)

Full length Ae aegypti OEH without the signal peptide (residue 23 to 149) was expressed in the bacterial vector pET32 (Novagen) that has an ampicillin resistance gene (selection marker), thioredoxin (for disulfide bond formation), a histidine tag (for purification using nickel column) and an enterokinase site (for digesting long OEH from other attached proteins) adjacent to the ligation independent cloning site (Fig.3.1B). The pET-32 vector with the full length OEH construct was transformed into the Rosseta Gami (Novagen) E coli competent cells. The cells were lysed and the recombinant OEH peptide was separated from the cell extracts by using affinity column elution method by serial increase of imidazole concentration. Eluted proteins was purified through 30K Centricon gel filtration column, desalted and digested overnight with enterokinase enzyme (EMD Millipore, cat# 69066) at room temperature to separate the long form rest of the fusion proteins (Fig.3.1B).
d. Comparison of gonadotropic activity of short and long OEH in vivo

Synthetic short (sOEH) and recombinant long OEH (rlOEH) were used in this experiment to compare its bioactivity to ILP3. Females (3-5 day old) were blood-fed on an anesthetized rat. Within 1 h post blood meal (PBM), females were decapitated and injected with 0.5 µl of either Aedes Beyenbach saline or different concentration (0.2, 2, 10 and 20 pmol) of synthetic or recombinant OEH. After 24 hours, the injected females were dissected, and longitudinal yolk lengths were measured under Olympus SZ-60 stereo microscope outfitted with an ocular micrometer (20 µm increments).

e. Comparison of ecdysteroidogenic activity of short and long OEH in vitro

Two or four pairs of nonoogenic ovaries were dissected from 3-5 day old sugar-fed females and transferred to 50 µl Aedes Beyenbach saline or amino acid rich SF900 medium in a cap of a 500 µl microcentrifuge tube placed into a 24 well flat bottom RIA plate (Costar®). Each well contains 200 µl deionized water. Different doses (0.1, 0.5, 1, 5 and 10 pmol) in 10 µl of sOEH or rlOEH or ILP3 were added into incubation medium except in controls where 10 µl of the same incubation medium was added. The ovaries were incubated for 6 hours at 27° C in a rotating water bath. Following incubation, 50 µl of saline media was removed from each cap by pipette and stored in 500 µl microcentrifuge tubes and stored at –80º C.

For RIA, samples were incubated overnight with [3H] ecdysone and anti-ecdysteroid rabbit serum (AS 4919, a gift from P. Poncheron, Université P. et M. Curie, Paris, France) which recognizes both ecdysone and 20-hydroxyecdysone equally (Porcheron et al., 1989). Each RIA tube contained 50 µl of a stock [23, 24-3H (N)] ecdysone solution (³H- ecdysone; 12,000-13,000 counts/minute (cpm)/50 µl; PerkinElmer, Boston, MA), 50 µl of antiserum diluted to 1:35,000-
45,000 (final dilution for bound to free [3H]ecdysone cpm ratio (B/F) = 1), and 50 μl of sample or ecdysone standard. Separate, triplicate tubes were set up for total cpm [3H] ecdysone, non-specific binding of [3H]ecdysone, diluted antibody and [3H]ecdysone alone to monitor B/F, and each of the ecdysone standards (4, 8, 16, 31, 62, 125, 250, 500, 1000 and 2000 pg) for every RIA. After overnight incubation at 4° C, bound and free radiolabeled ecdysone were separated in tubes by the ammonium sulfate method, and pellets in tubes were dispersed in water and scintillation fluid and counted in a scintillation counter (Beckman). For each RIA, a standard curve was plotted from the averaged B/F (Y axis) and log values for the ecdysone standards (X axis). The quantity of immunoreactive ecdysteroid in samples was calculated from a regression equation for the linear portion (typically 16-1000 pg) of the standard curve; samples were diluted when necessary to stay within this range. Sample values reported for each tissue treatment are presented as "ecdysteroid pg", because the secreted ecdysteroids are unknown, and the values are means of triplicate treatments from three experiments.

f. Late serine protease assay

Females (3-5 days old, sugar-fed) were blood-fed on an anesthetized rat. Within 1 h PBM, mosquitoes were decapitated and injected with two doses (5 and 10 pmol per 0.5 μl) of OEH. The same volume (0.5 μl) of Aedes saline was injected in the decapitated (negative control) and the intact (positive control) mosquitoes. ILP3 (20 pmol/ 0.5 μl) was also injected in decapitated mosquitoes for comparison. Eight to ten females per treatment were kept in a small cage lined with moist paper towel. The intact mosquitoes were provided with soaked cotton for drinking water. After 24 h, mosquitoes were dissected, and the color of the blood bolus was observed. A total of five midguts (one midgut /tube) were taken into 200 μl of Tris buffer (0.02M, pH 8.0;
with 0.02M CaCl$_2$) for each treatment. Midguts in solutions were then sonicated (low 3-4 medium pulse) and centrifuged at 13000 x g for 5 min. Supernatants were collected, and 10 µl of that supernatant was added to 90 µl of Tris-buffer. Then 200 µl of 4 mM BApNA (N $\alpha$ Benzoyl-L-Arginine-p-Nitroanilide) was added to both samples and standards and incubated at RT for 15 min on a rocker. After incubation 75 µl of both standards and samples were loaded on a 96 well plates and absorbance was read at $\lambda$ 405 nM.

g. Quantification of late serine protease transcript

Four midguts from each treatment group were collected in PBS and frozen at -80 ºC if not used immediately for RNA extraction. High Pure RNA isolation kit (Roche) with on-column DNase digestion was used for RNA extraction. Total RNA was quantified with a Nanodrop-1000 (Thermoscientific). Up to 1 µg of total RNA was used for cDNA synthesis (iScript cDNA synthesis kit, BioRad). The cDNA was again quantified and normalized for each treatment sample. Equal amount of diluted cDNA was used in 10 µl qRT-PCR reaction mixture. Serine protease VI (5G1) primer pair (AaeSPVI, GQ398048; forward 5’AGGAATGCCACA AGGCTTACT TGA 3’; reverse 5’CCATAACCCCGAGGATACCCT 3’) and ribosomal protein S7 primer pairs (AaeS7, AY380336 forward 5’ ACCGCCGTCTACGATGCCA 3’; reverse 5’ ATGGTGTTGCTGTGGTTTCTT 3’) were used to amplify the specific target transcript (Isoe et al., 2009). The qRT-PCR reaction mixture containing iQ SYBR Green Supermix (BioRad; 5 ml/tube), template cDNA and specific primer pairs were run on a Real Time-PCR thermal cycler (Rotor-Gene Q, Qiagen) for 40 cycles under the following conditions: denaturation at 95°C for 20 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. All reactions were run in quadruplicate with the reference housing keeping gene being ribosomal
S7 RNA. Real-time PCR data were analyzed by the ΔΔCt method as outlined by a previous study (Gulia-Nuss et al., 2011).

**h. Quantification of Vg transcript**

While collecting the midguts from each treatment group, 4 abdomens were also collected in PBS for RNA isolation from the same animals. Total RNA was extracted as described above from midguts and reverse transcribed to cDNA. As template 1 μl of 10 times diluted cDNA was used in 10 μl of qRT-PCR mixtures containing iQ SYBR Green Supermix (BioRad; 5 ml/tube). *Ae. aegypti* Vg primers (Genbank Accession Number U02548; forward 5’ CAGAAGTT CGGTGCTCCTTC 3’; reverse 5’ TTCGAAGCCGAAGTCTGTAGT 3’ from Gulia-Nuss et al., 2011) and ribosomal protein S7 primers ; AeS7, AY380336, forward 5’ CCCGGAGCCCTACCTATAAATAT3’; reverse 5’ GCAGCACAAGATGATTTAGCAC 3’ (Bian et al., 2008) were used to amplify the specific target. The reaction mixtures were run on a real time PCR thermal cycler (Rotor-Gene Q, Qiagen) for 40 cycles with the same reaction conditions as for SPVI gene and the data were analyzed as described above.

**i. Detection of Vg protein by Western blot**

Abdomen walls (2 per sample) containing fat body were collected in Laemelli sample buffer and frozen at -80°C. After thawing on ice, the tissue samples were homogenized with plastic pestles and sonicated at 3-4 medium pulses and heated at 90°C water bath for denaturing. Immediately after heat denaturation, samples were plunged into ice and centrifuged at 13,000 x g for 5 min and 0.1 of a tissue equivalent of the sample supernatants were loaded per lane on 4-20% Tris-glycine gel and run at 100 V at 4°C until the dye front reaches the bottom of the gel. Protein bands on the gel were transferred to 0.2 μm nitrocellulose membrane for 3 h at 50 V at
4°C in transfer buffer (12 mM Tris-base, 96mM glycine, 20% MeOH). After the transfer, the membrane was dried overnight. Next day, the membrane was blocked with ECL blocking agent (0.1 g/ 10ml) and treated overnight at 4°C with Vg primary antibody (rabbit anti Ae. aegypti Vg R2, 1:100,000 dilution). The following processing of the immunoblot was done as described in chapter 2. (see 2.2 b).

j. In vitro incubation experiment

Fat body and midguts were dissected from sugar-fed mosquitoes and cultured in SF900 medium, and the peptides (OEH and ILP3) were added and incubated for 3 h to see if these peptides can affect the mRNA expression and activity of midgut serine proteases or vitellogenin.

i. Midgut culture

Six midguts were dissected from 3-5 day old sugar-fed mosquitoes and placed into 1.5 ml microtube caps with 75 µl of SF900 medium. The caps were placed in the wells of a 24 well, flat bottom plates with each well containing 200 µl of deionized water. OEH (30 pmol/ 15 µl) or ILP3 (30 pmol/ 15 µl) or 15 µl SF900 (for control) added into incubation medium. The plate was incubated in a humidified box in a rotating water bath at 27°C for 3 h. After incubation, midguts were washed in Aedes saline twice. Four midguts were collected in PBS for RNA isolation and 1 midgut each (in duplicates) was collected in 200 µl of Tris buffer (0.02M, pH 8.0; containing 0.02M CaCl₂) for measuring late serine protease activity. Late late serine protease transcript levels were quantified by real-time PCR and late serine protease-like activity in the midgut was measured by late serine protease assay as described above for the in vivo experiment.
ii. Fat body culture

Four abdomen walls (cut opened, like flat sheet) were dissected from 3-5 day old females and incubated with OEH and ILP3 as with midguts describes above. Following incubation and washing twice with Aedes saline, 2 abdomens were collected in PBS for RNA isolation and 2 abdomens were collected in 1x Laemelli sample buffer for resolving the proteins in SDS-PAGE. Vg transcripts were quantified by real-time PCR and protein expression was detected by Western blot as described before.

k. Metabolic Assays

i. In vivo experiments

Newly emerged adult females were provided with water for 5 days, and on day 6, they were given access to a 10% sucrose solution for 20 min. Ten min later, females with swollen abdomens indicative of feeding were decapitated and injected with different doses (1, 10, 20 and 40 pmol per 0.5 µl) of OEH, ILP3, or ILP4 except for controls. Intact (non-decapitated) and decapitated females injected with only Aedes saline served as a positive and the negative control respectively. After injection, mosquitoes were housed in small cages, lined with moist paper towel at 27ºC. The intact mosquitoes were provided with soaked cotton for drinking water. After 24 h, 2 females (in triplicate) per treatment were frozen (−80 ºC), and later microseparated to measure stored glycogen, trehalose and lipid as described (Brown et al., 2008).

ii. Microseparation

Two females were placed in a 1.5 ml centrifuge containing 100 µl of saturated Na₂SO₄ and 200 µl of MeOH and frozen at −80 ºC. After thawing, the mosquitoes were homogenized with
plastic pestles and the homogenates were transferred to glass tube (labeled as A) and then rinsed with 1 ml of 1:1 MeOH/CHCl₃ (chloroform). The, tube A was vortexed and centrifuged at 2500 rpm for 5 min and supernatant was transferred to a new tube (labeled as B1) without disrupting the pellet. The extraction method was repeated once more and with addition of 1 ml of 1:1 MeOH/CHCl₃ and the supernatant was collected in B1. The pellet in the A tube stored at 4 ºC until used for the next step. Then, 1.4 ml CHCl₃ and 0.5 ml nanopure water was added, vortexed and centrifuged at 2500 rpm for 5 min and the organic material (lower phase) was transferred to a 10 ml glass conical tube (labeled B2) and the tube was placed at 4 ºC until used for the next step. Next, 1 ml of CHCl₃ followed by 0.5 ml nanopure water was added to B2 tube, vortexed and centrifuged at 2500 rpm for 5 min. After centrifugation, organic fraction (lower phase) was removed from B2 and pipetted through the silicic acid columns [columns were prepared with a small amount of glass wool plug and 200 mg of 100 mesh silicic acid (dried over night at 60 ºC) in a Pasteur pipette] into a tube, labeled as B3. After the samples ran through the column, the columns were washed with 2ml of CHCl₃. The aqueous phase from B2 was added to B1, which was stored at 4 ºC. Tube A was taken out of ice and 0.5 ml of 66% EtOH saturated with Na₂SO₄ was added and centrifuged at 2500 rpm for 5 min. The aqueous supernatant was transferred to tube B1. This step was repeated again with 0.25 ml of 66% EtOH saturated with Na₂SO₄. The content of B2 was added to B1 and the tube renamed as tube C was kept in the fume hood to volatilize the MeOH off at RT. Tube A was then warmed at 55 ºC for 5 min followed by 0.15 ml of Nanopure water was added vortexed and placed in a heating block at 90-100 ºC for 20 min. Following heating, tube A was removed from the heating block, 0.5 ml 100 % EtOH was immediately added to it, vortexed until the pellet was resuspended. Next, tube A was centrifuged at 3000 rpm for 5 min and supernatant was transferred to 1.5 ml microcentrifuge tube (tube D) or
discarded if the protein assay was not performed. Finally, after finishing this microseparation process, tube A containing glycogen was stored in the fume hood at RT. Tube B3 containing lipid fraction and the tube C containing soluble sugar (mostly trehalose) was dried in the heating block at 65 °C for 4-5 days.

iii. Glycogen assay

The dried pellet in the tube A was solubilized by adding 200 µl of 25% EtOH and vortexed well. Then 200 µl of anthrone reagent (150 ml H₂O + 380 ml of H₂SO₄, allowed to cool down and then 750 mg anthrone was added, turned into yellow-green color, stored in dark) was added to both sample and standards (0, 5, 10, 20, 50, 100, 200 µg glycogen in 200 µl 25% ethanol). Samples and standards were incubated in heating block at 90 ºC for 2 min. All the samples were kept in dark to protect from light. After heating, the samples and standards were cooled down at 4 ºC for 15 min. All samples and standards were vortexed and 100 µl of each was dispensed in 96-well plates in triplicate. The absorbance was read at 625 nm. From the standard line, the glycogen content was quantified for each triplicate samples.

iv. Trehalose assay

After complete drying at 65 ºC for 4-5 days, contents of tube C was dissolved in 0.2 ml of 25% EtOH followed by addition of, 25 µl of 1N HCl was added to both samples and standards (0, 5, 10, 20, 50, 100, 200 µg in 200 µl 25% ethanol) and heated at 90 ºC for 7 min. Next, 75 µl of 1 N NaOH was added and heated at 90 ºC for 7 min. After this, 2 ml of anthrone reagent was added, vortexed, and heated at 90 ºC for 20 min and then cooled down at 4 ºC for 15 min. The samples and the standards were vortexed before dispensing at 100 µl volume to a 96-well plate
in triplicate and the plate was read at 625 nm as done for glycogen. From the standard line equation, the amounts of trehalose in triplicate samples were calculated.

v. Lipid assay

After complete drying for 4-5 days, the samples in the B3 tube and Triolein standards (0, 5, 10, 20, 50, 100, 200 µg in 1 ml of chloroform) were dissolved by adding 1 ml chloroform. Then, both samples and standards were dried completely in the heating block at 90-100 ºC. After drying, 100 µl of 95-98% sulfuric acid was added, vortexed, and heated at 90-100 ºC for 10 min. After heating, the tubes were cooled down at room temperature for 10 min and then 2 ml of vanillin reagent (0.15 g vanillin was dissolved in 25 ml hot water with a stir bar, cooled down and then 100 ml 85% H3PO4 was added a mixed well and stored at 4ºC) was added and vortexed. Then the tubes were placed in a dark place for 15 min to allow the pink-red color to develop. Finally the samples and standards were vortexed and dispensed at 100 µl to a 96-well plate in triplicates and the plate was read at 525 nm and the lipid content of the samples were calculated based on the standard line.

3.3 Results

a. Long and short form of OEH induce gonadotropic and ecdysteroidogenic activity with no apparent difference

A previous study (Brown et al., 1998) showed that short form OEH induces yolk deposition in blood-fed decapitated mosquitoes and stimulates ecdysteroid production by the non-oogenic ovaries in vitro. In this study, we used synthetic short OEH and bacterially expressed long OEH in the gonadotropic and ecdysteroidogenic bioassays. ILP3 was also used in the same experiments for comparison. Except for the lowest does (0.2 pmol), no significant difference in
gonadotropic activity (yolk deposition) was observed between short and long form of OEH as determined at 24 h and 48 h PBM (Fig.3.2 A & B). Both short and long OEH stimulated similar level of ecdysteroid production by ovaries in vitro with no apparent difference (Fig.3.3. B). ILP3 exhibited similar levels of bioactivity in both gonadotropic and ecdysteroidogenic bioassays (Fig.3.2 & 3.3).

b. OEH and ILP3 can additively stimulate ecdysteroid production by the ovaries in vitro

Because both OEH and ILP3 exhibited independent bioactivities in both in vivo and in vitro bioassays, we examined the possibility that if OEH and ILP3 can additively affect ecdysteroid production by the ovaries. We used suboptimal doses (1pmol and 5 pmol) of both rlOEH and ILP3 alone or in combination along with no peptide control. Our results (Fig. 3.4) showed that ILP3 produced higher ecdysteroid production response than rlOEH at the same dose as shown before. Interestingly, when a similar (1pmol OEH + 1 pmol ILP3 or 5 pmol OEH + 5 pmol ILP3) or lower dose of ILP3 was paired with OEH (5 pmol OEH + 1 pmol ILP3), the ecdysteroid production additively increased but when higher doses of ILP3 (5 pmol) was used in combination with a lower dose of OEH (1 pmol), no additive effect was found in comparison to the single dose (5 pmol ILP3). This indicated the subtle effect of lower concentration of OEH did not make any significant contribution to the combined effect with higher dose of ILP3 (Fig. 3.4).

c. OEH restored late serine protease activity and gene expression in the midgut of blood-fed decapitated females

rlOEH (5 pmol and 10 pmol) restored late serine protease activity in the midgut of blood-fed decapitated females, similar to ILP3 (20 pmol) injected females and saline injected intact controls (Fig.3.5 A). A similar trend was observed for the SPVI transcript levels. Decapitated
OEH injected females induced relatively 3 fold higher transcript abundance in the midgut than decapitated control females and this upregulation was also similar to ILP3 injected and saline injected intact controls (Fig.3.3 B). To confirm that the injected peptides (r1OEH or ILP3) were bioactive, yolk deposition was examined in the same individuals from which midguts were dissected for late serine protease experiments. Significant difference in yolk deposition in OEH and ILP3 injected females in comparison to decapitated controls indicating the peptides injected were bioactive (Fig. 3.5 C).

d. OEH does not increase late serine protease activity or SPVI transcript expression in the midgut in vitro

The in vivo study showed that OEH rescued late serine protease activity and SPVI transcript expression in the blood-fed decapitated females at 24 PBM. We wanted to see if OEH has direct effect on the isolated midguts in presence of amino acid medium. ILP3 treatment increased SPVI transcript expression in the midgut in vitro as reported previously (Gulia-Nuss et al., 2011) but OEH or ILP3 treatment did not increase SPVI transcript expression level in the non-blood-fed midgut in vitro (Fig. 3.6A). Neither OEH nor ILP3 treatment increased the late serine protease activity after 3 h compared to the control midguts (Fig. 3.6B).

e. OEH rescues vitellogenin synthesis in the fat body of blood-fed decapitated females

Ae. aegypti females being an anautogenous species, obtain amino acids from the digested blood meal for synthesis of the major yolk protein, vitellogenin (Vg). Because ecdysteroid is the key regulator of Vg synthesis in fat body and OEH stimulates ecdysteroid production by the ovaries, we asked if OEH can induce Vg synthesis in the fat body of the blood-fed females. To test that in an alternative way is to see if OEH can restore Vg synthesis in blood-fed decapitated
f. OEH upregulates vitellogenin transcription but not translation in the fat body in vitro

The in vivo decapitation experiment showed that like ILP3, OEH restored Vg synthesis in the blood-fed decapitated mosquitoes. We wanted to see if OEH had a similar effect on the isolated fat body in presence of amino acid rich medium, SF900, mimicking the blood-fed condition. Our result shows that OEH and ILP3 both increase Vg transcription although the transcript level is much higher in ILP3 treated fat body than in OEH treated fat body (Fig.3.8A). ILP3 but not OEH induced Vg protein translation in fat body (Fig. 3.8B).

g. OEH increases carbohydrate storage but does not increase lipid storage in the body tissue

Previous studies (Brown et al., 2008; Wen et al., 2010) demonstrated that ILP3 but not ILP4 has a role in nutrient storage in the body tissue after ingestion of sugar meal. We followed the same approach to examine if OEH was also involved in nutrient storage in sugar-fed decapitated females. Our results showed that like ILP3, OEH had a hypoglycemic effect which means it increases the glycogen storage and reduces the soluble sugar, trehalose in the tissue, in a dose dependent manner (Fig.3. 9A &B). Like ILP4, OEH does not increase lipid in the body tissue (Fig. 3.10). Thus our results showed some overlapping functions of OEH with ILPs in metabolism and nutrient storage in the body tissues.
3.4. Discussion

After purification and identification of a gonadotropic neuropeptide from female head extract, Brown et al. named it ovary ecdysteroidogenic hormone I (or OEH-I) assuming that more than one OEH-like peptide may exist and be released from the female brain after blood meal ingestion (Brown et al., 1998). Later studies did not identify any other OEH-like peptide but identified the transcripts of a different class of peptides called insulin-like peptides (ILPs) from the head of female Ae. aegypti (Riehle et al., 2006). At least two ILPs, ILP3 and ILP4, were shown to stimulate yolk deposition in blood-fed decapitated females and ecdysteroid production by nonoogenic ovaries in vitro (Brown et al., 2008; Riehle et al., 2006; Wen et al., 2010). The identification of overlapping functions for these two families of peptides (OEH and ILPs) has made our understanding of the endocrine regulation of mosquito reproduction more complex than expected. Here in this study, we compared the bioactivity of long and short OEH peptide and characterized the functions for comparison to that of ILP3.

Our results did not identify any notable difference between the bioactivities of short and long OEH which may strengthen the speculation that the short form might be derived from long form after a post-translational modification after cleavage at 108th position, a potential endoproteinase site (Brown et al., 1998). It also implies that the C-terminal amino acids (108 to 149) are not essential for its bioactivity. The similar bioactivity and the ease of producing recombinant peptide over synthetic peptide led us to use recombinant bacterially expressed full length OEH peptide for the rest of the experiments. Our results also showed that ILP3 is more potent than OEH in stimulating ecdysteroid production in ovaries but together the peptides
additively increased this process indicating signaling by these peptides potentiate each other’s function. Also there may be some cross-talk between OEH and ILP3 signaling.

Digestion of the blood meal is essential for acquiring nutrients to mature the eggs. A previous study (Gulia-Nuss et al., 2011) showed that decapitation impaired midgut digestion and late serine protease activity in the midgut of blood-fed, decapitated females but it was restored by injecting ILP3. Our hypothesis was that OEH could regulate midgut late serine protease activity, given the above functional overlap. Earlier studies reported about 12 serine protease-like genes that were expressed in the Ae. aegypti midgut. Some of these genes are always turned on and others are turned on by the induction of blood meal ingestion (Brackney et al., 2010). Previous studies (Felix et al., 191; Noriega et al., 1996) showed that in Ae. aegypti, blood feeding caused a biphasic induction of serine protease activity in the midgut: early serine protease activity (which lasts from 0 to 6 h) and late serine protease activity (which lasts from 12-18 h PBM). The early serine protease gene is translationally regulated whereas the late late serine protease gene is transcriptionally regulated (Barillas-Mury et al., 1995; Brandon et al., 2008; Noriega et al., 1996; Noriega and Wells, 1999). Although there are two phases of late serine protease activity in the course of digestion, they are not dependent on each other. Studies have shown that translation of early late serine protease gene is not necessary for the transcription of late late serine protease gene (Brackney et al., 2010). Transcription of the late serine protease genes (AaLT, AaSPVI and AaSPVII) is not equally important for the serine protease activity in the midgut. Only, the knockdown of AaSPVI gene was found to have caused reduced serine protease activity in the midgut (Brackney et al., 2008). For that reason, we chose SPVI for examining transcript expression in the blood-fed decapitated females, injected with OEH peptide. As expected, OEH injected blood-fed decapitated females restored the late serine protease activity and SPVI
transcript expression to the same level as in ILP3 injected or nearly same level of intact females. This suggests the regulation of serine protease activity in the blood filled midgut of *Ae. aegypti* by more than one player.

To test further if this effect of OEH on the midgut is a direct effect, we performed an *in vitro* experiment in which non blood-fed midguts were bathed in amino acid rich medium, SF900 alone or in the presence of OEH or ILP3. We did not see any significant difference in late serine protease-like activity in the peptide treated vs control midguts, but there was a 2 fold increase in SPVI transcript expression in ILP3 treated midguts as reported in a previous study (Gulia-Nuss et al., 2011). Taken together, *in vitro* and *in vivo* results indicate the fact that OEH and ILP3 regulate late serine protease activity in the midgut but this function requires the presence of a blood meal which provides other signals that are not limited to the amino acids.

Blood meal digestion is followed by the uptake of amino acids in the fat body for synthesizing Vg, the major yolk protein which is taken up by oocytes for egg maturation. Insulin signaling was shown to stimulate vitellogenin synthesis in the fat body of *Ae aegypti* (Roy et al., 2007). Our own study also showed that knockdown of mosquito insulin receptor (MIR) delayed the expression of Vg in the fat body, which was correlated with the fact that the MIR knockdown female had ovaries with less yolk deposition compared to that in control female ovaries (Gulia-Nuss et al., 2011). Being a ligand of the MIR, ILP3 is a potent stimulator of Vg synthesis in the fat body. Our decapitation experiment had shown that OEH could restore Vg transcript and protein in the fat body almost to the same level as in ILP3 injected blood-fed decapitated and intact blood-fed females. We further tested whether OEH had a direct affect on fat body or other signals are also required to stimulate Vg synthesis in fat body in an *in vitro* experiment. Both
OEH and ILP3 treatment upregulated Vg transcript expression in amino acid rich SF900 medium, but only ILP3 induced translation of Vg. The same can be correlated with higher transcript expression in ILP3 treated fat body than in OEH treated fat body suggesting that OEH and ILP3 may require different amplifying signals for initiating Vg synthesis in the fat body.

Previous studies (Brown et al., 2008; Wen et al., 2010) have shown that ILP3 and ILP4 differently regulate nutrient storage in the fat body. ILP3, the closest relative of vertebrate insulin, binds to the MIR and reduces trehalose and increases glycogen storage in sugar-fed decapitated *Ae. aegypti* females. ILP3 was also shown to increase lipid store in the body tissue, which is an indirect function of mammalian insulin (Teleman, 2010). Another ILP family member, ILP4, which does not bind to the MIR, was shown to have no effect on glycogen and lipid storage (Wen et al., 2010). Because OEH shares many redundant functions with ILP3, we wanted to see if OEH has any role in nutrient storage. Our results indicate that OEH has a role in carbohydrate storage but not in lipid storage in the body tissue suggesting OEH has some redundant functions in the regulation of metabolism. *Locusta migratoria* neuroparsin was shown to have hypertrehalosomic and hyperlipemic functions like the adipokinatic hormone (AKH), although is less potent than AKH (Moreau et al., 1988), which is not the same function of OEH what we found in *Aedes aegypti* females. Characterization of OEH signaling pathway in future will throw more light on how OEH regulates carbohydrate storage in the sugar-fed decapitated females.
3.5. References


Figure 3.1. Structure of native *Ae. aegypti* OEH and the expression strategy for recombinant OEH. (A) Schematic diagram showing the OEH prepropeptide. The first 22 amino acids (labeled in green) form the signal peptide. Excluding the signal peptide, long OEH (propeptide) is 127 amino acids long. There is an arginine (R) at 108\textsuperscript{th} position which is thought to be an endoproteinase site. Upon cleavage at that position, a truncated form of the peptide (86 amino acids) is generated, called, short OEH. (B) Expression of long OEH in pET-32. The recombinant peptide has a thioredoxin (Trx) domain for proper folding, a histidine tag (His) for purification by nickel column chromatography and an enterokinase enzyme site (Ek) for cleavage of OEH peptide from fusion domain.
Figure 3.2. Comparison of gonadotropic activity of sOEH vs rlOEH vs ILP3. *Ae. aegypti* females were decapitated within 1 h PBM and injected once with 0.5 µl of sOEH, rlOEH and ILP3 at four different concentrations (0.2-20 pmol) except for the controls. Females injected with saline only served as a negative control, while normal non-decapitated (intact) females served as a positive control. (A) Yolk uptake (µg ± SE) by the primary oocytes was measured 24 h post injection. A minimum of 12 females were analyzed per treatment from three independent experiments. (B) Yolk uptake (µg ± SE) by the primary oocytes was measured at 48 h post injection. A minimum of 9 or more females were analyzed per treatment from three independent experiments. Different letters above a given treatment indicate means that significantly differ from one another (for 24 h, $F_{13, 187} = 30.79$, $P < 0.0001$; for 48 h, $F_{13, 156} = 33.02$, $P < 0.0001$) and Tukey’s multiple comparison test, $\alpha = 0.05$).
**Figure 3.3. Comparison of edysteroiodogenic activity of sOEH vs rI0EH vs ILP3.** Ovaries from non-oogenic sugar fed females were incubated for 6 hrs in the two different mediums containing different doses (0.1-10 pmol) of sOEH, rI0EH and ILP3 except for the controls, followed by quantification of ecdysteroids in the medium by radioimmuno assay. Ovaries incubated in the respective mediums without peptide served as negative control.

(A) Ecdysteroids (pg ± SE) produced per ovary pairs incubated in Beyenbach saline medium with the given doses of each peptide. (B) Ecdysteroids (pg ± SE) produced by ovaries incubated in SF900 medium with the given doses of individual peptides. This result is a summary of minimum of 5-12 replicates from 4 or more independent experiments. Different letters above a given treatment indicate means that significantly differ from one another (for Beyenbach saline, \( F_{15, 79} = 6.238, P < 0.0001 \) and Tukey’s multiple comparison test, \( \alpha = 0.05 \); for SF900, \( F_{15, 110} = 33.83, P < 0.0001 \) and Tukey’s multiple comparison test, \( \alpha = 0.05 \)).
Figure 3.4. OEH and ILP3 additively stimulated ecdysteroid production by the ovaries in vitro. Ovaries were dissected from non-oogenic sugar fed females and incubated for 6 h in the medium containing suboptimal doses (1 and 5 pmol) of rLOEH or ILP3 alone or in combination, followed by quantification of ecdysteroids in the medium by radioimmunoassay. Ovaries incubated in medium without peptide served as the negative control. Data were analyzed from a minimum 9 replicates from 3 independent experiments. Different letters above a given treatment indicate means that significantly differ from one another (F_{9,71} = 33.83, P < 0.0001 and Tukey’s multiple comparison test, α = 0.05).
**Figure 3.5. OEH rescues late serine protease activity and SPVI expression in blood fed decapitated midgut.** Females were blood fed, decapitated within 1 h PBM, injected with peptides and midguts were collected at 24 h PBM. (A) Trypsin-like activity was measured at 24 h PBM in the midgut of blood-fed decapitated peptide injected females. Midgut samples from saline injected intact (non-decapitated) and decapitated females served as positive and negative controls respectively. Data represents a summary of 15 replicate samples per treatment from 3 independent experiments. Different letters above a given treatment indicate means that significantly differ from one another (F\(_{4, 70} = 30.61, P < 0.0001\) and Tukey’s multiple comparison test, \(\alpha = 0.05\)). (B) Relative quantitative PCR analysis shows relative transcript abundance of late trypsin (SPVI) transcript in female midguts injected with 5 pmol or 10 pmol of rIOEH or 20 pmol of ILP3. Transcript levels were standardized to 1 for the saline-treated sample (negative control) while transcript abundance for the other treatments is expressed relative to the negative control. (C) To demonstrate that the peptides injected were bioactive, yolk deposition (\(\mu g \pm SE\)) was also measured in same females from each treatment group. A replicate of 30 ovary pairs from females per treatment were analyzed from three independent experiments. Different letters above a given treatment indicate means that significantly differ from one another (F\(_{4, 338} = 959.2, P < 0.0001\) and Tukey’s multiple comparison test, \(\alpha = 0.05\)).
Figure 3.6. OEH does not stimulate late serine protease (SPVI) transcript expression or activity in the dissected midgut in vitro. (A) Real-time PCR shows that only ILP3 treatment increased SPVI transcript expression about 2 fold in midguts in vitro. There is no difference in transcript expression of control and OEH treated midguts. Transcript levels were standardized to a level of 1 for saline treated midguts (negative control), while transcript levels for other treatments are expressed relative to the negative control. The result is the summary of 12 replicates for each treatment from a minimum of 4 or more independent experiments. (B) There is no significant difference in late serine protease activity in the rOEH (20 pmol) or ILP3 (20 pmol) treated midguts compared to that of control midguts (F2, 24 = 1.484, P = 0.2468, Tukey’s multiple comparison test, α = 0.05). This result is a summary of minimum 9 replicates from 3 independent experiments.
Figure 3.7. OEH rescues vitellogenin (Vg) synthesis in the fat body of blood fed decapitated females. Females were blood fed, decapitated within 1 h PBM, injected with peptides, and fat body samples were collected at 24 h PBM. (A) Relative quantitative PCR shows comparative Vg transcript expression in the fat body of the females injected with rLOEH (5 and 10 pmol) and ILP3 (20 pmol). Transcript levels were standardized to 1 for the saline-injected blood fed decapitated female fat body samples (negative control) while transcript abundance for the other treatments is expressed relative to the negative control. Fat body samples from intact (non-decapitated) blood fed females served as positive control. The result is the summary of 12 replicates for each treatment from a minimum of 4 or more independent experiments (B) Western blot of fat body from blood fed decapitated females injected with ILP3 (20 pmol) and rLOEH (5 and 10 pmol) showed partial rescues of Vg protein synthesis in comparison to the blood fed intact (non-decapitated) saline injected mosquito (positive control). Fat body samples from decapitated saline injected females served as negative control. Each lane was loaded with 1/10 th of abdomen equivalent protein.
Figure 3.8. OEH stimulated Vg transcript expression in fat body in vitro. Fat body were dissected from non-oogenic females and incubated with OEH and ILP3 for 3 h. Unlike ILP3, OEH did not stimulate translation of Vg protein in the fat body in vitro. (A) Real-time PCR shows that OEH (20 pmol) and ILP3 (20 pmol) treatment increased Vg gene transcript expression about 4 fold and 17 fold respectively. This result is a summary of 12 replicates for each treatment from a minimum of 4 independent experiments. (B) Unlike ILP3 (20 pmol), OEH (20 pmol) treatment did not induce Vg protein translation in the fat body in vitro. The Vg blot is a representative of the results from minimum of 3 replicate experiments and each lane was loaded with 3/4 th fat body equivalent protein.
A

Relative Vg transcript abundance

Control  rIOEH  ILP3

B

kDa

Vg (~ 220 kDa)
Figure 3.9. Effect of OEH on carbohydrate and lipid storage in sugar fed decapitated females. Mosquitoes were starved for 5 days and on day 6, sugar fed for 20 min, decapitated 10 min after sugar feeding and injected with OEH or ILP3 or ILP4 at different doses (1, 10, 10 and 40 pmol) per 0.5 µl saline or saline only (negative control). Intact (non-decapitated) females served as a positive control. (A) Glycogen level was measured per female at 24 hrs post injection. Data represents a minimum of 9 replicate samples per treatment from 3 independent experiments. (B) Trehalose level was measured per female at 24 hrs post injection. Data represents a minimum of 8 replicate samples per treatment from 3 independent experiments. (C) Lipid level was measured per female at 24 hrs post injection. Data represents a minimum of 8 replicate samples per treatment from 3 independent experiments. Different letters above a given treatment indicate means that significantly differ from one another (glycogen, F13,112 = 54.41, P < 0.0001; trehalose, F13,104 = 53.33, P < 0.0001; lipid, F13,105 = 23.14, P < 0.0001) and Tukey’s multiple comparison test, α = 0.05).
4.1. Introduction

OEH was the first peptide shown to stimulate ecdysteroid production by non-oogenic ovaries *in vitro* and yolk deposition in the oocytes of blood-fed decapitated *Aedes aegypti* females (Brown et al., 1998). Later, two members of the insulin-like peptide (ILP) family, ILP3 and ILP4, were also shown to stimulate the same processes as OEH (Brown et al., 2008; Wen et al., 2010). Additionally, it was shown that ILP3 binds to the mosquito insulin receptor (MIR) on the ovary membrane and requires the expression of MIR for its activity (Brown et al., 2008). In contrast, ILP4 does not bind to the MIR, even though MIR expression is required for its activity (Wen et al., 2010). Instead, ILP4 binds to a low molecular weight protein (~ 55 kDa), which either may be a scaffold protein for the MIR or a different receptor.

We know that the ovary is a primary target tissue of OEH or ILPs, and ovary incubation with each of these peptides should activate signaling cascades that result in ecdysteroid production. The signal transduction pathways involved in ovarian ecdysteriodogenesis are not well characterized in insects. Previous studies showed that factors released from the female brain of two dipteran species (*Ae. aegypti* and *Phormia regina*) stimulated cAMP accumulation in the ovary (Maniere et al., 2004; Maniere et al., 2000; Riehle and Brown, 1999; Shapiro, 1983). It was also demonstrated that Rp-cAMPS, an antagonist of cAMP, could not inhibit ovarian
ecdysteroid production stimulated by MNC released factors but partially inhibited ecdysteroid production stimulated by the factors released from other parts of the female brain (Maniere et al., 2004). This indicated that head factors stimulate ovarian ecdysteroid production in the blowfly in both cAMP dependent and independent pathways. Cyclic AMP is the second messenger of G-protein coupled receptors, which activates membrane-bound adenyl cyclase through the activation of Gas subunit (Fig. 1A).

So far, no OEH receptor or binding protein has been identified. In this study we investigated if OEH mediated ecdysteroid production in the ovaries occurs through the cAMP pathway. NKH 477 (colforsin dapropate hydrochloride) is a water soluble derivative of forskolin, an activator of adenyl cyclase that has been used as a positive control for cAMP production in many studies (Furukawa et al., 1996; Kofman and Bersudsky, 2000; Nakashima et al., 2005).

In mammals, the target of rapamycin (TOR) pathway (Fig. 4.1B) plays a well characterized role in nutrient sensing and the regulation of metabolism (Howell and Manning, 2011). Mosquito orthologs of TOR and its downstream signaling proteins have been implicated in stimulatin yolk protein synthesis through amino acid sensing after blood meal (Hansen et al., 2004). Recently, amino acid sensing and TOR signaling were shown to work in coordination with ILP3 to regulate ecdysteroid synthesis and egg maturation in female Ae. aegypti (Gulia-Nuss et al., 2011). Amino acid medium boosted the ecdysteroidogenic response of ILP3 in saline buffered medium. The rationale behind using the amino acid rich medium for ovary incubation is to mimic the physiological situation in vivo where ovaries are bathed in hemolymph enriched with amino acids derived from blood meal ingested by the female. Because, the ingestion of blood meal not only provides amino acids to the females but also triggers the release of OEH.
from female brain into the hemolymph, we examined here if OEH has a similar coordination with TOR signaling as shown for ILP3. Rapamycin, a macrolide antibiotic and a specific TOR signaling inhibitor, was used to abolish TOR activation due to presence of amino acids in the incubation medium.

Insulin receptor signaling through activation of PI3K/Akt pathway (Fig.1B) has also been implicated in ovarian ecdysteroidogenesis in *D. melanogaster, P. regina* and *Ae. aegypti* (Brown et al., 2008; Graf et al., 1997; Maniere et al., 2004; Riehle and Brown, 1999, 2002; Tu et al., 2002). Recently ILP3 was shown to stimulate ecdysteroid production through binding to the MIR on the ovary membrane (Brown et al., 2008; Wen et al., 2010). In the past, bovine insulin, in combination with known chemical inhibitors or activators was used for studying MIR activation (Riehle and Brown, 1999, 2002). In this study, we used chemically synthesized *Ae. aegypti* ILP3, to examine activation of the MIR in ovaries. PQIP or cis-3-[3-(4-methyl-piperazin-1-yl)-cyclobutyl]-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine is an inhibitor of vertebrate insulin-like growth factor-I receptor (Fig.1.B) (Ji et al., 2007). In our *in vitro* signaling study we tested PQIP to see if it can block MIR mediated ecdysteroidogenesis.

The midgut is the tissue where the blood meal is digested, and in the fat body, yolk protein vitellogenin is produced. So, midgut and fat body are very important tissues in the process of egg maturation in females. In this chapter, we also examined the activation profile of signaling proteins in these tissues in response to OEH or ILP3 *in vitro* to identify if there is any tissue specific difference in signaling pathway activation.
4.2. Materials and Methods

a. Peptides

Bacterially expressed full length recombinant Ae. aegypti OEH peptide and synthetic Ae. aegypti ILP3 were used as peptide stimulators. For details about the peptides, please see the Materials and Methods section of chapter 3.

b. cAMP assay

Ten ovary pairs were dissected in Aedes saline with 1x protease inhibitor (PI) from 3-5 day old sugar-fed mosquito and transferred to a cap of 1.5 ml microtube containing 100 µl of SF900 medium. Twenty µl of rLOEH or ILP3 in SF900 (final conc = 0.33 µM) or 20 ul of 50 mM NKH 477 (Sigma-Aldrich, cat # N3290, dissolved in nano pure water, final conc = 1mM) or 20 ul SF900 (in control). Two µl of cAMP phosphodiesterase inhibitor, isobutyl methylxanthine or IBMX (Sigma-Aldrich, cat # I5879) dissolved in absolute EtOH, final conc = 0.83 mM), were added to inhibit the degradation of cAMP produced in the ovaries. Each treatment was done in triplicate. Ovaries were incubated for 30 min at 27°C in the water bath chamber with slow agitation. After incubation, caps were attached to their corresponding tubes, and centrifuged (at 4°C) at 1000 g for 1 min. Supernatant was removed, and 100 µl sample diluent was added into that, vortexed (on ice water) and incubated for 10 min at room temperature (RT). After incubation, samples were sonicated and frozen at -80°C. The samples were then thawed on ice, vortexed and centrifuged at 1000 x g at 4°C for 15 min. Supernatant was collected and stored at -80°C until further use for ELISA.
A cyclic AMP chemiluminescence ELISA (enzyme-linked immunosorbent assay) kit (Arbor Assays, cat # K019-C1) was used to measure cAMP levels in ovary extracts. Reagents (wash buffer, sample diluents, cAMP conjugate, cAMP standards) were prepared as per kit instruction. Plate primer (50 ul) was added into all wells used, then 75 µl of sample diluents were added into the non-specific binding (NSB) wells and 50 ul of sample diluent into wells to act as maximum binding (B0 or 0 pg/ml) wells. Next, 50 ul of samples or standards were added into wells in the plate. After that, 25 ul of the diluted DeteX® cAMP conjugate was added to each well. Finally, 25 ul of DeteX® cAMP antibody was added to each well, except the NSB wells. The plate was gently tapped to ensure that reagents were adequately mixed. The plate was covered with the plate sealer and kept on shaker at RT for 2 h. The wells turned from very pale yellow to pale pink during incubation. After incubation, the plate was washed 4 times with wash buffer and tapped on a dry absorbent towel to dry out the wash buffer. Then, 100 ul of the mixed chemiluminescence substrate (A & B) added to each well. Immediately after adding the substrate, luminescence generated from each well was read in a chemiluminescent plate reader using 0.1 second read time per well. The standard curve was generated and based on that regression line equation, cAMP content of the each treated sample was calculated and plotted on a graph.

c. In vitro ecdysteroidogenic bioassay

Ecdysteroidogenic bioassays were conducted as described in chapter 3 (see 3.2.e). For determining the effect of amino acids on the ovaries, we used two incubation mediums, Beyenbach saline medium (noted as BS), amino acid rich medium (Roy et al., 2007) and SF900 insect cell culture medium (SFM-II, Gibco, cat # 10902-088). Ten µl containing 0.2, 2 and 20
pmol of OEH or ILP3 was added to the caps except in the control. To block the effect of TOR signaling, 100 pmol rapamycin (LC laboratories, Woburn, MA) was added along with OEH or ILP3 as a separate treatment. To block the insulin receptor signaling, 5 nmol PQIP (OSI Pharmaceuticals Inc, New York) was added in the same way along with OEH and ILP3 peptide treatment. The ovaries were incubated for 6 h at 27°C in a rotating water bath. Following incubation, 50 µl of saline media was removed from each cap and stored until quantification of ecdysteroids level by RIA as described in chapter 3. For TOR signaling experiments, we first used an amino acid rich medium (AA), (a gift from Alexander Raikhel, University of California, Reverside). Later, we compared commercially available insect cell culture media for activation of ovarian ecdysteroid production in response to OEH or ILP3. We found, insect cell culture medium SF900 was as good as Raikhel’s medium and was used in all other experiments.

d. Tissue incubation and signaling protein extraction

Twenty ovary pairs, midguts or six abdomen walls (containing fat body) were dissected in ice cold Aedes saline (with 1x PI) from 3-5 day old regular sugar-fed mosquitoes, and transferred to a cap of 1.5 ml microtube containing 100 µl of SF900 or BS medium with 1x Halt protease and phosphatase inhibitor (PPI) cocktail (Thermo scientific, cat # 78440). OEH and ILP3 were added in 20 µl volume at concentration (20 pmol/10 µl). The same volume of SF900 or BS medium was added in the control. For ovary incubation, PQIP or rapamycin was used along with OEH or ILP3 to block TOR and insulin signaling respectively. Then caps were placed in a covered petridish in a humidified box incubated in a water bath with slow agitation at 27°C for 30 minutes. After incubation, each cap was attached to a microtube and centrifuged (at 4°C) at 1000 x g for 1 min. Supernatant was removed and 100 µl of ice cold IP lysis buffer (Pierce cat #
with 1x PPI was added and kept on ice for 5 min. Ovaries in lysis buffer were homogenized (for 30 sec - 1min) with plastic pestle and the pestle was rinsed with 100 µl IP lysis buffer. Lysate was freeze-thawed 3x, vortexed and sonicated at low pulses (3-4 times). The homogenate mixture was transferred to a 100K molecular weight cut-off column (Pal Life Sciences, Ann Arbor, MI) and centrifuged at 13,000 x g for 35-40 mins. About 20 µl of retentate (fractions of protein extract retained on the top of the column after centrifugation) and about 180 µl filtrate (fraction of the protein extract passed through the column) were collected and stored at -80 ºC until further processing for SDS-PAGE and Western blot.

**e. Immunoblot of signaling proteins**

Protein content of the retentate and filtrate were measured (Bradford Coomassay kit, Thermo scientific, cat # 23200) and 120 µg of protein was loaded on 4-20% Tris-glycine gel and transferred to a nitrocellulose membrane (0.2 µm) as described in Chapter 3. After developing the blots with respective signaling antibodies, it appeared that the signaling proteins were mostly retained in the retentate fraction even though the molecular weight of the proteins was less than 100K which indicates formation of a molecular complex of scaffold proteins. We only had specific antibody only for *Ae. aegypti* insulin receptor, but not for other downstream signaling proteins. So phospho-antibodies for *Drosophila* insulin and TOR signaling proteins were tested for detection of *Ae. aegypti* proteins. The ones that showed consistent immunoreactivity at the expected molecular weight protein bands were used in this *in vitro* signaling study. All the primary antibodies were anti-rabbit and used at 1:1000 dilution: [ Invitrogen: anti phospho tyrosine (cat # 61-5800) ; Cell signaling: phospho Drosophila -Akt (S505, cat # 4054), phospho-Drosophila p70 S6 kinase (Thr398, cat # 9209), phospho GSK-3α/β (Ser21/9) (cat # 9331), phospho-4E-BP1 (Thr 37/46) (cat # 2885), phospho PKA (cat# 5661), phospho-FOXO
(cat#9464), human phospho-MAPK (Thr202/Tyr204) (cat # 9101), β tubulin antibody (abcam cat # ab6046). The goat-anti rabbit secondary antibody conjugated with horseradish peroxidase was used at a 1:20K dilution. After the antibody treatment, the blot surface was covered with 800 ul of 1:1 mixture of reagent A and reagent B of ECL Amersham Advance chemiluminescence substrate (GE Healthcare, cat # RPN2135) and exposed in Gel Logic 4000 PRO (Carestream Health Inc, Rochester, NY) for different exposure times (starting from 10 seconds to 3 mins) to optimize visualization by increasing signal vs noise ratio. To illustrate the signaling status of different signaling proteins at different experimental treatments, the same blot was stripped and treated with IR antibody and other anti-phospho antibodies as described above. Stripping was done at 60°C for 1 hour on a heated rocker with the stripping buffer (0.2 M Glycine, pH 2.5, 0.05% Tween, 0.5% β mercaptoethanol). Following stripping, the blot was washed 3x with TBST and dried overnight before using it for the next antibody.

4.3. Results

a. OEH does not increase cAMP production in the ovaries

A possible signaling mechanism for OEH mediated ovarian ecdysteroid production is binding to a GPCR which activates cAMP production in the ovaries. First we showed that OEH, ILP3, and the cAMP activator, NKH 477, all stimulate ecdysteroid production by the ovaries in comparison to the control (Fig. 4.2.A). Then, we determined if stimulation of ecdysteroid production was correlated with a high level of cAMP in the ovaries in presence of the peptides or NKH 477. Our results showed (Fig. 4.2B) that there was a significant cAMP accumulation only in the NKH 477 treated ovaries over the control but neither in OEH nor ILP3 treated ovaries.
b. OEH induced ecdysteroid production can be modulated through the target of rapamycin (TOR) pathway

Amino acid rich medium boosts ILP3 stimulated ecdysteroid production in vitro (Gulia-Nuss et al., 2011). Here, we compared the ecdysteroidogenic response of OEH in both saline medium and the amino acid supplemented medium (AA). We found that like ILP3, OEH stimulated ecdysteroid production by ovaries increased in a dose dependent manner in saline medium. The same doses of OEH boosted ecdysteroid production by ovaries up to 3 fold (at 20 pmol) in AA medium (Fig. 4.3). Addition of rapamycin (100 pmol) blocked the boosted response to amino acids, indicating that OEH-mediated ecdysteroid production could be modulated by amino acid sensing signaling via the TOR pathway.

c. OEH stimulates ecdysteroid production in ovaries without being dependent on insulin receptor signaling

Both ILP3 and OEH stimulate ecdysteroid production by the ovaries in vitro, and it was previously demonstrated that ILP3 required the expression of MIR on the ovary membrane to stimulate ecdysteroid production (Brown et al., 2008). Here, we show that both OEH and ILP3 stimulated ecdysteroid production by the ovaries in a dose responsive manner. Presence of PQIP, an inhibitor for mammalian IGF-IR completely shut down ILP3 stimulated ecdysteroid production in the ovaries whereas OEH-stimulated ecdysteroid production remained unaltered in presence of PQIP (Fig. 4.4). This result indicates that OEH mediated ecdysteroid synthesis works through a pathway independent of insulin receptor signaling.
d. OEH, ILP3 and cAMP activate different signaling pathways

As mentioned previously, both cAMP and insulin signaling pathways were shown to be involved in ecdysteroid production in dipteran species. Here we chose important regulatory proteins in both cAMP and insulin signaling pathways as shown in the schematic diagram [Fig. 4.1 A & B] for checking their activation (phosphorylation) status in response to OEH, ILP3 or cAMP stimulation. We ran both the retentate and filtrate fractions separately assuming that the proteins having molecular weight less than 100K will only be found in filtrate. As expected, we observed that the insulin receptor protein (M.W. 500 kDa) in the retentate fraction, but interestingly another signaling protein, phospho-Akt (M.W. 55 kDa), which should have passed through100K was mostly retained in the retentate fraction [Fig 4.5 A], suggesting that the signaling proteins in the cellular compartments might be attached to adjacent signaling proteins forming a scaffold and thus blocking their passage through molecular weight cut off filter. We see that ILP3 but not OEH nor cAMP could stimulate MIR phosphorylation. Both OEH and ILP3 activated proteins downstream of insulin receptor, e.g. Akt, ribosomal S6 kinase and GSK-3α/β, and 4EBP1 compared to the control (Fig. 4.5B). Human anti phospho-PKA antibody did not recognize mosquito orthologue protein even in presence of cAMP. Taken together, these results primarily demonstrate that OEH, ILP3 and cAMP work through different signaling pathways.

e. Effects of inhibitors: MIR and TOR signaling

i. ILP3 activated the MIR and its downstream signaling proteins

ILP3, being a ligand for the MIR, should activate MIR and its downstream signaling proteins as illustrated in a schematic diagram [Fig. 4.1B]. As expected, ILP3 treatment resulted
in phosphorylation of the MIR and other downstream proteins (Akt, ribosomal S6 kinase, GSK-3α/β). The IGF-IR inhibitor, PQIP, completely blocked phosphorylation of the MIR thus inhibiting the activation of downstream proteins (Fig. 4.6). The TOR inhibitor, rapamycin, in contrast, did not affect MIR phosphorylation but abolished activation of ribosomal S6 kinase, which is the major signaling regulator of the TOR pathway. In saline medium, ILP3 did not active S6 kinase indicating ILP3 signaling also requires amino acid to activate the TOR protein (Fig. 4.7). ILP3 phosphorylates glycogen synthase kinase (GSK-3α/β) which is downstream of p-S6K. PQIP inhibited phosphorylation of GSK-3α/β in ILP3-treated ovaries. Rapamycin did not inhibit phosphorylation of GSK-3α/β in the ILP3 treated ovaries. Phosphorylation of 4EBP1 is comparatively higher in ILP3 treated ovaries than in the control ovaries. Little or no phosphorylation of 4EBP1 was evident in the control ovaries in saline buffered medium (Fig. 4.7) but amino acids in SF900 medium activated 4EBP1 even in the control ovaries (Fig. 4.6).

ii. OEH did not activate the MIR but activated Akt and other downstream proteins

OEH treatment did not result in MIR phosphorylation, but Akt was phosphorylated although to a lesser extent compared to ILP3 (Fig. 4.6). OEH also resulted in the phosphorylation of other signaling proteins (S6K, GSK-3α/β and 4EBP1) downstream of Akt. PQIP did not inhibit OEH induced activation of Akt and other downstream signaling proteins. Rapamycin blocked the activation of S6K in the OEH treated ovaries. Like ILP3, OEH did not stimulate S6K in the ovaries incubated in saline buffered medium. Rapamycin had no effect on the phosphorylation of GSK-3α/β in OEH treated ovaries as in ILP3 treated ovaries.
f. OEH boosted ILP3 signaling in vitro

In the in vitro signaling study, OEH alone did not phosphorylate MIR, but when ovaries were treated with OEH and ILP3 together, phosphorylation of MIR was increased relative to ILP3 treatment alone (Fig. 4.8). This enhancement of MIR phosphorylation was also reflected in the phosphorylation of Akt. Our bioassay result in the chapter 3 showed similar additive effect of OEH and ILP3 on ovary ecdysteroid production when a low dose of both the peptides was combined which might correlate with this boosting effect of OEH on ILP3 mediated activation of MIR. Although in this signaling study, we used 20 pmol of each OEH and ILP3 alone or in combination.

g. OEH and ILP3 signaling are not the same in midgut and fat body

Apart from the ovary, midgut and fat body are the important target tissues for OEH and ILP3. So, we wanted to examine if there is any difference in OEH or ILP3 signaling specific to these tissues. ILP3 treatment resulted in the phosphorylation of MIR and Akt in both midgut and fat body as in ovary. But activation of downstream proteins in midgut is not same as in the fat body. In fat body, S6K and 4EBP1 were activated as in ovaries in response to OEH or ILP3, but they were not activated in midgut in vitro (Fig. 4.9. A & B). This result demonstrates that there are differences in activation of signaling proteins specific to a tissue which may be related to the functional activity of that tissue.

4.4. Discussion

We know that in Ae. aegypti females, three peptides (OEH, ILP3 and ILP4) stimulate the ovary to produce ecdysteroids. Recent studies (Brown et al., 2008; Wen et al., 2010) have
elucidated ILP signaling in ovary. Nothing is known about the OEH signaling pathway, and how OEH activates ecdysteroid production in ovary is still not understood. In this study, we centered our focus to understand OEH signaling in comparison to ILP3 signaling in ovaries and other tissues involved in egg maturation process.

One study showed that a partially purified head extract stimulated ovary ecdysteroid production and also increased cAMP in the isolated ovaries within 30 seconds of incubation. This action was similar to a cAMP analogue that stimulated ecdysteroid production by ovaries (Shapiro, 1983). A neuropeptide, prothoracicotropic hormone (PTTH) which stimulates ecdysteroid synthesis in the prothoracic gland of the tobacco horn worm, *Manduca sexta*, also increases cAMP by activating a cAMP dependent protein kinase (Smith et al., 1986; Smith et al., 1984, 1985). In silk worm, *Bombyx mori*, cAMP was also reported to be an inducer of ecdysteroid production in the prothoracic gland (Yamanaka et al., 2005; Yamanaka et al., 2006). In contrast, a recent study (Rewitz et al., 2009) has shown that PTTH activates Torso, a tyrosine kinase receptor, and thereby activates downstream MAPK/Erk signaling in the prothoracic gland of silk worm *B mori*. In the blow fly, some head factors that stimulated ecdysteroid production ovaries also increased cAMP accumulation in ovaries (Maniere et al., 2004; Maniere et al., 2000). These previous findings led us to investigate if OEH stimulated ecdysteroid synthesis in ovary via production of cAMP. Our result showed that OEH like ILP3 does not activate ecdysteroid production via cAMP production in the ovaries *in vitro* (Fig. 4.2 A & B) indicating the head factors, previously (Shapiro, 1983) shown to increase in cAMP production in ovary were neither OEH nor ILP3. To further test activation of cAMP in response to OEH or ILP3, we examined the phosphorylation of protein kinase A (PKA), a key signaling
protein downstream of cAMP. Unfortunately, because anti-human PKA antibody did not recognize the mosquito orthologue protein, we were not able to detect the phosphorylation status of PKA. Another possibility is that PKA was not at all phosphorylated, but that possibility may not be true because PKA should be activated in the presence of cAMP irrespective of receptor activation.

Blood meal ingestion not only provides female mosquitoes with nutrients, in particular, amino acids, for making eggs but also sends cues to the female brain for release of neuropeptides to initiate and coordinate the egg maturation process. The utilization of nutrients in female mosquitoes is tightly regulated by the endocrine pathways. Previous work (Gulia-Nuss et al., 2011) has shown that ILP3 stimulated insulin receptor signaling and TOR signaling coordinately regulate egg maturation in Ae. aegypti. In that study, amino acid rich medium increased ILP3 mediated ecdysteroid production by ovaries a few folds in vitro. Here, our results showed a similar boosting effect of amino acid on OEH- stimulated ecdysteroid production, which was inhibited by the TOR inhibitor, rapamycin (Fig. 4.3). This finding implies that OEH cross-talks with TOR signaling in presence of amino acids as does ILP3. Moreover, it also suggests that OEH and ILP3 may follow some common pathway to coordinate with TOR to regulate ecdysteroid synthesis in the ovary.

OEH and ILP3 independently stimulated ecdysteroid production by isolated ovaries in vitro and yolk deposition in the blood-fed decapitated females in vivo. Functional redundancy of OEH and ILP3 in activation of digestion in midgut, Vg synthesis and carbohydrate metabolism in the fat body was demonstrated in chapter 3. Here our experimental results showed that OEH did not activate cAMP production in ovaries. These findings altogether raised the question if
OEH uses MIR signaling like ILP3 to activate these processes. To examine that possibility, in vitro ecdysteroidogenic bioassay was done in the presence of MIR inhibitor PQIP. Our result showed that PQIP inhibited ILP3-stimulated ecdysteroid production, but OEH-stimulated ecdysteroid production remained unaltered indicating OEH does not work through MIR for ecdysteroid production in the ovaries.

Our results indicate that OEH neither works through a GPCR/cAMP pathway nor does it work through the MIR to stimulate ecdysteroid production. A previous study (Wen et al., 2010) showed that even though ILP4 did show binding with the MIR but it required the expression of MIR for its activity. Both ILP3 and ILP4 activated MIR signaling proteins in ovaries even though they have different receptor binding activity. To further confirm our results that OEH does not activate MIR signaling pathways, we examined the activation (phosphorylation) status of MIR and some of its important downstream signaling proteins in the isolated ovaries in vitro. Surprisingly, we observed that although OEH does not phosphorylate the MIR, but phosphorylation of the proteins downstream of insulin receptor was similar as that of ILP3. We did not see any change in MAPK kinase activation in presence of OEH or ILP3 (Fig. 4.5B) as shown before for ILP3 and 4 (Wen et al., 2010). Although OEH, ILP3 and cAMP activate different signaling pathways, but overall comparisons suggest that OEH signaling converges with ILP3 signaling at some point downstream of the MIR.

With our knowledge from the previous experiments that OEH does not activate MIR but activates other downstream proteins, we repeated the same in vitro signaling experiments in the presence or absence of PQIP. As shown in the previous experiment, OEH did not phosphorylate
the MIR as did ILP3 in the ovary. This is in agreement with the result (Fig. 4.5) from the earlier experiment which showed that PQIP inhibited ecdysteriodogenic response of ILP3 but not that of OEH (Fig.4.3). Although, OEH does not activate the MIR, Akt, a protein kinase B, a key regulatory protein activated by phosphoinostide 3-kinase (PI3K) at downstream of MIR is phosphorylated in response to OEH. Akt activation by OEH was not inhibited by PQIP which is also indication that OEH does not interact with the MIR.

We have already shown that OEH works in coordination with TOR signaling to activate ecdysteroid production by ovaries. Akt was characterized as a key regulator of insulin/insulin-like growth factor mediated activation of TOR signaling (Kim and Guan, 2011). Activated Akt phosphorylates TOR binding inhibitory proteins such as teuberous sclerosis complex 2 (TSC2, a component of TSC1/2 complex) and proline rich Akt substrate, 40 kDa (PRAS40) thus removes the inhibitory signal for activating TOR signaling (Kim and Guan, 2011). Activated TOR protein further activates the ribosomal protein, S6K by phosphorylation. So, phosphorylation of S6K is an indicator of active TOR signaling. Our result shows that OEH as like as ILP3 phosphorylated S6K in the ovary but interestingly, PQIP did not inhibit the phosphorylation of S6K in the OEH treated ovaries as opposed to its nearly complete inhibition of phosphorylation of S6 kinase in the ILP3 treated ovaries. As expected, rapamycin (a selective inhibitor of TOR protein), inhibited S6K phosphorylation in both OEH and ILP3 treated ovaries. Another downstream mediator of TOR signaling, 4E-BP1 (a repressor of eukaryotic elongation factor EF1), is required for protein translation. Our result shows that 4EBP1 is phosphorylated in all the treatments including controls and is not inhibited by rapamycin. We think that phosphorylation of 4EBP1 in controls was due to the presence of amino acid in SF900 medium. This is in agreement with the findings.
of previous studies (Roy and Raikhel, 2011) which showed that in the fat body of Ae. aegypti, amino acid mediated phosphorylation of 4EBP1 is insensitive to rapamycin and does not occur through TORC1. Our speculation was indeed true because in the saline buffered medium, 4EBP1 was phosphorylated in the OEH or ILP3 treated ovaries and not in the controls. So, these results confirm our previous findings that both OEH and ILPs stimulate TOR pathway, and amino acid rich medium, SF900, boosted that affect. This signaling study further indicated that the activation of TOR by OEH may occur through different receptors.

Midgut and fat body are important tissues for reproduction in female mosquitoes. We have shown in chapter 3 that ILP3 but not OEH stimulated trypsin transcript expression in midgut and robust expression of Vg mRNA and protein in fat body in the in vitro culture condition in SF900 medium. So, we examined if that difference is reflected in signaling pathway activation. We found, as in ovaries, ILP3 but not OEH phosphorylated the MIR and OEH weakly phosphorylated Akt compared to ILP3 in midgut and fat body. Interestingly, no TOR downstream signaling proteins (S6K and 4EBP1) were phosphorylated in midgut (Fig. 4.9A) whereas activation of TOR signaling proteins in fat body was the same as in ovary incubated in SF900 medium (Fig. 4.9.B). Because the fat body is the nutrient storage tissue, it is quite relevant that it will have an active TOR signaling but in midgut, the purpose of OEH or ILP3 signaling is mainly to regulate digestion of the blood meal, TOR signaling may not be that important in the perspective of non-bloodfed environment. So our results indicate that based on tissue function, OEH and ILP3 activates relevant signaling proteins as required for the tissue specific expression of certain genes.
Activation of Akt may occur through different classes of receptors including tyrosine kinase receptors, cytokine receptors, G-protein coupled receptors, intergrin receptors and transforming growth factor beta (TGFβ) receptors. In our signaling study, we examined if OEH could phosphorylate smad proteins, which are downstream activators of TGFβ receptor signaling but we did not find any significant difference in phosphorylation of smad2/3 proteins in the OEH treated ovaries compared to the control or the ILP3 treated ovaries (data not shown here) indicating OEH does not work through TGFβ signaling.

So far, it is very clear that OEH does not bind to or activate the MIR. There are several classes of GPCRs which activate Akt. We have only examined cAMP mediated pathway but it is possible that OEH may work through other classes of GPCRs which activate Ca\(^{2+}\) signaling, which needs to be investigated. It can be concluded from the present study that OEH does activate Akt and TOR signaling. Although both OEH and ILP3 have many redundant functions, they appear to work through different receptors. Future studies need to be directed to find out the missing link between OEH and Akt.

4.5. References


Figure. 4.1. Schematic diagrams of signaling transduction pathways. (A) G-protein coupled receptor (GPCR) signaling mediated through cAMP. Gαβγ, trimeric G-protein complex; GαS, activated Gα subunit; GTP, guanosine tri-phosphate; GDP, guanosine di-phosphate; cAMP, 3’-5’cyclic adenosine mono phosphate; 5’ AMP, 5’ adenosine monophosphate; PDE, phosphodiesterase enzyme; PKA, protein kinase A; IBMX, isobutyl methylxanthine; NKH 477, colforsin daphropate hydrochloride. (B) Insulin and amino acid sensitive TOR signaling. ILP3, insulin-like peptide 3; MIR, mosquito insulin receptor; RTK, receptor tyrosine kinase; IRS, insulin receptor substrate, PIP2, phosphatidyl inositol (4,5) bisphosphate; IP3, inositol triphosphate; PI3K, phosphoinositide 3 kinase; Akt, a protein kinase B; TORC1, target of rapamycin class 1 protein; p70S6K, ribosomal protein kinase (mol wt. 70 kDa); 4EBP1, a repressor and binding protein for eukaryotic translation initiation factor1; GSK3-β, glycogen synthase kinase 3 alpha/beta; RAS, a GTPase family protein; SOS, son of sevenless; GRB2, growth factor receptor-bound protein 2; MAPK/ERK, mitogen activated protein kinase/ extra-cellular signal regulated kinase.
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**Figure. 4.2. OEH mediated ecdysteroid production and induction of cAMP.** (A) Ecdysteroid production was measured in the ovary in SF900 medium alone (control) and in presence of OEH or NKH 477 or ILP3. OEH, NKH 477, and ILP3 stimulated ecdysteroid production to a different extent compared to control. (B) Only NKH477 significantly stimulated cAMP production compared to control, OEH and ILP3. Different letters above a given treatment indicate means that significantly differ from one another (for ecdysteroid, F₃,₁₂ = 15.63, P < 0.0001 and Tukey’s multiple comparison test, α = 0.05; for cAMP, F₃,₁₁ = 6.796, P = 0.0074 and Tukey’s multiple comparison test, α = 0.05 ).
Figure. 4.3. OEH stimulated ecdysteroid production and modulation through TOR signaling. OEH stimulates ovaries in a dose-responsive manner to produce ecdysteroids in Beyenbach saline (BS) and amino acid rich saline medium (AA). Amino acid boosted ecdysteroid production up to 3 fold relative to that of OEH in saline medium. TOR inhibitor, rapamycin, blocked the effect of amino acid medium, indicating that OEH signaling can be modulated through amino acid sensing via TOR signaling. Asterisks indicate significant differences between the treatments (F_{12, 143} = 94.96, P < 0.0001).
Figure. 4.4. OEH stimulated ecsyteroid production does not occur through insulin receptor signaling. OEH and ILP3 both stimulated ecdysteroid production in ovaries in a dose-responsive manner in SF900 medium. However, PQIP completely inhibited the ILP3 response but not that of OEH, indicating that OEH mediated ecdysteroid production is not occurring through insulin receptor activation. Asterisks indicate significant differences between the treatments ($F_{13, 38} = 20.68$, $P < 0.0001$). Absence of asterisks indicates that there is no significant difference between the treatments.
Figure 4.5. Ovary incubation with OEH, ILP3 and cAMP and activation of signaling proteins. (A) Two representative blots for MIR and phospho-Akt antibody staining from both retentate and filtrate fractions. [M= prestained protein ladder, C = control, O = OEH (20 pmol), I = ILP3 (200 pmol), A = cAMP (100µM) (B) Summary of screened phosphorylated proteins. OEH did not phosphorylate the MIR but activated other signaling proteins downstream of the MIR: Akt, S6 kinase, and GSK-3α/β. β-tubulin antibody staining was used a loading control.
**Figure. 4.6.** Ovary incubation in SF900 medium with OEH or ILP3 alone or in the presence of PQIP and rapamycin (RAPA). Equal amount of protein (120 µg/lane) was loaded in each lane of a gel and immunoblotted with different antibodies on the representative blot after subsequent stripping. OEH does not phosphorylate the MIR as does ILP3, but OEH weakly phosphorylates Akt. PQIP does not inhibit OEH-mediated phosphorylation of Akt. RAPA inhibited ILP3 and OEH mediated phosphorylation of ribosomal p70 S6 kinase. PQIP inhibited the phosphorylation of S6 kinase completely in ILP3-treated ovaries and partially in OEH-treated ovaries. PQIP but not rapamycin inhibited ILP3 induced activation of GSK-3. Phosphorylation of 4EBP1 was higher in treatments than control but remain unaltered in different treatments.
Figure. 4.7. Ovary incubation with OEH or ILP3 in Beyenbach medium and activation of signaling proteins. Equal amount of protein (120 µg/lane) was loaded and immunoblotted with different antibodies on the same blot after subsequent stripping. OEH does not phosphorylate MIR but phosphorylates Akt. No S6K activation was detected for both OEH and ILP3 treated ovaries. 4EBP1 was activated in both ILP3 and OEH treated ovaries compared to control.
Figure. 4.8. Combined effect of OEH and ILP3 on insulin receptor signaling. Equal amount of protein (120 µg/lane) was loaded and immunoblotted with different antibodies on the same representative blot after subsequent stripping. OEH does not activate MIR but boosted the activation of MIR signaling in ovary when used together with ILP3. Phosphorylation of Akt seems to have increased when OEH was paired with ILP3 in vitro.
Figure. 4.9. OEH and ILP3 signaling in midgut and fat body in SF900 medium. Representative blot summary from at least three independent experiments. Equal amount of protein (120 µg/lane) was loaded and immunoblotted with different antibodies on the same blot after subsequent stripping. (A) In midgut, ILP3 phosphorylated MIR and Akt but not other downstream proteins S6K or 4EBP1. OEH did not phosphorylate IR, S6K and 4EBP1 but weakly phosphorylated Akt (B) In fat body, OEH and ILP3 signaling are similar as in ovaries incubated in SF900 medium (Fig. 4.6).
CHAPTER 5
CHARACTERIZATION OF OEH RECEPTOR OR BINDING PROTEIN IN OVARY

5.1. Introduction

The Ovary is the major target tissue of OEH in female Ae. aegypti. Despite the progress in understanding the functions of OEH in ovary and other tissues, it is still not known how OEH initiates these functions, i.e. which receptor it activates to regulate the biochemical processes, responsible for the described functions. According to the standard definition of a peptide hormone, it must bind to a receptor on the cell membrane that activates a signaling cascade to elicit its bioactivity. Later studies identified two insulin-like peptides, ILP3 and ILP4, which also stimulated yolk deposition and ovary ecdysteroid production (Brown et al., 2008; Wen et al., 2010). As expected, ILP3 was shown to bind to the mosquito insulin receptor (MIR) and its bioactivity required the expression of MIR on the ovary membrane (Brown et al., 2008). ILP4, less potent than ILP3 in bioactivity, did not show any binding to the MIR, rather it showed dose-dependent binding to a low molecular weight (approx 55 kDa) protein, which has not been characterized yet (Wen et al., 2010).

This diverse nature of ligand-receptor interactions indicates that redundant functions do not necessarily mean the involvement of the same receptor. In this study, we took a similar approach with radiolabeled ligand-receptor binding and cross-linking experiments to identify if any receptor or binding protein for OEH is present on the ovary membrane. We also investigated the
alternative possibility that OEH crosses the cell membrane and localizes into the cytoplasm or nucleus.

5.2. Materials and Method

a. Mosquito rearing: UGAL strain of Aedes aegypti was maintained as described in chapter 2 (See 2.2.a).

b. Radio-receptor binding experiment

i. Ovary dissection and isolation of ovary membrane

Sixty ovary pairs were dissected from 3-5 day old sugar-fed mosquitoes in buffered medium [50 mM HEPES, 1x Hanks balanced salt solution (HBSS), and 1x protease inhibitor (PI)] and put in a 1.5 ml microtube cap containing 100 µl of the medium. The caps with ovaries were kept in ice cold until all 300 ovary pairs were dissected. These ovaries were pooled into a 1.5 ml microtube by centrifuging at 5000 x g for 1 min. The supernatant was removed and 100 µl of homogenization buffer [50 mM Tris-Hcl (pH 7.5), 250 mM sucrose and 1x PI] and was added. Then the ovaries in the tube were homogenized manually with a blue tip generator for 60 sec and then for 2 min with a motorized generator (brief pause at every 30 sec) in an ice bath. After homogenization, 400 µl of homogenization buffer was used to rinse the generator. After sonication at 3 brief low pluses, the homogenates were centrifuged at 2000 x g for 5 min. Supernatant were transferred to a HIGH G-Force tube on ice and centrifuged at 48,000 x g for 1 hr. The supernatant was discarded and the pellet was dissolved in 200 µl binding buffer [50 mM HEPES (pH 7.6) + 1x HBSS+ 3% BSA + 0.1x PI]. The membrane solution was stored at -80°C until use.
ii. Radiolabeling of OEH

Synthetic OEH (short form) was radiolabeled with $^{125}$I by the lactoperoxidase-hydrogen peroxide method and was purified by HPLC (Crim et al., 2002).

iii. Optimization of the membrane binding

Different amounts of membrane as determined by protein content (e.g. 100, 200, and 300 µg) and a fixed amount of radiolabeled OEH (e.g. 400,000 cpm per reaction) were incubated with 1 µM unlabeled OEH. Based on total and specific binding, the amount of membrane (µg) and the concentration of radiolabeled OEH (cpm) per reaction were optimized for binding experiments.

iv. Determination of OEH binding kinetics

Keeping the amount of membranes and radiolabeled OEH fixed, different concentrations of unlabeled OEH were used to displace the radiolabeled OEH. The more unlabeled OEH in the reaction, the more displacement of radiolabeled OEH should occur. The concentration of unlabeled OEH that displaced 50% of the bound radiolabeled OEH displaced was calculated as the IC$_{50}$.

c. Ovary incubation and immunoblot

Eight pairs of ovaries were dissected in Aedes saline with 1x PI from 3-5 day old sugar-fed females and transferred into 50 µl of Beyenbach saline (BS) in a 0.5 ml microtube cap placed
into 24 well, flat bottom RIA plates (Costar®) containing 200 μl deionized water. Then, 10 μl of rLOEH (40 pmol total amount) or 10 μl of BS (control) were added to respective caps and the plate was incubated in a humidified box at 37° C for 30 min, 1 h and 2 h in a rotating water bath. After incubation, ovaries were collected by attaching the caps to the microtubes and centrifuging at 1000 rpm for 1 min. The incubation medium was collected and stored at – 80 °C for lyophilization. The control and treated ovaries were washed 2 times with BS, homogenized in Tris-tricine sample buffer and stored at -80ºC until further use. The lyophilized medium and ovary samples were resuspended with denaturing Tris-tricine sample buffer (Nusep Tris-tricine, 125 mM DTT), and the ovary samples were sonicated at low pulses. Samples and standards were heated at 65°C for 45 min, put into ice immediately and centrifuged at 13,000 xg for 2 min. After centrifugation, the sample supernatant was loaded on to a 16.5% Tris-tricine gel (Criterion, BioRad) for gel electrophoresis at 90 V at 4°C. Afterwards, the proteins were transferred to 0.1 μm nitrocellulose membrane for 1 h at 45 V at 4°C in transfer buffer (12 mM Tris-base, 96 mM glycine, 20% MeOH) and the blot was dried overnight. The membrane was washed with TBST for 5 min and then blocked with ECL blocking agent (0.1 gm/ 10ml) and treated overnight at 4°C with OEH primary antibody (rabbit anti AaeOEH 304C) (Brown and Cao, 2001) at a 1:10,000 dilution. The next day, the membrane was washed three times with TBST (each wash for 20 min). After washing, the membrane was treated with a secondary goat anti-rabbit antibody conjugated with horseraddish peroxidase (diluted to 1:20,000 final conc.). The membrane was washed 3 times (each washing for 20 min) with TBST. ECL advance chemiluminescence reagents were added to the membrane blot and images were taken with the GeneGnome (SynGene) system.
d. Aag2 cell incubation and immunoblot

This same incubation experiment was done with the Aag2 cell line that was established from *Aedes aegypti* cells. Incubation was done with OEH in the same concentration as with ovary in presence or absence of serum in the medium for 1 h. Then, the medium was collected and the cells were washed 3 times with the same incubation medium and lysed with lysis buffer and homogenized. The protein concentration of the cell extract was determined. Equal amounts of protein from the ovaries and lyophilized supernatant were dissolved in Tris-tricine sample buffer for electrophoresis and blotting as described above.

e. Ovary incubation and immunocytochemistry

Four ovary pairs were dissected in *Aedes* saline and transferred to 100 μl of BS in a 1.5 μl microtube cap placed into 12 well, flat bottom RIA plates (Costar®) containing 200 μl deionized water. Then, 20 μl of BS or OEH (240 pmol/20 final conc. 40 pmol) was added into caps. Each OEH treatment was done in duplicate. The ovaries were incubated at 37° C in a humidified chamber with slow agitation for three different time points (30 min, 1 h and 2 h). At the end of each time period, the medium was taken out and 120 ul of fresh BS was added. For tissue fixation, 30 μl of 20% paraformaldehyde was added in each cap (final conc. 4% paraformaldehyde) and kept on rocker at RT for 2 h. Permeabilization was done in 0.2% Triton X-100 in paraformaldehyde solution (4% paraformaldehyde in BS) for 15 min on rocker at RT. Following permeabilization, the ovaries were washed three times (5 min each at RT) and blocked with a blocking solution (BS-GS-T: BS containing 0.1% Tween-20 and 5% goat serum) for 2 h,
on a rocker at 4ºC. After blocking, the ovaries were incubated overnight with anti-rabbit OEH antibody (304C) in blocking solution (1:1000 dilution) on a rocker at 4ºC. For immunocytochemistry control an MP-1 antibody (1:800 dilution) was used on midguts. After overnight incubation with primary antibody, ovaries were washed 3 times with BS-GS-T (each for 30-40 min), on a rocker at 4ºC. Then, the ovaries were treated with goat-anti rabbit secondary antibody (Alexa Fluor™ 488 fragment of goat anti-rabbit IgG (H+L); Molecular Probes, A11070, lot no. 73B2-1) 1/2000, overnight, on a rocker at 4ºC. After secondary antibody treatment, ovaries were washed 3 times with BS-T (BS with 0.1% Tween-20), for 30-40 min each, on a rocker at 4ºC and mounted on slides with 1:1 BS/glycerol and observed on a confocal microscope (Leica TCS SP II microscope, Leica Microsystem, Heidelberg, GmbH, Germany).

f. Ovary incubation, cell fractionation and immunoblot

A total of 100 ovary pairs (20 ovary pairs per cap, 5 replicates) from 3-5 day old sugar-fed females in five replicates were dissected in BS with 1x PI and transferred to 100 µl BS in a 1.5 ml microtube cap and then placed into 12 well, flat bottom RIA plates (Costar®) containing 200 µl of deionized water. Then, 20 µl of rlOEH (40 pmol total) and BS (control) was added to it and incubated for 1 h at 27º C in a rotating water bath. After incubation, the ovaries were spun down into a 1.5 ml microfuge tube by centrifuging at 500 x g for 1 min. Ovaries from 5 caps were pooled in one 1.5 ml microtube. Using the pipette the supernatant was removed carefully leaving the ovaries as dry as possible. Then, 100 µl of ice cold cytoplasmic extraction reagent-I (CER I) was added to the pellet and the tubes were vortexed vigorously on the highest setting for 15 sec to fully resuspend the tissue pellet and the tubes were incubated on ice for 10 min. Following incubation, 11 µl of ice cold CER II was added to the tubes and vortexed for 5 sec on the highest
setting. After vortexing, the tubes were again incubated on ice for 1 min. Then the tubes were vortexed for 5 sec on the highest setting and centrifuged for 5 min at maximum speed (approx 16,000 xg) in a refrigerated microcentrifuge. The supernatant (the cytoplasmic fraction) was immediately transferred to a clean pre-chilled tube and the tube was stored in –80 °C until further use. The insoluble pellet was resuspended in 50 μl of nuclear extraction reagent (NER) and vortexed for 15 sec every 10 min and then put back on ice and thus continued for a total of 40 min. Then the tubes were centrifuged at approx 16,000 xg in a refrigerated microcentrifuge for 10 min. The supernatant (which contains mostly the nuclear fraction) was transferred to a clean pre-chilled tube on ice. The nuclear fraction was stored in –80 °C until further use. After thawing, protein concentration of both cytoplasmic and nuclear fractions was determined by Coomassie Plus Bradford assay kit (Thermo scientific, Cat # 23236). The same amount of protein was dissolved in Tris-tricine sample buffer and loaded on a 16.5 % Tris-tricine gel for electrophoresis and blotting as described above.

5.3. Results

a. Radiolabeled OEH did not bind to isolated ovary membranes

The radioreceptor binding study was designed as described in previous studies that characterized binding of ILPs to the MIR (Brown et al., 2008; Wen et al., 2010). Radiolabeled OEH showed no binding to isolated ovary membrane (Fig. 5.1). However, in control assays, ILP4 bound to the ovary membrane and was displaced by unlabeled ILP4. Modification of the binding medium or subtraction of BSA from the binding medium and different temperatures (room temperature vs 4°C) did not improve OEH binding to the ovary membranes but boosted ILP4 binding (Fig. 5.1).
b. Immunocytochemistry did not detect OEH on the ovary membranes

Because OEH did not bind to isolated ovary membrane in the radioreceptor binding experiment, we took an immunocytochemical approach to assess whether any binding of OEH to the ovary membranes, occurred. The confocal cross section images of the ovarioles showed primary follicle, secondary follicle and the germarium (Fig. 5.2, 1 h incubation). Some punctate staining was observed on the membrane surrounding primary follicles of both OEH treated, and control ovaries indicating cross-reactivity of OEH antiserum with follicle proteins.

c. OEH immunoreactivity was observed in ovary and Aag2 cell extracts after incubation with OEH

We also assessed whether OEH binds to ovary membrane or enters the ovarioles during incubation. Interestingly, we observed some OEH binding or uptake to ovaries (Fig. 5.3). We also noticed that the duration of incubation (30 min, 1 h and 2 h) did not affect the level of OEH immunoreactivity in the ovary extract. Robust and similar OEH immunoreactivity in the incubation medium of OEH treated ovaries compared to no ovary (only peptide) control medium indicated no apparent degradation of OEH during incubation. In addition, we used the Aag2 cell line for the same incubation experiment to see if binding or uptake of OEH occurred. The culture medium of Aag2 cells usually contains serum, so we did this experiment both in the presence and absence of serum to rule out any serum interference on OEH binding. OEH immunoreactivity was detected in the extract of cells treated with OEH. The level of OEH immunoreactivity was higher in the cells incubated without serum compared to those with serum,
indicating serum may affect binding or uptake (Fig.5.4). We also observed some cross-reactivity of OEH antiserum with higher molecular weight proteins in both control and OEH treated Aag2 cells.

d. OEH immunoreactivity was observed in cytoplasmic and nuclear fractions of OEH treated ovaries

Both ovary and Aag2 cell incubation studies indicated that there might be some uptake of OEH. We fractionated the control and the OEH treated ovaries into cytoplasmic and nuclear fractions and both fractions were tested for OEH immunoreactivity on immunoblot. Our result shows that both cytoplasmic and nuclear fractions had OEH immunoreactivity, and the cytoplasmic fraction seemed to have more immunoreactivity than its nuclear counterpart (Fig. 5.5).

5.4. Discussion

Based on the nature of peptide hormone-receptor interactions in both vertebrates and invertebrates, it is likely that OEH binds to a membrane receptor to activate downstream signaling in the mosquito ovary. Like ILP3, OEH stimulates ovary ecdysteroid production and this functional redundancy and the characterization of the MIR as ILP3 receptor (Brown et al., 2008) indicated a possibility that OEH can bind to the MIR which is abundantly expressed in the ovary membrane, irrespective of the blood meal ingestion (Riehle and Brown, 2002). ILP4 also stimulates (but less potent than ILP3) ecdysteroidogenic activity in ovaries, but did not bind to the MIR in ovary membranes, rather bound to a different membrane protein (~ 55 kDa) which has not been characterized yet (Wen et al., 2010). Here we show that the same strategy for
identifying OEH receptor or binding protein on ovary membrane was not successful upon repeated trials. Modifications of the binding medium and temperature did not increase OEH binding to the ovary membrane indicating that there are two possibilities: either OEH does not bind to proteins in the ovary membrane or the binding conditions were not optimal. The integrity and preservation of three dimensional structures of both ligand and receptor are very important for proper binding. We radiolabeled synthetic short OEH to use as a ligand for membrane binding assays because previous studies in chapter 3 indicated no apparent difference in the bioactivities of short and long form of OEH. There are 12 cysteine residues in the short OEH, indicating up to six intrachain disulfide bonds could exist and play roles in proper folding of the peptide (Brown et al., 1998). A reason for not seeing any OEH binding with the ovary membrane could be due to altered peptide conformations, which might have occurred during radiolabelling of OEH. Although unlikely, this possibility cannot be ruled out until radiolabelled sOEH is tested.

Another way to demonstrate the interaction of OEH with ovary membrane receptors is by incubating whole ovaries with unlabeled OEH followed by immunostaining with OEH antisera. Our immunocytochemistry results did not reveal any OEH specific immunostaining in both control and OEH treated ovaries. The OEH antiserum cross-reacted with some nonspecific proteins on the membrane of primary follicle and on the secondary follicles. This result supports the outcome of the ovary membrane binding experiment with radiolabeled OEH.

To straighten out the nonspecific cross-reactivity of OEH antiserum, we repeated the same incubation experiment but used the immunoblot method to detect OEH binding or uptake in the ovary. A previous study to determine OEH expression during a gonotrophic cycle (Fig 2.2,
chapter 2) revealed that even though OEH transcript was expressed in the ovary throughout the gonadotropin cycle, no OEH protein was detected in ovary until around 18 h PBM and gradually increases up to 48 h PBM. It is unlikely that ovary synthesizes OEH at that late period considering the time frame of the egg maturation cycle. So, a reasonable justification for the gradual increase of OEH in ovaries may be uptake from hemolymph rather than binding to ovary membrane. Contrary to our immunocytochemistry results, OEH immunoreactivity was detected only in the extracts of the OEH treated ovary suggesting some level of binding or uptake. Aag2 cells line expresses all known ILPs except ILP5, the MIR. They also expresses other signaling pathway proteins such as Akt, ribosomal S6 kinase, extracellular signal regulated kinase (Erk) and mitogen-activated protein kinase (MAPK). Because of the expression of both ligand (ILP3) and receptor (MIR), it is thought that ILP signaling works in an autocrine or paracrine manner in Aag2 cells. ILP3 and ILP4 exhibit similar binding kinetics with Aag2 cell membranes as they do with ovary (unpublished data). Our idea behind using Aag2 cells in this study is justified for two reasons: (i) to facilitate OEH binding or uptake for greater contact with cell membrane (in monolayer cells) which is somewhat restricted in tissues because of extracellular matrix and other adhesion proteins and (ii) to understand if OEH binding or uptake is ovary specific, meaning there may be some specific transporter protein on ovary membrane which brings OEH into the cytoplasm.

The results showing possible uptake of OEH into follicle cells led us to examine which part of the cell, OEH localizes to after crossing the cell membrane. Ovary incubation and cell fractionation followed by immunoblot detection revealed the presence of OEH in both cytoplasm
and nuclear compartments. This experiment was not repeated enough times to make any conclusive statement about the distribution of OEH in the cellular compartments.

In summary, the identity of an OEH receptor or binding protein remains unknown but there is a hint that OEH binds to or crosses the ovary membrane. Cross-reactivity of OEH antiserum with other ovary proteins indicates an immunoblot or immunocytochemistry approach may not identify proteins that interact with OEH in ovary, thus different approaches like incubating radiolabeled OEH with whole ovary followed by measurements of radioactivity in the ovary or cross-linking and autoradiography may identify OEH binding protein or uptake.

5.5. References


Figure. 5.1. OEH binding with ovary membrane. I$^{125}$-OEH was incubated with ovary membranes and total binding (TOT) was measured. To determine nonspecific binding (NSB), the membrane bound I$^{125}$-OEH was competed with 1µm unlabeled sOEH peptide. I$^{125}$-ILP4 was used as positive control. I$^{125}$-OEH does not show binding with ovary membrane in the standard conditions. I$^{125}$-ILP4 shows binding to ovary membrane and displacement by 5 µM unlabeled ILP4. Modification in the binding medium or adding BSA or changing temperature did not improve OEH binding with ovary membrane, whereas it improved binding of I$^{125}$-ILP4 to ovary membranes.
Figure. 5.2. Immunocytochemistry of the ovary following incubation with OEH for 1 h.

Dissected ovaries were incubated with rLOEH (A) in Beyenbach saline (B). After incubation, ovaries were fixed, permeabilized for immunocytochemistry with anti-rabbit OEH primary antibody and goat-anti rabbit secondary antibody conjugated with Alexafluor 488. The confocal cross section images of ovarian follicles showing a primary follicle, secondary follicle and gerrmerium. There is no difference in staining between rLOEH treated ovaries (A) and control ovaries (B).
Figure 5.3. Uptake or binding of OEH by ovaries in vitro. Dissected ovaries were incubated alone (control) or with rOEH (40 pmol) in Beyenbach medium for different durations (30 min, 1 h and 2 h). Following incubation, the medium was collected and lyophilized and ovaries were washed and homogenized. Lyophilized medium and ovary extracts were run on a 16.5% Tris-tricine gel, transferred to 0.1 µm nitrocellulose membrane, and treated with an anti-rabbit OEH primary antibody.
Figure 5.4. Immunoblot Aag2 cell extract after incubation with OEH. Aag2 cells were incubated with rLOEH (40 pmol) for 1 hour in HQ insect cell medium in the presence or absence of fetal bovine serum (FBS). After incubation, lyophilized medium (supe) and cells extracts were run on a 16.5% Tris-Tricine gel and transferred to a 0.1µm nitrocellulose membrane, and treated with anti-rabbit OEH primary antibody. Aag2 cells treated with OEH showed some binding or uptake of OEH compared to control cells. In absence of FBS, OEH uptake or binding increased. OEH antibody cross-reacted non-specifically with higher molecular weight proteins in Aag2 cells.
Figure. 5.5. Ovary incubation with OEH and cell fractionation. Ovaries were incubated with rLOEH (40 pmol) for 1 h. Cytoplasmic and nuclear fractions were separated from ovary extracts, run on 16.5 % Tris-tricine gel, transferred to a 0.1μm nitrocellulose membrane, and treated with an anti-rabbit OEH primary antibody. (A) OEH is found in both cytoplasmic and nuclear extracts in the rLOEH treated ovaries. (B) Each cytoplasmic or nuclear fraction was tested for their specific markers proteins (cytoplasm = tubulin, nucleus = Histone H1).
CHAPTER 6

CONCLUSIONS

Disrupting egg maturation in females is one promising strategy for controlling the mosquito reproduction. Like other anautogenous species, egg maturation process in *Ae. aegypti*, is tightly regulated by endocrine signals which come from the female brain in response to blood feeding. OEH was the first neuropeptide shown to stimulate yolk deposition in blood fed decapitated females and ovary ecdysteroid production *in vitro* in *Ae. aegypti*. Later studies identified ILPs, which also exhibited similar gonadotropic and ecdysteroidogenic functions in *Ae. aegypti* females. The receptor and some important functions of ILP3 were recently characterized. With this information as a reference, I characterized OEH expression, function and signaling mechanism.

My results demonstrate the colocalization of OEH with ILP3 in some MNCs of the female brain. Transcript and protein expression profiles of OEH in different body parts at different life stages and in different female tissues during egg maturation indicated that the brain is the main source of OEH in *Ae. aegypti*. In addition to the brain, OEH mRNA was also detected in the ovary but protein is not detected until 18 h PBM which suggests a possible uptake of OEH from the hemolymph. During isolation and identification of OEH, a truncated version of the full length peptide was shown to be bioactive in both gonadotropic and ecdysteroidogenic bioassays. This study demonstrates no significant difference in the bioactivity of the full length peptide and truncated peptide supporting the hypothesis that full length OEH peptide is a
prepropeptide and the truncated version is the mature form after endogenous processing. The dose response study revealed that at suboptimal doses OEH and ILP3 acted additively to stimulate ovary ecdysteroid production in vitro.

My dissertation also reveals that OEH stimulates blood digestion in the midgut and Vg synthesis in the fat body of blood fed decapitated females. Unlike ILP3, OEH does not increase late serine protease expression in dissected midguts nor does it induce Vg translation in the isolated fat body in vitro. In sugar fed decapitated females, OEH stimulates glycogen storage in the fat body and reduces trehalose levels in the hemolymph like ILP3. Unlike ILP3 but similar to ILP4, OEH has no effect on lipid storage.

I attempted to identify an OEH receptor or binding protein in the ovaries and Aag2 cells using a radioreceptor binding approach, but was not successful. Several modifications of the binding medium and temperature did not improve the binding of radiolabeled OEH to the ovary membrane. However, in vitro incubation studies with whole ovary or Aag2 cells suggested some uptake or binding of OEH by the cells. Further, fractionation of the OEH treated ovaries demonstrated both cytoplasmic and nuclear localization of OEH in the cells. Because the receptor for OEH remains unknown, I took a different approach to characterize OEH signaling. Ovaries incubated with OEH in the presence of insulin receptor kinase inhibitor, PQIP did not show any inhibition in ecdysteroid production as opposed to the complete inhibition found in ILP3 treated ovaries, suggesting OEH mediated ecdysteroid production does not occur through MIR signaling. Like ILP3, OEH boosts ecdysteroid production by ovaries in the presence of amino acid medium. Inhibition of this response by rapamycin confirmed that OEH mediates
activation of TOR signaling in the presence of amino acids. Further, ovary incubation with OEH did not phosphorylate the MIR but did phosphorylate the downstream protein kinase Akt. OEH also stimulates phosphorylation of TOR downstream signaling proteins, ribosomal S6 kinase and 4EBP1 which again confirm that OEH activates TOR signaling. Activation of signaling proteins by OEH differs in a tissue specific manner which is likely functionally significant. OEH treatment shows no increase in the cAMP level in the ovary indicating OEH does not bind to a GPCR that activates cAMP production. Altogether, my study suggests that OEH and ILP3 work through different receptors but both activate downstream elements of the insulin signaling pathway.