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Neuronal Mechanisms that Regulate Vitellogenesis in the Fruit Fly

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ABSTRACT: Vitellogenesis is the process by which yolk accumulates in developing oocytes. The initiation of vitellogenesis represents an important control point in oogenesis. When females of the model insect *Drosophila melanogaster* molt to become adults, their ovaries lack mature vitellogenic oocytes, only producing them after reproductive maturation. After maturation, vitellogenesis stops until a mating signal re-activates it. Juvenile hormone (JH) from the endocrine organ known as the corpora allata (CA) is the major insect gonadotropin that stimulates vitellogenesis, and the seminal protein sex peptide (SP) has long been implicated as a mating signal that stimulates JH biosynthesis. In this review, we discuss our new findings that explain how the nervous system gates JH biosynthesis and vitellogenesis associated with reproductive maturation and the SP-induced post-mating response. Mated females exhibit diurnal rhythmicity in oogenesis. A subset of brain circadian pacemaker neurons produce Allatostatin C (AstC) to generate a circadian oogenesis rhythm by indirectly regulating JH and vitellogenesis through the brain insulin-producing cells. We also discuss genetic evidence that supports this model and future research directions.

Key words: Vitellogenesis, Juvenile hormone, Sex peptide, Allatostatin C, Ecdysis triggering hormone

초 록: 난황형성과정(Vitellogenesis)은 발달하는 난모세포에 난황이 축적되는 과정으로, 이 과정의 개시는 알형성과정(oogenesis)을 제어하는 주요 메커니즘이다. 곤충생리학 모델인 노랑초파리(*Drosophila melanogaster*)에서 난황형성과정은 성충으로 우화한 직후 시작하여 성적 성숙이 일어나는 2-3일간 지속된다. 성숙한 난모세포가 충분히 만들어지고 성적 성숙이 종료되면, 짝짓기 후 알형성과정이 다시 시작될 때까지 난황형성과정은 멈춘다. 수컷 초파리의 정액 단백질인 성 펩타이드(Sex peptide, SP)는 짝짓기의 신호로서 알라타체(corpora allata)를 자극해 유약호르몬(Juvenile hormone, JH) 생합성 및 분비를 유도하며, 혈림프(hemolymph) JH 농도의 증가는 난황형성과정을 자극한다. 최근 연구 결과에 따르면, SP수용체 뉴런은 자궁 내막의 수상돌기를 통해 교미 중 정액과 함께 자궁으로 전달된 SP를 감지함으로써, 축삭돌기를 통해 중추신경계인 복부신경절에 짝짓기 신호를 보내는데, 이러한 중추신경계 SP 신호가 JH 생합성 및 분비, 그리고 난황형성과정을 유도하는 것으로 밝혀졌다. 짝짓기 후 암컷에서의 난황형성과정은 일주기 리듬을 보이는데, 노랑초파리의 일주기 리듬은 중추 신경계 뉴런들에 의해 제어된다. 본 종설은 성적 성숙, 짝짓기 신호, 그리고 일주기 리듬에 따라 난황형성과정을 제어하는 신경 메커니즘에 관한 최근 연구 성과를 다룬다.

검색어: 난황형성과정, 유약호르몬, 섹스펩타이드, 알라토스타틴C, 탈피촉진호르몬

Drosophila melanogaster females have a pair of ovaries, each of which contains 15-20 polytrophic ovarioles (Riddiford, 1993). Each ovariole represents an independent egg assembly line with progressively developing egg chambers (i.e., follicles). The anterior tip of each ovariole, referred to as the germarium, contains a population of germline stem cells (GSC), their niche, and early-stage follicles. In oogenesis, a GSC divides asymmetrically, producing a daughter cell that then divides four times to produce a cyst of 16 cystocytes. One of these cystocytes will become the oocyte, while the others become nurse cells. This oocyte-nurse cell complex is surrounded by a

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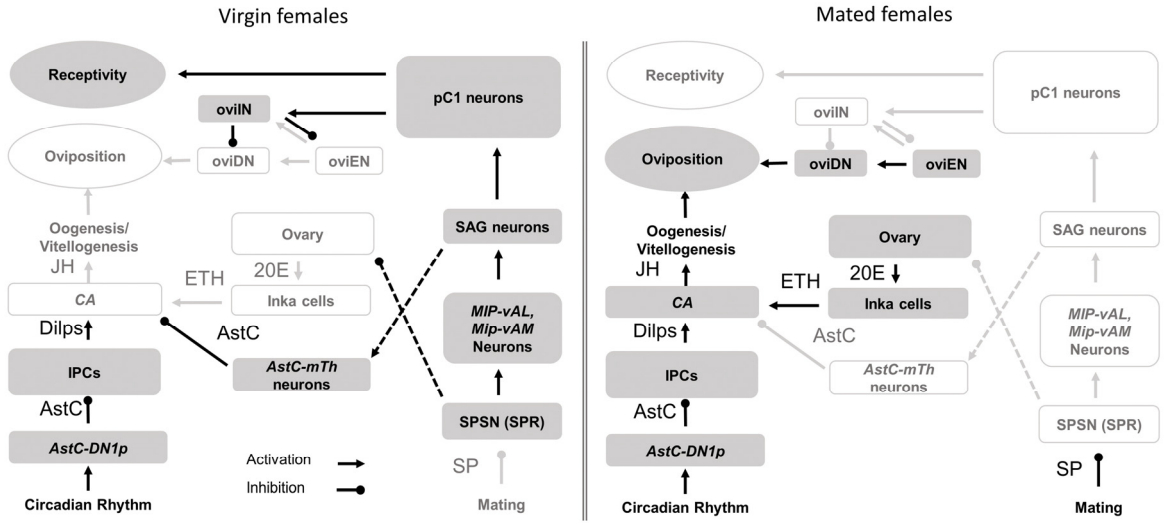

Fig. 1. A schematic that explains the neuronal and endocrine regulation of vitellogenesis before and after mating. In virgin females (left), SPSNs activate *Mip-vAL/vAM* and SAG neurons in sequence. The SAG neurons stimulate *AstC-mTh* neurons indirectly (dotted arrow) to secrete AstC into circulation. Circulating AstC then sets up a vitellogenesis blockade by inhibiting JH biosynthesis in the CA. In their activated state, SAG neurons also activate brain pC1 neurons. This activation inhibits oviposition via the oviIN/oviEN-oviDN network and promotes mating receptivity in virgin females. In mated females (right), SP silences SPSNs via SPR, consequently inhibiting *Mip-vAL/vAM* and SAG neurons in sequence. This inhibition relieves the AstC-induced vitellogenesis blockade by silencing *AstC-mTh* neurons. SP also elevates 20E levels by silencing SPSNs via an unknown pathway (dotted line). This 20E stimulates the endocrine Inka cells to secrete ETH. The resulting ETH induces JH biosynthesis in the CA. *AstC-DN1p* neurons, a subset of the brain circadian pacemaker neurons that express AstC, inhibit IPCs rhythmically, generating a circadian oogenesis rhythm. Black and gray lines indicate active and inactive signaling states, respectively. Filled boxes indicate cells or neurons in an activated state, whereas empty boxes indicate those in an inactivated state.

layer of follicular epithelial cells to comprise a stage 1 follicle. During development, this complex of cells moves toward the posterior tip of the ovariole, passing through pre-vitellogenesis (stages 1-7) and vitellogenesis (stages 8-14). Vitellogenesis occurs in two steps-yolk protein (YP) synthesis by the fat body and follicle cells and YP uptake by the developing oocyte.

Vitellogenesis initiation is an important control point in oogenesis and is subject to complex control by two major gonadotropic hormones-juvenile hormone (JH) and 20-hydroxyecdysone (20E). 20E stimulates YP synthesis in the fat body (Jowett and Postlethwait, 1980), while JH stimulates the synthesis and uptake of YP by the ovary (Postlethwait and Handler, 1979; Jowett and Postlethwait, 1980). JH is essential for the continuing development of vitellogenic follicles past stages 8 and 9 but unnecessary for stage 10 oocytes to complete development. The increased 20E titer observed in flies subjected to nutrient deprivation induces follicle degeneration at stages 8 and 9 (Terashima et al., 2005). Applications of 20E induce oocyte apoptosis, while applications of JH suppress follicle degeneration. Thus, the JH and 20E balance determines whether oocytes pass through the mid-oogenesis checkpoint at stage 9 or instead undergo apoptosis (Soller et al., 1999).

During the life history of *Drosophila*, there are at least three major episodes of vitellogenesis. The first begins shortly after eclosion and continues during reproductive maturation. The second is induced by mating to sustain robust egg-laying activity in mated females. The last one occurs with circadian rhythmicity, supporting a diurnal oogenesis and egg-laying rhythm. We recently published two research articles that uncover the neuronal and endocrine mechanisms that generate these vitellogenesis episodes (Zhang et al., 2022, 2021). This article will discuss our major findings in the context of previous studies (Fig. 1).

Vitellogenesis during Reproductive Maturation

Drosophila females molt into the adult stage with ovaries lacking vitellogenic follicles (Spradling, 1993). Vitellogenesis begins after molting (i.e., eclosion) and continues throughout reproductive maturation, taking 2-3 days. Early vitellogenic follicles (stages 8-11) appear as early as 12 hours post-eclosion and accumulate quickly to reach a maximum within 24 hours.

Later-stage vitellogenic follicles (stages 12-14) begin to appear 24 hours after eclosion and continue to increase for 3-4 days before reaching a maximum (Zhang et al., 2022). The temporal changes in the number of early vitellogenic follicles seem to be positively correlated with JH titer with a delay of ~24 hours. In *Drosophila* females, JH titers begin to increase shortly before eclosion, reaching a maximum shortly after eclosion and then decreasing over the next 1-2 days (Bownes and Rembold, 1987). Thus, it is likely that signals that initiate eclosion (i.e., adult ecdysis) also induce JH secretion and vitellogenesis.

Ecdysis in insects and some other arthropod species is triggered by ecdysis-triggering hormone (ETH), which is synthesized and secreted by the peritracheal gland Inka cells. ETH was first isolated in the hawkmoth *Manduca sexta* and later in the fruit fly *Drosophila melanogaster* (Žitňan et al., 1996; Park et al., 2002). ETH induces ecdysis motor programs by activating ETH receptors (ETHR) in the central nervous system (CNS). The *ETHR* gene encodes two ETHR isoforms via alternative splicing (Iversen et al., 2002; Park et al., 2003). These ETHR mRNAs (ETHR-A and ETHR-B) are localized in distinct central neuron subsets in both moths and flies (Kim et al., 2006a, 2006b; Daubnerová et al., 2021). ETHR is also expressed in the CA of the silk moth *Bombyx mori* and the hawkmoth *M. sexta* (Yamanaka et al., 2008). As with *Drosophila,* these moth species also exhibit a transient rise in JH at ecdysis (Baker et al., 1987; Niimi and Sakurai, 1997). CA expression of ETHR in dipteran species has also been observed in the yellow fever mosquito *Aedes aegypti* (Areiza et al., 2014). Consistent with an allatotropic function for ETH, the application of ETH to CAs isolated prior to eclosion stimulates JH biosynthesis. Moreover, ETHR-RNAi reduces JH synthesis in CAs isolated from 1-day-old females. Further biochemical evidence suggests ETH increases the activity of juvenile hormone acid methyltransferase (JHAMT), a key JH biosynthetic enzyme in the mosquito CA. The mechanism underlying this ETH-induced activation of JHAMT in the CA, however, remains unclear.

In *Drosophila*, the CA-specific depletion of ETHR reduces JH biosynthesis in both males and females (Meiselman et al., 2017). Likewise, ablation of the Inka cells, the sole source of ETH, reduces adult JH titers. This loss of JH is also associated with reduced fecundity. Both CA-specific ETHR RNAi and Inka cell ablation each led to a 30-35% reduction in egg production in mated females. Conversely, topical application of the JH mimic methoprene restores fecundity to normal levels in females with impaired ETH signaling. This observation confirmed the relationship between ETH signaling and JH activity. Virgin females lacking ETH exhibit impaired vitellogenesis. The ovaries of females with ablated Inka cells contain a normal number of pre-vitellogenic oocytes (stages 1-7) but significantly fewer vitellogenic oocytes (stages 9-13). This reduction in vitellogenic oocytes coincides with increased stage 9 oocytes undergoing apoptosis. Thus, a loss of ETH signaling leads to reduced JH biosynthesis, resulting in a systemic imbalance in the JH-20E ratio. This then leads to a failure of oocytes to pass through the mid-oogenesis checkpoint (Soller et al., 1999; Pritchett et al., 2009). Notably, Inka cell ablation and CA ablation each result in comparable reductions in egg production (Meiselman et al., 2017), suggesting ETH is the obligatory allatotropin critical for vitellogenesis during reproductive maturation.

Kramer et al. (1991) discovered that Allatostatin-C (AstC) in M. sexta inhibits JH biosynthesis in isolated CAs. Wang et al. (2012) also ascribed an allatostatic function to AstC in *D. melanogaster* when they found RNAi-induced depletion of either AstC or AstC receptors increased JH titers. We evaluated the role of AstC in vitellogenesis during reproductive maturation by comparing the number of vitellogenic oocytes in the ovaries of AstC-deficient and control females shortly after eclosion (Zhang et al., 2022). We found AstC deficiency advances vitellogenesis initiation by ~12 hours, suggesting AstC temporally decouples eclosion and vitellogenesis, presumably by delaying the JH titer increase. It is interesting to consider why *Drosophila* females have evolved a mechanism for delaying reproductive maturation.

AstC is expressed in a relatively large number of cells in the brain and ventral nerve cord (VNC) (Zhang et al., 2021). *AstC-mTh* neurons, a pair of AstC-positive cells located in the mesothoracic ganglion, seem to regulate vitellogenesis progression during reproductive maturation (Zhang et al., 2022). As with AstC deficiency, the silencing of *AstC-mTh* neurons advances vitellogenesis initiation by ~12 hours. Conversely, we found activating *AstC-mTh* neurons inhibits egg production in virgin females. Importantly, this inhibition does not occur in females

treated with the JH mimic methoprene, supporting a causal relationship between AstC and JH biosynthesis. Moreover, we observed increased *AstC-mTh* neural activity as females progressed through reproductive maturation. Using the TRIC (i.e., transcriptional reporter of intracellular Ca^{2+}) technique, which increases EGFP expression in response to intracellular Ca^{2+}, we were able to label *AstC-mTh* neurons.

When pharate adults are ready to emerge, the endocrine Inka cells secrete ETH. ETH enters the circulatory system and acts directly on the CNS to generate a motor pattern required for adult molting. In addition, an increase in ETH in the hemolymph induces JH biosynthesis in the CA and elevates blood JH levels. This induces vitellogenesis during reproductive maturation, which begins shortly after eclosion. Prior to eclosion, *AstC-mTh* neurons also begin secreting AstC. This inhibits JH biosynthesis in the CA and delays the ETH-induced JH peak by ~12 hours. As female complete reproductive maturation, ETH levels drop to their lowest point and *AstC- mTh* neurons augment their secretory activity, terminating JH biosynthesis and vitellogenesis.

Post-mating Vitellogenesis

After reproductive maturation, vitellogenesis stops until mating triggers its re-activation. The seminal protein sex peptide (SP, ACP70A) is a mating signal that stimulates JH biosynthesis and vitellogenesis (Soller et al., 1997). This 36-mer amidated peptide is synthesized in the male accessory gland (MAG). Upon its transfer to females during copulation, SP enters the circulation and induces many behavioral and physiological changes. These post-mating changes contribute to sustained and robust egg laying and refractoriness to further mating (Chen et al., 1988; Chapman et al., 2003; Pilpel et al., 2008). Intriguingly, SP induces JH biosynthesis in CAs isolated from virgin females (Moshitzky et al., 1996), suggesting SP may act on the CA hormonally to stimulate JH biosynthesis and vitellogenesis.

After identifying SPR in a genome-wide RNAi screen, Yapici et al. (2008) found SPR-deficient females behave like wild-type females that have copulated with SP-less males, laying only as many eggs as unmated females. The *SPR* gene encodes a G protein-coupled receptor (GPCR) with broad expression across the CNS. SPR is expressed in a group of sensory neurons-the SPR-positive sensory neurons (SPSNs)-that innervate the lumen of the uterus and project axons into the tip of the abdominal ganglion (Abg) (Häsemeyer et al., 2009; Yang et al., 2009). *SPR-RNAi* in SPSNs recapitulates most, if not all, SP-induced post-mating responses, including robust egg laying and refractoriness to mating. SPSNs relay the SP signal into the Abg sequentially into two subsets of Myoinhibitory peptide neurons (i.e., Mip-vAL and -vAM) and into SP abdominal ganglion (SAG) neurons (Feng et al., 2014; Jang et al., 2017). These findings indicate the SP signal enters the CNS via a neuronal pathway. Within the CNS, the SP signal seems to diverge from the SAG neurons to regulate each component of the post-mating response. For example, the SAG-pC1-oviIN/oviEN-oviDN pathway regulates oviposition but not ovulation (Wang et al., 2020).

Mating-induced vitellogenesis begins 12 hours post-mating, when the number of stage 10 follicles rises compared with virgin females (Zhang et al., 2022). It takes ~12 hours for pre-vitellogenic stage 7 follicles to become stage 10 follicles (Jia et al., 2016). Thus, the increase in stage 10 follicles indicates vitellogenesis begins immediately upon mating. Consistent with a role for SP in stimulating vitellogenesis, females mated with SP-less males exhibit no increase in stage 10 follicles.

SP silences SPSNs and the downstream Mip-vAL and SAG neurons in mated females because SPR is coupled to the inhibitory trimeric G-proteins, Gai or Gao (Feng et al., 2014; Jang et al., 2017). Thus, forced activation of SAG neurons should cause mated females to exhibit virgin-like neural activity, and forced silencing of SAG neurons should cause virgin females to exhibit mated female-like neural activity. We found, as expected, that forced activation of SAG neurons suppresses post-mating vitellogenesis in mated females by ~50% (Zhang et al., 2022) and forced silencing of SAG neurons enhances vitellogenesis in virgin females. Thus, SP seems to stimulate vitellogenesis via a neuronal route that includes the SAG neurons. Like the SAG neurons, *AstC-mTh* neurons respond to SP by downregulating their neural activity. Moreover, forced activation of *AstC-mTh* neurons similarly suppresses mating-induced vitellogenesis by ~50%. Functional epistasis suggests the *AstC-mTh* neurons function downstream of the SAG neurons. For example, simultaneous silencing of SAG

neurons and activation of *AstC-mTh* neurons overrides the vitellogenesis-stimulating effect of SAG silencing.

Forced activation of SAG neurons or *AstC-mTh* neurons in mated females leads to a ~50% reduction in post-mating vitellogenesis and in the number of stage 10 oocytes (Zhang et al., 2022). In mature virgin females, *AstC-mTh* neurons continue to supply inhibitory inputs to the CA, suppressing its production of JH. In mated females, SP reduces *AstC-mTh* neuronal activity by silencing SAG neurons. Thus, the reduced activity of *AstC-mTh* neurons in mated females disinhibits the CA, thereby permitting it to produce JH and elicit post-mating vitellogenesis. But disinhibition alone (i.e., silencing *AstC-mTh* neurons) in virgin females does not stimulate vitellogenesis. Notably, ETH seems essential for eliciting post-mating vitellogenesis, because ETHR-RNAi in the CA reduces the number of stage 10 oocytes by ~50% (Zhang et al., 2022). SP induces 20E biosynthesis via the neuronal SP response pathway (Ameku and Niwa, 2016), and 20E can activate ETH expression and secretion from adult Inka cells (Meiselman et al., 2017). Together, these lines of evidence led us to our current model for this phenomenon in which SP stimulates post-mating vitellogenesis by simultaneously enhancing ETH-induced stimulation of the CA by activating 20E production and reducing AstC-induced inhibition of the CA by silencing *AstC-mTh* neurons.

Generating the Circadian Vitellogenesis Rhythm

In *Drosophila*, the light and dark (LD) cycle generates an egg-laying rhythm by influencing oogenesis and oviposition (Allemand, 1976a, 1976b). While oviposition depends on light cues, oogenesis cycles with circadian rhythmicity. Thus, oogenesis is maintained even in the absence of environmental timing cues (i.e., in constant darkness or DD). Remarkably, we recently found that the number of vitellogenic stage 8 follicles cycles under DD conditions, rising to a peak at circadian time (CT) 14 and then falling again (Zhang et al., 2021). This rhythm is markedly attenuated in females lacking either the key molecular clock protein PERIOD (PER) or AstC. Of note, restoring AstC expression specifically in DN1p neurons, a small subset of PER-expressing clock neurons in the dorsal brain, rescues the rhythm of AstC-deficient mutants. AstC from DN1p neurons represses vitellogenesis by inhibiting JH, but it does so indirectly via the brain median neurosecretory cells or insulin-producing cells (IPCs). There are two GPCR receptors for AstC: AstC-R1 and AstC-R2. RNAi-mediated knockdown of either of these receptors in the IPCs almost completely abolishes the vitellogenesis rhythm. The IPCs produce three of the eight *Drosophila* insulin-like peptides (Dilps): Dilps 2, 3, and 5 (Ikeya et al., 2002; LaFever and Drummond-Barbosa, 2005). The actions of these Dilps are mediated by a single insulin receptor (InR) (Boucher et al., 2014), and knockdown of InR in the CA reduces its production of JH (Tatar et al., 2001). In addition, the Dilps produced by the IPCs act directly on the ovarian germline and promote GSC proliferation and follicle growth (LaFever and Drummond-Barbosa, 2005). Because AstC from the DN1p neurons regulates CA activity indirectly via the IPCs, it is also involved in GSC proliferation and follicle growth in oogenesis.

Future Research Directions

Across *Drosophila* species, the time required for a female to reach reproductive maturity varies widely. For example, *D. mettleir* females are ready to mate within a few hours after eclosion, whereas *D. pachea* females take several weeks to exhibit sexual receptivity. Vitellogenesis should also progress according to schedule in a species-specific manner. Comparisons of the neurons and signaling molecules discussed in this review across species will provide valuable insights into how evolution programs differential rates of vitellogenesis in the nervous system.

In *D. melanogaster*, the SP mating signal stimulates vitellogenesis by simultaneously enhancing ETH-induced stimulation of the CA and reducing AstC-mediated inhibition of the CA. The genetic evidence supporting this model is highly compelling, but SP occurs only in some *Drosophila* species. ETH and AstC, however, are highly conserved in all insect species. Thus, it remains unclear whether and how the mating signal stimulates vitellogenesis via the ETH and AstC pathways in insect species that lack SP.

The AstC-positive DN1p neurons generate the circadian vitellogenesis rhythm (Zhang et al., 2021). The DN1p neurons are a part of the circadian pacemaker neuron network, which

integrates light, temperature, and nutrition information (Shafer, 2006; Zhang et al., 2010). Intriguingly, all these environmental cues can induce a reproductive dormancy in *Drosophila* that is characterized by a pronounced suppression of vitellogenesis (Saunders et al., 1989; Ojima et al., 2018; Nagy et al., 2019). Thus, it is possible that the AstC-producing DN1p neurons are responsible for inducing reproductive dormancy in this species.

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Statements for Authorship Position & Contribution

Kim, Y.-J.: GIST, Professor; Wrote the manuscript
Zhang, C.: GIST, Post-doctoral fellow; Wrote the manuscript

All authors read and approved the manuscript.

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