UNIVERSITY OF CALIFORNIA
RIVERSIDE

# Influence of Hormonal State on Behavioral Plasticity in the Fruit Fly *Drosophila melanogaster*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

Sang Soo Lee

December 2017

Dissertation Committee:
Dr. Michael E. Adams, Chairperson
Dr. Anandasankar Ray
Dr. Naoki Yamanaka

The Dissertation of Sang Soo Lee is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

**Acknowledgements**

Foremost I would like to gratefully acknowledge my graduate advisor, Dr. Michael E. Adams. Dr. Adams provided me the opportunity to carry out my graduate research with his thoughtful considerations and genuine love of science. I could overcome my personal adversities by his ceaseless considerations.

I greatly appreciate my dissertation committee members, Dr. Anandasankar Ray and Dr. Naoki Yamanaka for their critical reviews on my dissertation. Moreover, they always provided critic advices during the fly lab meetings during my years at UCR, and those were valuable for accomplishing my research. Their love of science with exceptional expertise made them important role models for me to stay in academia.

I also appreciate my qualification committee members, Dr. Scott N. Currie, Dr. Khaleel Razak, and Dr. Wendy Saltzman. They provided truly helpful suggestions from a variety of perspectives to paint the broader picture.

I would like to thank all faculty members who offered me various knowledge and experience. Dr. Scott N. Currie, Dr. Peter W. Hickmott, and Dr. Khaleel Razak taught me to build up the intellectual foundation of neuroscience. Dr. Anupama Dahanukar, Dr. Shou-Wei Ding and Dr. Xuemei Chen helped me to understand basic genetics. Dr. Alexander Raikhel taught me basic knowledge of insect endocrinology. Laboratory courses of Dr. Margarita C. Curras-Collazo, Dr. Todd A. Fiacco, and Sara Mednick provided me a series of new technical experience to expand my knowledge. I also thank Dr. Jun-Hyoung Cho and Dr. Sachiko Haga-Yamanaka for their kindness and concern. Their generous advices had been big supports for me when I encountered challenges.

I thank the formal and current members in Adams laboratory, especially Dr. Do-Hyoung Kim, Dr. Ryan Arvidson, Robert Hice, Matthew Meiselman, Yike Ding, Sarah Frankenberg, Haroun Mohammad, Cebrina Nolan, and Jason Higa. In addition, I also want to acknowledge all my assistants and neuroscience graduate students who were joined in my research: Natalie Karapetians, Tedrick Mangasarian, Marina Barsoum, Shylla Taqi, Alfredo Vasquez, Wenny Wong, Tyler Bailey, and Anu Venkatesh. While working with and teaching them, I also tried many new things and learned a lot. I wish them all a great luck for their future.

I also deeply appreciate for efforts of all my collaborators. Dr. Fernando Gabriel Noriega and Dr. Crisalejandra Rivera-Pérez in Florida International University contributed to the measurement of juvenile hormones in adult flies. Some of their contribution is described in our recent publication

(Meiselman et al., 2017). Although my Dissertation does not include the topic, Dr. B. Hyle Park, Minh Q. Tong, Md. Monirul Hasan, Dr. Md. Rezuanul Haque, Dr. Md. Shahidul Islam in Department of Bioengineering supported the optical coherence tomography (OCT) technique to investigate the combination imaging system for simultaneous phase-resolved OCT and fluorescence microscopy (Tong et al., 2017).

I would like to thank all other scholars for their critical supports, especially Dr. Young-Joon Kim, Dr. Aki Ejima, and Dr. Leslie C. Griffith for intellectual discussion of behavioral assays. I also appreciate the generosity and kindness of many investigators for providing their precious fly lines.

I would like to appreciate Dr. Jung-Ha Lee, my formal advisor at Sogang University in South Korea. In addition to the continued support, he taught me how to build the scientific insight, so I could establish my own philosophical base.

I want to express my deep appreciation to my parents Won-Cheol Lee and Ki-In Kim, and to my grandparents Doo-Hwan Kim, Yeong-Hee Cho, and Bok-Kyo Kim for their love, confidence, and support. I also thank all my family in South Korea, especially to my parents in law Hyo-Jin Go and Yeon-Pa Hwang, and to my sister Soo-Jin Lee.

Lastly, I would like to give the greatest appreciation and to attribute the glory to my other half, Yeo Rim Go. My achievement wouldn’t be possible without her endless encouragement, unconditioned trust, and sacrifice.

**Dedication**

*To Yeo Rim,*

*To my precious May and Mark,*

*To my parents,*

*And to my grandfather Hang-Bok Lee in heaven.*

*"務實力行,*

*Devote your life to true knowledge."*

ABSTRACT OF THE DISSERTATION

Influence of Hormonal State on Behavioral Plasticity
in the Fruit Fly *Drosophila melanogaster*

by

Sang Soo Lee

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, December 2017
Dr. Michael E. Adams, Chairperson

Developing insects shed old cuticle at the end of each stage by performing a sequence of patterned muscle contractions, a process referred to as ecdysis. This behavioral sequence is initiated by endocrine peptides called “ecdysis triggering hormone” (ETH). Although ETH signaling components persist in the adult fruit fly *Drosophila melanogaster*, their function(s) during adulthood have not been studied.

To elucidate functions of ETH signaling in adult behavior, I investigated possible roles of ETH-driven hormonal state in learning and memory processes of male *Drosophila* by using a simple social learning paradigm, courtship conditioning. First, I show that ETH regulates short-term memory performance of males recently rejected by mated females. Adult ETH signaling regulates memory retention through maintenance of juvenile hormone (JH) production. The requirement of JH for normal memory performance is confined to a critical period during the first three days of adult life. JH targets dopaminergic neurons to maintain the short-term courtship memory (STM).

Next, I show that ETH signaling also modulates long-term memory (LTM) formation through pathways distinct from those regulating STM. ETH is necessary and sufficient to induce memory formation through *de novo* protein synthesis. RNAi knockdown experiments reveal that ETH

signaling regulates LTM through both direct and JH synthesis-mediated indirect modulation of memory circuits. As described for emotional memory circuits in the limbic system of mammals, actions of ETH and JH converge mainly in specific mushroom body neuropils to regulate LTM. Since ETH induces calcium mobilization in memory circuit neurons, I propose that the essential nature of hormonal state in regulation of memory circuits involves calcium-dependent mechanisms.

Although ETH promotes calcium mobilization in target cells, the intracellular signal transduction machinery induced by ETH receptors is largely based on logical inference. To investigate the specific steps in ETH receptor (ETHR)-induced signaling within target neurons, I used the pupal ecdysis sequence of *Drosophila* as a bioassay system. I identified a series of putative downstream G protein-coupled receptor (GPCR) signaling steps in pupal ETHR-expressing bursicon neurons. I also show that ETH-driven internal calcium mobilization is dependent on calcium influx from the extracellular space, possibly by promoting TRP channel activities, which provides a new link between the ETHR-driven intracellular cascade and membrane channels important for induction of electrical activity in target neurons.

Lastly, I describe that neurons expressing the neuropeptide crustacean cardioactive peptide (CCAP) have different functions in adult male and female *Drosophila*. In males, activity of CCAP neurons is important in gender recognition. Chemical signaling from CCAP neurons is required for suppression of abdominal contractions during the courting. In females, CCAP neurons are important for oviposition. In particular, regulate oviposition and choice of oviposition site. This finding contributes new insight into neuropeptide-based sexual dimorphic behaviors.

In summary, my dissertation work reveals functions for circulating peptides acting on the CNS to regulate behavioral plasticity of *Drosophila*.

## Table of Contents

Page

## List of Tables

Page

**List of Figures**

Page

# CHAPTER I

## General Introduction

### 1. Principle scope of endocrine system in neuroethology

The behavior of animals is based on processing of information within the nervous system. Through activity in peripheral and central nervous systems (PNS and CNS), acquired information is finely tuned to trigger appropriate behavioral output, as is the case for data processing in a computational module (Figure I.1A). Among numerous biological elements affecting neuronal excitability and communication between functional units, circulating and locally acting hormones are important factors in promotion and modulation of behaviors. Many hormones act globally through the blood stream, but also affect local circuits with distinct time windows and/or neural populations (Figure I.1B, adapted from Tinbergen, 1951). My graduate studies have focused mainly on endocrine mechanisms regulating innate and acquired behavioral plasticity using the model organism, *Drosophila melanogaster*.

### 2. *Drosophila* as a model organism for studying neuroethology

The fruit fly, *Drosophila melanogaster* has more than a hundred-year history in genetic study (Morgan, 1910). Genetic approaches dominated the first 50 years (1910–1960), focusing on the principle of inheritance. During the more recent period (1960–present), newly developed genetic tools have made *Drosophila* a prime model organism for a broad range of biological fields studying multi-cellular eukaryotes (Bellen et al., 2010). The nature of *Drosophila* provides numerous benefits for biological studies. Flies have a short generation period (10-12 days) and economic advantages. It is also one of the first organisms with a fully sequenced genome: approximately 13,600 protein-coding genes are localized on four chromosomes (Adams et al., 2000).

Cross-genomic comparisons show that many genes and cellular processes of the nervous system are conserved among *Drosophila* and mammals, including humans (Adams et al., 2000; Rubin et al., 2000; Yoshihara et al., 2001). Their nervous system is composed of about 250,000 neurons and supporting cells (glia) relative to the billions of neurons found in higher mammalian brains. Although simpler, *Drosophila* has high similarity neuroethologically to its mammalian counterparts, from simple valence (preference and avoidance to environment) to the complex cognitive behaviors such as learning and memory.

As described above, the ability to manipulate gene expression in *Drosophila* is a powerful tool for biological studies. The most common and well-known gene manipulation technique is the GAL4/UAS binary system (Figure I.2A) (Caygill and Brand, 2016). The insertion of the yeast GAL4 transcriptional activator-coding gene downstream of an endogenous promotor sequence of interest results in expression of GAL4 in spatially restricted fashion. Thus, when flies expressing GAL4 are crossed to flies bearing a transgene downstream of the GAL4-binding UAS-sequence, progeny express the transgene in the GAL4-specific spatial pattern. The advantage of this system is that the GAL4 driver and target transgene are carried in different parental lines, thus ensuring their viability and enabling a combinatorial approach with different driver and target lines. One limitation of this system is lack of the temporal control over the target gene expression. Recently, newly developed alternative gene expression systems address this issue, including the TARGET system (temporal and regional gene expression targeting) and Geneswitch, making use of hormone-inducible chimeric GAL4 activators (Figure I.2B).

### 3. The *Drosophila* neuroendocrine system in behaviors: beyond developmental events

Neuroendocrine control mechanisms are observed in all animal species that possess the nervous system. For decades, studies have revealed a great degree of similarity of neuroendocrine

mechanisms in distinct model species, even between invertebrates and vertebrates. Although these circulating molecules play critical roles during development of the nervous system, they also modulate excitability of mature nervous systems, thereby effecting changes of behavior. In this section, I will briefly introduce neuroendocrine regulation of developing and mature nervous systems, mainly related to behavioral controls of *Drosophila melanogaster*.

### 3.1. Ecdysteroids

In insects, the major steroid hormones are ecdysteroids, which play essential roles in programming developmental transitions such as molting and metamorphosis. Ecdysteroids are biosynthesized and released from the prothoracic gland (PG) during embryonic, larval, and even pharate adult development (Niwa and Niwa, 2014; Selcho et al., 2017). Although ecdysteroids also play various roles in the adult, sources of these steroids during this stage are not clear.

The timing of molting and metamorphosis are determined by dynamic fluctuations of circulating ecdysteroids. The active form, 20-hydroxyecdysone (20E) binds to a heterodimeric nuclear hormone receptor complex of proteins encoded by *Ecdysone Receptor* (*EcR*) and *ultraspiracle* (*usp*) genes. This heterodimeric complex binds to specific promoter sequences called ecdysone response elements to regulate gene expression (Koelle et al., 1991; Yao et al., 1992). Recently, a novel membrane protein was identified as the co-receptor for both ecdysteroids (20E) and dopamine (DA), revealing that binding of 20E to a G protein-coupled receptor (GPCR) type promotes rapid cellular signaling independent of new gene expression (Srivastava et al., 2005).

Like the function of steroids in the developing mammalian nervous system, ecdysteroids play a critical role for neuronal wiring in *Drosophila* (Boulanger et al., 2011; Kuo et al., 2005; Lee et al., 2000; Schubiger et al., 1998). EcR expression in the mushroom body (MB), the main memory center in the fly brain, determines the remodeling process of the MB neuropils during metamorphosis. For example, abnormalities of MB wiring in mutant flies during this period of

development leads to impairment of learning and memory in adults (Redt-Clouet et al., 2012). Besides regulation of developmental events, ecdysteroid signaling also regulates diverse adult behaviors, such as sleep (Ishimoto and Kitamoto, 2010), courtship (Ganter et al., 2011), and learning and memory (Ishimoto et al., 2009; Ishimoto et al., 2013).

### 3.2. Juvenile hormones

The sesquiterpenoid juvenile hormone (JH) also is an important regulator of molting, metamorphosis and reproduction in insects. JH is synthesized and secreted by an endocrine gland called the corpora allata (CA) (Jindra et al., 2013). Presence of JH maintains the "status quo" larval body plan during the juvenile growth phase and prevents precocious metamorphosis. Upon disappearance of JH during the last larval instar, fluctuation in 20E levels program pupal and subsequently adult development. During adulthood, JH returns in a broad range of insect species to regulate reproductive potential. In females, the major role of JH is to regulate ovary maturation and vitellogenesis, which includes expression of yolk protein precursors and vitellogenins (Vg), as well as receptor-driven uptake of these proteins by oocytes (Raikhel et al., 2005). Studies have reported the significant role of JH in caste differentiation of eusocial insects (Oliveira et al., 2017). Recent studies revealed that, in addition to promoting vitellogenesis, JH also modulates pheromone synthesis (Bilen et al., 2013) and immune responses (Schwenke and Lazzaro, 2017) in female *Drosophila*. Although the role of JH in males is less clear, several lines of evidence from previous studies suggested that JH is also essential for sperm motility (Hiroyoshi et al., 2017) and accessary gland protein synthesis (Braun and Wyatt, 1995; Yamamoto et al., 1988).

JH also regulates insect behaviors by targeting both peripheral and central nervous systems (PNS and CNS). A considerable number of studies reported that JH plays important roles in regulation of female oviposition behaviors (Cayre et al., 1994; Renucci et al., 1992), learning and memory (Maleszka and Helliwell, 2001; McQuillan et al., 2014), and sexual behaviors (Gadenne

et al., 1993; Lin et al., 2016; Manning, 1967; Ringo et al., 1991; Ringo and Pratt, 1978; Teal et al., 2000; Wijesekera et al., 2016; Wilson et al., 2003) in various insect species including *Drosophila melanogaster*.

Recent genetic approaches using *Drosophila* have enabled dissect of individual neural targets of JH underlying behavioral control. Proteins encoded by the paralogous genes *Methoprene-tolerant* (*Met*) and *germ-cell expressed* (*gce*) function as authentic JH receptors, which may function as transcription factors with or without JH binding. During both metamorphosis and reproduction, these paralogs are partially redundant in many instances (Jindra et al., 2013; Jindra et al., 2015). Both Met and gce appear to be widely expressed in the CNS (Baumann et al., 2017), and a recent report documents Met expression in olfactory neurons, where it regulates pheromone sensing of male flies (Lin et al., 2016).

### 3.3. Neuropeptide hormones

*Drosophila melanogaster* expresses at least 42 genes encoding neuropeptide precursors and peptide hormones (Nassel and Winther, 2010). Cellular responses to neuropeptide signaling depend on activation of membrane-bound receptors and the second messenger pathways they initiate. Most neuropeptides activate GPCRs (Brody and Cravchik, 2000; Ewer, 2005; Hewes and Taghert, 2001), whereas insulin-like peptides (ILPs) and prothoracicotropic hormone (PTTH) target receptor tyrosine kinases (Fernandez et al., 1995; Rewitz et al., 2009) and eclosion hormone (EH) activates receptor guanylate cyclases (Chang et al., 2009).

A huge variety of neuropeptides regulate *Drosophila* behaviors, including molting, circadian rhythms, feeding, aggression, and sexual behaviors (Nassel and Winther, 2010). Although most neuropeptide signaling molecules target neurons for behavioral control, some modulate behaviors through indirect hormone-hormone interactions; e.g., through regulation of 20E and JH secretion.

Some significant findings regarding neuropeptide regulation of *Drosophila* adult behaviors are listed in Table I.1.

**4. Ecdysis triggering hormone (ETH)**

During my graduate studies, I focused mainly on the role of ecdysis triggering hormone (ETH) signaling in regulation of adult *Drosophila* behaviors. In Section 4.1, I will first provide background knowledge for functions of ETH signaling in juvenile molting behaviors. Next (Section 4.2), our recent findings regarding ETH functions in adult reproduction will be introduced.

**4.1. Functions during juvenile development: a command peptide that schedules the ecdysis sequence**

Ecdysis is a common innate behavior essential for shedding of old cuticle and proceeding to the next developmental events in a broad range of invertebrates, including insects. It has been reported that in some holometabolous insects including *Drosophila*, the ecdysis sequence is composed of three sequential phases, pre-ecdysis, ecdysis, and post-ecdysis (Kim et al., 2006a; Zitnan and Adams, 2005). These behaviors are characterized by centrally-patterned skeletal muscle contractions, which are regulated under precise neural control. Principal molecules determining the ecdysis sequence are small peptides named ecdysis triggering hormones (ETHs) released from endocrine Inka cells (Park et al., 1999; Zitnan et al., 1996). Circulating ETH initiates the ecdysis sequence by activating ETH receptors (ETHR) expressed in diverse neurons in the CNS. Identification of the *ETHR* gene in *Drosophila* and moth *Manduca sexta* revealed that *ETHR* gene encodes two variants via alternative splicing of 3' exons (ETHR-A and ETHR-B) of GPCR superfamily (Iversen et al., 2002; Kim et al., 2006b; Park et al., 2003).

Considerable studies showed that ETH signaling orchestrates neuronal ensembles by targeting diverse peptidergic neurons in juvenile CNS (Kim et al., 2015; Kim et al., 2006a; Kim et al., 2006b).

Genetic manipulation of *ETHR* gene expression in specific peptidergic neurons using *Drosophila* revealed sequential neural activities promoted by ETH signaling in determining pupal ecdysis sequence (Kim et al., 2015; Kim et al., 2006a). For example, activity of Kinin-producing neurons triggers pre-ecdysis behavior, which is characterized by anterior rolling contractions of the abdomen. Crustacean cardioactive peptide (CCAP) and Bursicon neurons are targets of ETH that terminate pre-ecdysis and initiate ecdysis behavior, an anteriolateral swinging of the abdomen following the head eversion. *In vitro* calcium imaging from the pupal CNS indicates that ETHR-mediated calcium responses of these neurons occur sequentially. Calcium mobilization in bursicon neurons coincides with onset of ecdysis behavior. Since binding of ETH to ETHR promotes intracellular calcium elevation, this GPCR signaling is considered to link the Gαq protein-mediated signaling cascade (Hall, 2000).

**4.2. Allatotropic role in adult *Drosophila***

Although previous studies reported that *ETH*/*ETHR* transcripts (Graveley et al., 2011) and ETH immunoactive Inka cells (Park et al., 2002) persist into the adult stage of *Drosophila*, the role of ETH signaling during adulthood had not been studied. Recent studies in the silkworm *Bombyx mori* (Yamanaka et al., 2008) and yellow fever mosquito *Aedes aegypti* (Areiza et al., 2014) provided evidence that ETH functions as an allatotropin, promoting JH synthesis in adult *Drosophila*.

We recently reported that ETH functions as an obligatory allatotropin adult *Drosophila* (Meiselman et al., 2017). ETH promotes calcium elevation in adult CA cells and possibly upregulates enzyme activities required for JH biosynthesis. Suppression of ETH signaling molecules downregulates JH levels in both sexes, thereby impairing reproductive potential as measured by ovary maturation, egg development, and yolk protein deposition in females, and accessary gland protein production in males. Moreover, adult ETH signaling likely provides a link between ecdysteroid and JH signaling. Similar with previous evidence gathered during larval and

pupal development (Cho et al., 2014), elevated ecdysteroids likely promote ETH synthesis, while declining steroid levels enable release of ETH during adulthood. Suppression of EcR expression in Inka cells impairs ETH-JH signaling-mediated phenotypes (Meiselman et al, 2017). Therefore, adult ETH signaling is an essential part of the steroid-peptide-JH endocrine network that regulates reproductive fitness of adult *Drosophila*.

**5. Contributions of the work described in this dissertation**

Although we discovered important functions of ETH signaling in reproduction of *Drosophila* (Meiselman et al., 2017), its role in regulation of behaviors has not been studied. As I described above, neuroendocrine systems play important roles in promotion and modulation of neural activities underlying behavioral controls. During my graduate period, I have focused mainly on ETH functions in regulation of cognitive behaviors. In particular, I found that adult ETH signaling plays important roles in learning and memory processes in male *Drosophila*. My dissertation thus provides evidence for direct and indirect ETH action on CNS circuits that affect memory performance. For example, the ETH-JH hormonal cascade is essential for short-term memory (STM) retention of male flies, whereas ETH directly targets neural circuits required for long-term memory (LTM). Since hormonal systems regulating animal cognition are largely unknown, my work introduces functional roles for neuroendocrine networks into this field.

Although our previous studies suggested that the binding of ETH to ETHR promotes calcium mobilization in neurons through a G protein-mediated secondary messenger pathway, how each relevent molecular unit regulates neural activity has not been studied. To address this question, I investigated roles of GPCR signaling molecules in regulation of neuronal activity by utilizing pupal ecdysis as a model. I found that timing of ETH-promoted calcium mobilization in bursicon neurons and proper scheduling of ecdysis onset depends mainly on influx of extracellular calcium rather

than release of calcium from intracellular stores. Expression of transient receptor potential (TRP) subtype channels is important for proper functioning of bursicon neurons, suggesting that the ETHR-initiated signaling cascade culminates with TRP channels activation. This finding provides new insights into the mechanism of GPCR signal transduction and neuronal activation.

Besides examining the role and mechanism of ETH signaling, I also investigated functions of CCAP neurons in adult *Drosophila* behavior. I found that activity of CCAP neurons is required for male sexual behaviors and for female oviposition. Although the underlying neural architectures for these functions remain unclear, my findings provide important clues for understanding sexually dimorphic behavioral plasticity.

**6. Specific aims and organization of the dissertation**

The main goal of my graduate studies has been to understand how the endocrine system influences molecular and neural bases of cognitive decision-making. To accomplish this goal, I identified hormone-neural signaling interactions required for *Drosophila* memories by utilizing behavior assays, genetics, and calcium imaging techniques. In Chapter II, I found that the ETH-JH cascade during the early adult stage is essential for STM retention following courtship conditioning: JH targets DA neurons to maintain STM. In Chapter III, I show that the ETH-JH hormonal cascade also regulates courtship LTM. Similar with my finding at Chapter II, ETH-mediated JH synthesis is required for appropriate memory performance. However, I provide evidence that both ETH and and ETH-JH signaling regulates memory circuits involving MB neurons, rather than DA neurons. Together, evidence presented in Chapter II and Chapter III demonstrates parallel endocrine signaling pathways essential for STM and LTM memory processes. In Chapter IV, I shift my focus to the ETHR-mediated intracellular signaling cascade in pupal bursicon neurons for ecdysis regulation to understand the basic role of GPCR signaling in neural activity. I found that the ETHR-

mediated secondary messenger cascade promotes intracellular calcium elevation in bursicon neurons through not only the $IP_3$-mediated internal calcium release, which has been considered as the conventional Gq-mediated pathway, but also via the calcium influx from the extracellular space. Calcium mobilization in bursicon neurons has distinct kinetics determined by both external and internal calcium sources. In Chapter V, I shift my focus to CCAP neurons, demonstrating new roles for CCAP neurons in adult reproductive behavior such as male courtship and female oviposition. Chapter VI summarizes my findings in this dissertation.

## References


Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. (2000). The genome sequence of Drosophila melanogaster. Science 287, 2185-2195.

Aigaki, T., Fleischmann, I., Chen, P.S., and Kubli, E. (1991). Ectopic expression of sex peptide alters reproductive behavior of female D. melanogaster. Neuron 7, 557-563.

Areiza, M., Nouzova, M., Rivera-Perez, C., and Noriega, F.G. (2014). Ecdysis triggering hormone ensures proper timing of juvenile hormone biosynthesis in pharate adult mosquitoes. Insect Biochem Mol Biol 54, 98-105.

Asahina, K., Watanabe, K., Duistermars, B.J., Hoopfer, E., Gonzalez, C.R., Eyjolfsdottir, E.A., Perona, P., and Anderson, D.J. (2014). Tachykinin-expressing neurons control male-specific aggressive arousal in Drosophila. Cell 156, 221-235.

Bader, R., Colomb, J., Pankratz, B., Schrock, A., Stocker, R.F., and Pankratz, M.J. (2007). Genetic dissection of neural circuit anatomy underlying feeding behavior in Drosophila: distinct classes of hugin-expressing neurons. J Comp Neurol 502, 848-856.

Baumann, A.A., Texada, M.J., Chen, H.M., Etheredge, J.N., Miller, D.L., Picard, S., Warner, R., Truman, J.W., and Riddiford, L.M. (2017). Genetic tools to study juvenile hormone action in Drosophila. Sci Rep 7, 2132.

Belgacem, Y.H., and Martin, J.R. (2006). Disruption of insulin pathways alters trehalose level and abolishes sexual dimorphism in locomotor activity in Drosophila. J Neurobiol 66, 19-32.

Bellen, H.J., Tong, C., and Tsuda, H. (2010). 100 years of Drosophila research and its impact on vertebrate neuroscience: a history lesson for the future. Nat Rev Neurosci 11, 514-522.

Bilen, J., Atallah, J., Azanchi, R., Levine, J.D., and Riddiford, L.M. (2013). Regulation of onset of female mating and sex pheromone production by juvenile hormone in Drosophila melanogaster. Proc Natl Acad Sci U S A 110, 18321-18326.

Boulanger, A., Clouet-Redt, C., Farge, M., Flandre, A., Guignard, T., Fernando, C., Juge, F., and Dura, J.M. (2011). ftz-f1 and Hr39 opposing roles on EcR expression during Drosophila mushroom body neuron remodeling. Nat Neurosci 14, 37-44.

Braun, R.P., and Wyatt, G.R. (1995). Growth of the male accessory-gland in adult locusts - roles of juvenile-hormone, Jh esterase, and Jh binding-proteins. Arch Insect Biochem 30, 383-400.

Brody, T., and Cravchik, A. (2000). Drosophila melanogaster G protein-coupled receptors. J Cell Biol 150, F83-88.

Carvalho, G.B., Kapahi, P., Anderson, D.J., and Benzer, S. (2006). Allocrine modulation of feeding behavior by the Sex Peptide of Drosophila. Curr Biol 16, 692-696.

Caygill, E.E., and Brand, A.H. (2016). The GAL4 System: a versatile system for the manipulation and analysis of gene expression. Methods Mol Biol 1478, 33-52.

Cayre, M., Strambi, C., and Strambi, A. (1994). Neurogenesis in an adult insect brain and its hormonal control. Nature (London) 368, 57-59.

Chambers, D.B., Androschuk, A., Rosenfelt, C., Langer, S., Harding, M., and Bolduc, F.V. (2015). Insulin signaling is acutely required for long-term memory in Drosophila. Front Neural Circuits 9, 8.

Chang, J.C., Yang, R.B., Adams, M.E., and Lu, K.H. (2009). Receptor guanylyl cyclases in Inka cells targeted by eclosion hormone. Proc Natl Acad Sci U S A 106, 13371-13376.

Cho, K.H., Daubnerova, I., Park, Y., Zitnan, D., and Adams, M.E. (2014). Secretory competence in a gateway endocrine cell conferred by the nuclear receptor betaFTZ-F1 enables stage-specific ecdysone responses throughout development in Drosophila. Dev Biol 385, 253-262.

Dierick, H.A., and Greenspan, R.J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. Nat Genet 39, 678-682.

Ewer, J. (2005). Behavioral actions of neuropeptides in invertebrates: insights from Drosophila. Horm Behav 48, 418-429.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., and Schlessinger, J. (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. EMBO J 14, 3373-3384.

Gadenne, C., Renou, M., and Sreng, L. (1993). Hormonal control of pheromone responsiveness in the male black cutworm Agrotis ipsilon. Experientia (Basel) 49, 721-724.

Galikova, M., Klepsatel, P., Xu, Y.J., and Kuhnlein, R.P. (2017). The obesity-related Adipokinetic hormone controls feeding and expression of neuropeptide regulators of Drosophila metabolism. Eur J Lipid Sci Tech 119.

Ganter, G.K., Panaitiu, A.E., Desilets, J.B., Davis-Heim, J.A., Fisher, E.A., Tan, L.C., Heinrich, R., Buchanan, E.B., Brooks, K.M., Kenney, M.T., et al. (2011). Drosophila male courtship behavior is modulated by ecdysteroids. J Insect Physiol 57, 1179-1184.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., et al. (2011). The developmental transcriptome of Drosophila melanogaster. Nature 471, 473-479.

Hall, I.P. (2000). Second messengers, ion channels and pharmacology of airway smooth muscle. Eur Respir J 15, 1120-1127.

Hentze, J.L., Carlsson, M.A., Kondo, S., Nassel, D.R., and Rewitz, K.F. (2015). The neuropeptide allatostatin A regulates metabolism and feeding decisions in Drosophila. Sci Rep 5, 11680.

Hewes, R.S., and Taghert, P.H. (2001). Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. Genome Res 11, 1126-1142.

Hiroyoshi, S., Reddy, G.V.P., and Mitsuhashi, J. (2017). Effects of juvenile hormone analogue (methoprene) and 20-hydroxyecdysone on reproduction in Polygonia c-aureum (Lepidoptera: Nymphalidae) in relation to adult diapause. J Comp Physiol A Neuroethol Sens Neural Behav Physiol.

Hyun, S., Lee, Y., Hong, S.T., Bang, S., Paik, D., Kang, J., Shin, J., Lee, J., Jeon, K., Hwang, S., et al. (2005). Drosophila GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron 48, 267-278.

Isaac, R.E., Li, C., Leedale, A.E., and Shirras, A.D. (2010). Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. Proc Biol Sci 277, 65-70.

Isabel, G., Martin, J.R., Chidami, S., Veenstra, J.A., and Rosay, P. (2005). AKH-producing neuroendocrine cell ablation decreases trehalose and induces behavioral changes in Drosophila. Am J Physiol Regul Integr Comp Physiol 288, R531-538.

Ishimoto, H., and Kitamoto, T. (2010). The steroid molting hormone Ecdysone regulates sleep in adult Drosophila melanogaster. Genetics 185, 269-281.

Ishimoto, H., Sakai, T., and Kitamoto, T. (2009). Ecdysone signaling regulates the formation of long-term courtship memory in adult Drosophila melanogaster. Proc Natl Acad Sci U S A 106, 6381-6386.

Ishimoto, H., Wang, Z., Rao, Y., Wu, C.F., and Kitamoto, T. (2013). A novel role for ecdysone in Drosophila conditioned behavior: linking GPCR-mediated non-canonical steroid action to cAMP signaling in the adult brain. PLoS Genet 9, e1003843.

Iversen, A., Cazzamali, G., Williamson, M., Hauser, F., and Grimmelikhuijzen, C.J. (2002). Molecular identification of the first insect ecdysis triggering hormone receptors. Biochem Biophys Res Commun 299, 924-931.

Jindra, M., Palli, S.R., and Riddiford, L.M. (2013). The juvenile hormone signaling pathway in insect development. Annu Rev Entomol 58, 181-204.

Jindra, M., Uhlirova, M., Charles, J.P., Smykal, V., and Hill, R.J. (2015). Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. PLoS Genet 11, e1005394.

Johard, H.A., Enell, L.E., Gustafsson, E., Trifilieff, P., Veenstra, J.A., and Nassel, D.R. (2008). Intrinsic neurons of Drosophila mushroom bodies express short neuropeptide F: relations to extrinsic neurons expressing different neurotransmitters. J Comp Neurol 507, 1479-1496.

Johard, H.A., Yoishii, T., Dircksen, H., Cusumano, P., Rouyer, F., Helfrich-Forster, C., and Nassel, D.R. (2009). Peptidergic clock neurons in Drosophila: ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. J Comp Neurol 516, 59-73.

Kahsai, L., Martin, J.R., and Winther, A.M. (2010). Neuropeptides in the Drosophila central complex in modulation of locomotor behavior. J Exp Biol 213, 2256-2265.

Kim, D.H., Han, M.R., Lee, G., Lee, S.S., Kim, Y.J., and Adams, M.E. (2015). Rescheduling behavioral subunits of a fixed action pattern by genetic manipulation of peptidergic signaling. PLoS Genet 11, e1005513.

Kim, Y.-J., Zitnan, D., Giovanni Galizia, C., Cho, K.-H., and Adams, M.E. (2006a). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Current Biology 16, 1395-1407.

Kim, Y.J., Zitnan, D., Cho, K.H., Schooley, D.A., Mizoguchi, A., and Adams, M.E. (2006b). Central peptidergic ensembles associated with organization of an innate behavior. Proc Natl Acad Sci U S A 103, 14211-14216.

Ko, K.I., Root, C.M., Lindsay, S.A., Zaninovich, O.A., Shepherd, A.K., Wasserman, S.A., Kim, S.M., and Wang, J.W. (2015). Starvation promotes concerted modulation of appetitive olfactory behavior via parallel neuromodulatory circuits. Elife 4.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67, 59-77.

Krashes, M.J., DasGupta, S., Vreede, A., White, B., Armstrong, J.D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in Drosophila. Cell 139, 416-427.

Kuo, C.T., Jan, L.Y., and Jan, Y.N. (2005). Dendrite-specific remodeling of Drosophila sensory neurons requires matrix metalloproteases, ubiquitin-proteasome, and ecdysone signaling. Proc Natl Acad Sci U S A 102, 15230-15235.

Lebreton, S., Mansourian, S., Bigarreau, J., and Dekker, T. (2016). The adipokinetic hormone receptor modulates sexual behavior, pheromone perception and pheromone production in a sex-specific and starvation-dependent manner in Drosophila melanogaster. Front Ecol Evol 3.

Lee, G., Bahn, J.H., and Park, J.H. (2006). Sex- and clock-controlled expression of the neuropeptide F gene in Drosophila. Proc Natl Acad Sci U S A 103, 12580-12585.

Lee, T., Marticke, S., Sung, C., Robinow, S., and Luo, L. (2000). Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in Drosophila. Neuron 28, 807-818.

Lin, H.H., Cao, D.S., Sethi, S., Zeng, Z., Chin, J.S., Chakraborty, T.S., Shepherd, A.K., Nguyen, C.A., Yew, J.Y., Su, C.Y., et al. (2016). Hormonal modulation of pheromone detection enhances male courtship success. Neuron 90, 1272-1285.

Luo, J., Lushchak, O.V., Goergen, P., Williams, M.J., and Nassel, D.R. (2014). Drosophila insulin-producing cells are differentially modulated by serotonin and octopamine receptors and affect social behavior. PLoS One 9, e99732.

Maleszka, R., and Helliwell, P. (2001). Effect of juvenile hormone on short-term olfactory memory in young honeybees (Apis mellifera). Horm Behav 40, 403-408.

Manning, A. (1967). The control of sexual receptivity in female Drosophila. Anim Behav 15, 239-250.

McQuillan, H.J., Nakagawa, S., and Mercer, A.R. (2014). Juvenile hormone enhances aversive learning performance in 2-day old worker honey bees while reducing their attraction to queen mandibular pheromone. PLoS One 9, e112740.

Meiselman, M., Lee, S.S., Tran, R.T., Dai, H., Ding, Y., Rivera-Perez, C., Wijesekera, T.P., Dauwalder, B., Noriega, F.G., and Adams, M.E. (2017). Endocrine network essential for reproductive success in Drosophila melanogaster. Proc Natl Acad Sci U S A 114, E3849-E3858.

Melcher, C., and Pankratz, M.J. (2005). Candidate gustatory interneurons modulating feeding behavior in the Drosophila brain. PLoS Biol 3, e305.

Monyak, R.E., Emerson, D., Schoenfeld, B.P., Zheng, X., Chambers, D.B., Rosenfelt, C., Langer, S., Hinchey, P., Choi, C.H., McDonald, T.V., et al. (2016). Insulin signaling misregulation underlies circadian and cognitive deficits in a Drosophila fragile X model. Mol Psychiatry.

Nassel, D.R., and Winther, A.M. (2010). Drosophila neuropeptides in regulation of physiology and behavior. Prog Neurobiol 92, 42-104.

Niwa, Y.S., and Niwa, R. (2014). Neural control of steroid hormone biosynthesis during development in the fruit fly Drosophila melanogaster. Genes Genet Syst 89, 27-34.

Oh, Y., Yoon, S.E., Zhang, Q., Chae, H.S., Daubnerova, I., Shafer, O.T., Choe, J., and Kim, Y.J. (2014). A homeostatic sleep-stabilizing pathway in Drosophila composed of the sex peptide receptor and its ligand, the myoinhibitory peptide. PLoS Biol 12, e1001974.

Oliveira, R.C., Vollet-Neto, A., Akemi Oi, C., van Zweden, J.S., Nascimento, F., Sullivan Brent, C., and Wenseleers, T. (2017). Hormonal pleiotropy helps maintain queen signal honesty in a highly eusocial wasp. Sci Rep 7, 1654.

Parisky, K.M., Agosto, J., Pulver, S.R., Shang, Y., Kuklin, E., Hodge, J.J., Kang, K., Liu, X., Garrity, P.A., Rosbash, M., et al. (2008). PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron 60, 672-682.

Park, Y., Filippov, V., Gill, S.S., and Adams, M.E. (2002). Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development 129, 493-503.

Park, Y., Kim, Y.J., Dupriez, V., and Adams, M.E. (2003). Two subtypes of ecdysis-triggering hormone receptor in Drosophila melanogaster. J Biol Chem 278, 17710-17715.

Park, Y., Zitnan, D., Gill, S.S., and Adams, M.E. (1999). Molecular cloning and biological activity of ecdysis-triggering hormones in Drosophila melanogaster. FEBS Lett 463, 133-138.

Peng, J., Chen, S., Busser, S., Liu, H., Honegger, T., and Kubli, E. (2005). Gradual release of sperm bound sex-peptide controls female postmating behavior in Drosophila. Curr Biol 15, 207-213.

Raikhel, A.S., Brown, M.R., and Belles, X. (2005). Hormonal control of reproductive processes. In Comprehensive molecular insect science Volume Three Endocrinology (Amsterdam, Boston etc.: Elsevier), pp. 433-491.

Redt-Clouet, C., Trannoy, S., Boulanger, A., Tokmatcheva, E., Savvateeva-Popova, E., Parmentier, M.L., Preat, T., and Dura, J.M. (2012). Mushroom body neuronal remodelling is necessary for short-term but not for long-term courtship memory in Drosophila. Eur J Neurosci 35, 1684-1691.

Ren, G.R., Hauser, F., Rewitz, K.F., Kondo, S., Engelbrecht, A.F., Didriksen, A.K., Schjott, S.R., Sembach, F.E., Li, S., Sogaard, K.C., et al. (2015). CCHamide-2 is an orexigenic brain-gut peptide in Drosophila. PLoS One 10, e0133017.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell 99, 791-802.

Renucci, M., Cherkaoui, L., Rage, P., Augier, R., and Strambi, A. (1992). Juvenile hormone exerts a primer effect on oviposition behaviour in Acheta domesticus. In insect juvenile hormone research: fundamental and applied approaches: chemistry, biochemistry and mode of action (Paris.: Institut National de la Recherche Agronomique), pp. 147-163.

Rewitz, K.F., Yamanaka, N., Gilbert, L.I., and O'Connor, M.B. (2009). The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. Science 326, 1403-1405.

Ringo, J., Werczberger, R., Altaratz, M., and Segal, D. (1991). Female sexual receptivity is defective in juvenile hormone-deficient mutants of the apterous gene of Drosophila melanogaster. Behav Genet 21, 453-469.

Ringo, J.M., and Pratt, N.R. (1978). A juvenile hormone analogue induces precocious sexual behavior in Drosophila grimshawi (Diptera: Drosophilidae). Annals of the Entomological Society of America 71, 264-266.

Rubin, G.M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M., and Harvey, D.A. (2000). A Drosophila complementary DNA resource. Science 287, 2222-2224.

Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., and Bender, M. (1998). Drosophila EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. Development 125, 2053-2062.

Schwenke, R.A., and Lazzaro, B.P. (2017). Juvenile hormone suppresses resistance to infection in mated female Drosophila melanogaster. Curr Biol 27, 596-601.

Selcho, M., Millan, C., Palacios-Munoz, A., Ruf, F., Ubillo, L., Chen, J., Bergmann, G., Ito, C., Silva, V., Wegener, C., et al. (2017). Central and peripheral clocks are coupled by a neuropeptide pathway in Drosophila. Nat Commun 8, 15563.

Shafer, O.T., Helfrich-Forster, C., Renn, S.C., and Taghert, P.H. (2006). Reevaluation of Drosophila melanogaster's neuronal circadian pacemakers reveals new neuronal classes. J Comp Neurol 498, 180-193.

Shohat-Ophir, G., Kaun, K.R., Azanchi, R., Mohammed, H., and Heberlein, U. (2012). Sexual deprivation increases ethanol intake in Drosophila. Science 335, 1351-1355.

Srivastava, D.P., Yu, E.J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T., and Evans, P.D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel Drosophila G-protein-coupled receptor. J Neurosci 25, 6145-6155.

Tanabe, K., Itoh, M., and Tonoki, A. (2017). Age-related changes in insulin-like signaling lead to intermediate-term memory impairment in Drosophila. Cell Rep 18, 1598-1605.

Teal, P.E., Gomez-Simuta, Y., and Proveaux, A.T. (2000). Mating experience and juvenile hormone enhance sexual signaling and mating in male Caribbean fruit flies. Proc Natl Acad Sci U S A 97, 3708-3712.

Terhzaz, S., Rosay, P., Goodwin, S.F., and Veenstra, J.A. (2007). The neuropeptide SIFamide modulates sexual behavior in Drosophila. Biochem Biophys Res Commun 352, 305-310.

Tinbergen, N. (1951). The Study of Instinct. New York: Oxford Univ. Press.

Wijesekera, T.P., Saurabh, S., and Dauwalder, B. (2016). Juvenile hormone is required in adult males for Drosophila courtship. PLoS One 11, e0151912.

Wilson, T.G., DeMoor, S., and Lei, J. (2003). Juvenile hormone involvement in Drosophila melanogaster male reproduction as suggested by the Methoprene-tolerant(27) mutant phenotype. Insect Biochem Mol Biol 33, 1167-1175.

Yamamoto, K., Chadarevian, A., and Pellegrini, M. (1988). Juvenile hormone action mediated in male accessory glands of Drosophila by calcium and kinase C. Science (Washington D C) 239, 916-919.

Yamanaka, N., Yamamoto, S., Zitnan, D., Watanabe, K., Kawada, T., Satake, H., Kaneko, Y., Hiruma, K., Tanaka, Y., Shinoda, T., et al. (2008). Neuropeptide receptor transcriptome reveals unidentified neuroendocrine pathways. PLoS One 3, e3048.

Yang, C.H., Rumpf, S., Xiang, Y., Gordon, M.D., Song, W., Jan, L.Y., and Jan, Y.N. (2009). Control of the postmating behavioral switch in Drosophila females by internal sensory neurons. Neuron 61, 519-526.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell 71, 63-72.

Yapici, N., Kim, Y.J., Ribeiro, C., and Dickson, B.J. (2008). A receptor that mediates the post-mating switch in Drosophila reproductive behaviour. Nature 451, 33-37.

Yoshihara, M., Ensminger, A.W., and Littleton, J.T. (2001). Neurobiology and the Drosophila genome. Funct Integr Genomics 1, 235-240.

Zitnan, D., and Adams, M.E. (2005). Neuroendocrine regulation of insect ecdysis. In Comprehensive molecular insect science Volume three Endocrinology (Amsterdam, Boston etc.: Elsevier), pp. 1-60.

Zitnan, D., Kingan, T.G., Hermesman, J.L., and Adams, M.E. (1996). Identification of ecdysis-triggering hormone from an epitracheal endocrine system. Science 271, 88-91.

**Table I.1. Previous findings for roles of *Drosophila* neuropeptides in regulation of adult behaviors.**

| Phenotype | Neuropeptides* | References |
|---|---|---|
| Locomotion | AKH | (Isabel et al., 2005) |
| | ILP | (Belgacem and Martin, 2006) |
| | sNPF | (Kahsai et al., 2010) |
| | TK | (Kahsai et al., 2010) |
| Feeding | AstA | (Hentze et al., 2015) |
| | AKH | (Galikova et al., 2017) |
| | CCHamide-2 | (Ren et al., 2015) |
| | Hugin | (Bader et al., 2007; Melcher and Pankratz, 2005) |
| | NPF | (Shohat-Ophir et al., 2012) |
| | sNPF | (Ko et al., 2015) |
| | SP | (Carvalho et al., 2006) |
| | TK | (Ko et al., 2015) |
| Circadian rhythm (sleep) | ILP | (Monyak et al., 2016) |
| | IPNamide | (Shafer et al., 2006) |
| | ITP | (Johard et al., 2009) |
| | MIP | (Oh et al., 2014) |
| | NPF | (Lee et al., 2006) |
| | PDF | (Hyun et al., 2005; Parisky et al., 2008; Renn et al., 1999) |
| | sNPF | (Johard et al., 2009) |
| | SP | (Isaac et al., 2010) |
| Sexual behavior | AKH | (Lebreton et al., 2016) |
| | NPF | (Lee et al., 2006) |
| | SIFamide | (Terhzaz et al., 2007) |
| | SP | (Aigaki et al., 1991; Peng et al., 2005; Yang et al., 2009; Yapici et al., 2008) |
| Aggression | ILP | (Luo et al., 2014) |
| | NPF | (Dierick and Greenspan, 2007) |
| | TK | (Asahina et al., 2014) |
| Learning and memory | ILP | (Chambers et al., 2015; Monyak et al., 2016; Tanabe et al., 2017) |
| | NPF | (Krashes et al., 2009) |
| | sNPF | (Johard et al., 2008) |

***Abbreviations:**

AKH, adipokinetic hormone; AstA, allatostatin A; ILP, insulin-like peptide; ITP, ion transport peptide; MIP, myoinhibitory peptide; NPF, neuropeptide F; PDF, pigment dispersing factor; sNPF, short neuropeptide F; SP, sex peptide; TK, tachykinin.

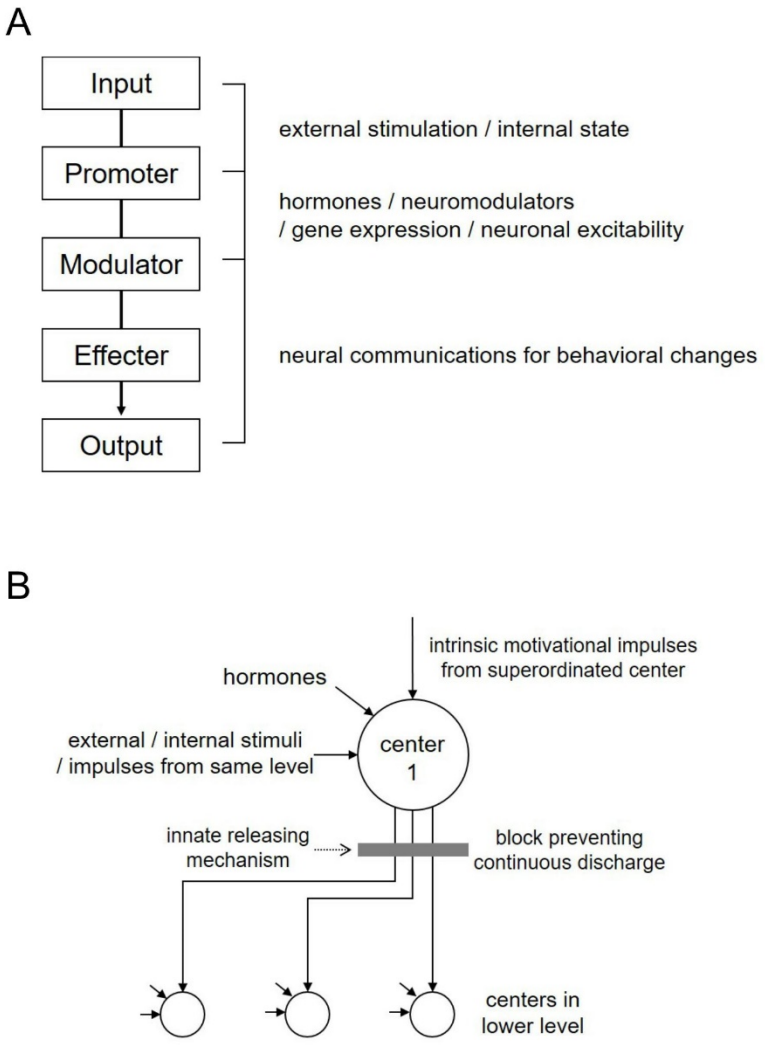



**Figure I.1. Hypothetical endocrine functions in regulation of animal behaviors.** (A) A model for sequential neural elements in regulation of animal behaviors. Hormones may play roles in promotion and/or modulation of neural functions through altering neuronal excitability or gene expression. (B) A model for endocrine actions at a single neural level to modulate hierarchical communication between layers. This model is adapted from Tinbergen (1951).

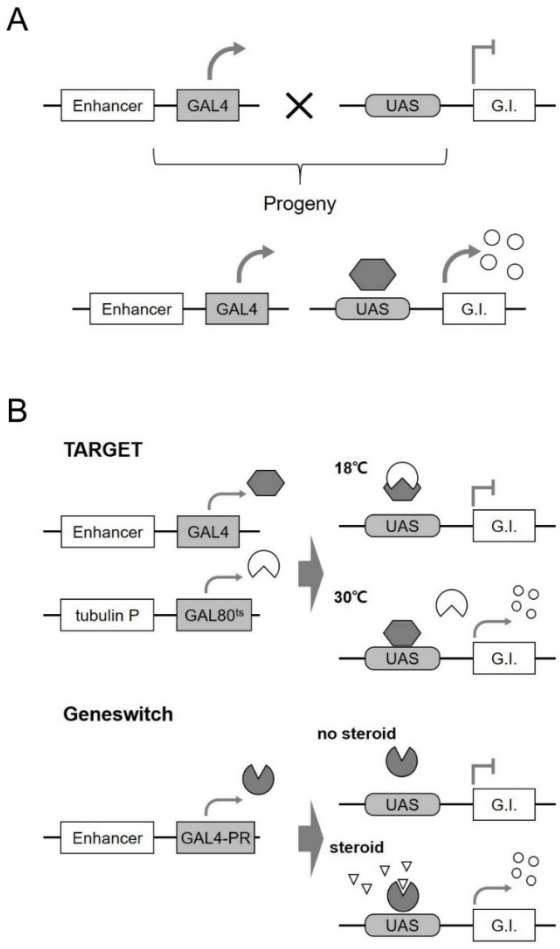



**Figure I.2. The GAL4-UAS, TARGET, and GeneSwitch systems in *Drosophila melanogaster*.** (A) The conventional GAL4-UAS system. The yeast transcriptional activator (GAL4) is driven in a specific spatial pattern by a defined promoter. The GAL4 protein binds to its cognate UAS binding site and constitutively activates transcription of the gene of interest (G.I.) downstream of UAS. (B) The TARGET system. In this system, the conventional GAL4-UAS system is conditionally regulated by the temperature-sensitive GAL4 inhibitor, $GAL80^{ts}$. At 18°C, transcription of G.I. is repressed, whereas this repression is relieved by a temperature shift to 30°C. (C) The ligand-inducible Geneswitch system. The DNA binding domain of the GAL4 protein is fused to the activation domain and a mutant progesterone-binding domain to generate the ligand-inducible chimeric activator. Only in the presence of steroid hormone will the Geneswitch molecule undergo a conformational change to an active form where it can bind to UAS and promote transcription of G.I.

## CHAPTER II

## A Hormonal Cascade during an Early-adult Critical Period Required for Courtship Memory Retention in *Drosophila*


### Abstract

Formation and expression of memories are critical for context-dependent decision-making. In *Drosophila*, a courting male rejected by a mated female subsequently courts less avidly when paired with a virgin female, a behavioral modification attributed to "courtship memory". Here we show the critical role of hormonal state for the maintenance of courtship memory. Ecdysis triggering hormone (ETH) is essential for courtship memory through regulation of juvenile hormone (JH) levels in adult males. Reduction of JH levels via silencing of ETH signaling genes impairs short-term courtship memory, a phenotype rescuable by the JH analog methoprene. JH deficit-induced memory impairment involves rapid decay rather than failure of memory acquisition. A critical period governs memory performance during the first three days of adulthood. Using sex peptide expressing "pseudo-mated" trainers, we find that robust courtship memory elicited in absence of aversive chemical mating cues also is dependent on ETH-JH signaling. Finally, we find that JH acts through dopaminergic neurons and conclude that an ETH-JH-dopamine signaling cascade is required during a critical period for promotion of social context-dependent memory.


### Introduction

The faculty to acquire and preserve information is essential for adapting to environmental changes and species propagation. Studies in both vertebrates and invertebrates have revealed the importance of hormones in learning and memory. Regarding the fruit fly *Drosophila melanogaster*, biogenic

amines contribute to diverse memory forms by influencing neuronal activity in the brain (Berry et al., 2012; Schwaerzel et al., 2003; Sitaraman et al., 2008; Wu et al., 2013). Recent studies have shown that the steroid 20-hydroxyecdysone contributes to memory formation and retention through distinct mechanisms (Ishimoto et al., 2009; Ishimoto et al., 2013). However, hormonal regulation of circuits mediating learning and memory in *Drosophila* is still poorly understood.

During juvenile development, insects perform periodic, hormonally-driven ecdysis behaviors that are obligatory for shedding of the old cuticle at the end of each molt. Ecdysis triggering hormones (ETH) released from epitracheal gland Inka cells initiate each ecdysis sequence via activation of ETH receptors (ETHRs), G protein-coupled receptors activating Gαq pathways in separate neuronal groups (Kim et al., 2015; Kim et al., 2006; Park et al., 2002b; Park et al., 2003; Park et al., 1999; Zitnan et al., 1996; Zitnan et al., 2003). Although Inka cells and transcripts of *ETH*/*ETHR*s are present in adult *Drosophila*, roles of ETH signaling in adult stage have not been described (Catalan et al., 2012; Graveley et al., 2011; Park et al., 2002a).

Release of the sesquiterpenoid JH from the corpora allata (CA) promotes juvenile body plan and its effects during the pre-adult period have been extensively studied in a broad range of insect species. In *Drosophila*, the Methoprene-tolerant (Met) gene encodes a bHLH-PAS protein, which functions as a JH receptor. Functions of Met in developmental and reproductive events are complemented by the paralog germ-cell expressed (gce) (Jindra et al., 2013). During adulthood, JH is re-purposed as a gonadotropic hormone, coordinating vitellogenesis, ovary maturation, pheromone synthesis of females, and mating behaviors of both males and females (Argue et al., 2013; Belgacem and Martin, 2002; Bilen et al., 2013; Lin et al., 2016; Postlethwait and Weiser, 1973; Ringo et al., 1991; Ringo et al., 1992; Sroka and Gilbert, 1974; Teal et al., 2000; Wijesekera et al., 2016; Wilson et al., 2003). In previous studies of the honeybee, JH determines social status and regulates olfactory memory of adult animals, possibly by affecting aminergic circuits in brain

(Harano et al., 2008; Maleszka and Helliwell, 2001; McQuillan et al., 2014; Sasaki et al., 2012). However, roles of JH in adult *Drosophila* behaviors remain largely undescribed.

Recent reports indicate that ETH functions as an allatotropin in mosquitoes (*Aedes aegypti*) and flies (*Drosophila melanogaster*) (Areiza et al., 2014; Meiselman et al., 2017). In this study, we found that the ETH-JH signaling cascade is required for memory performance of male *Drosophila* by applying a simple learning paradigm known as courtship conditioning. In this paradigm, male courtship intensity is modified by previous experience with a courtship partner (Siegel and Hall, 1979). Virgin females are highly receptive, but mated females are unreceptive because of the presence of sex peptide (SP) in the seminal fluid given by previous partner (Aigaki et al., 1991; Chapman et al., 2003). We provide evidence that ETH regulates courtship memory maintenance of male *Drosophila* through promotion of JH synthesis and activation of dopaminergic neurons. Together, our study thus reveals a hormonal cascade consisting of ETH-JH-dopamine to regulate a critical period for learning and memory during adulthood.

## Materials and Methods

**Fly Strains.** Flies were raised on standard-cornmeal-agar medium at room temperature. Crosses were maintained at room temperature on a 12-12 h light/dark cycle. Wild- type flies were Canton-S. To reduce variation arising from genetic background, we backcrossed all flies for at least five generations to the wCS strain. Many fly lines used in this study were kindly provided by colleagues and institutions as follows: *JHAMT-GAL4* and *UAS-Dicer;UAS-JHAMT RNAi* flies, Brigitte Dauwalder (University of Houston) (Wijesekera et al., 2016); *ETH-GAL4* flies, David Anderson (California Institute of Technology); *UAS-Met RNAi* and *UAS-gce RNAi* lines, Lynn Riddiford (Janelia Research Campus); *sex peptide* null mutant ($SP_0$) and *UAS-SP* flies, Barry Dickson (Janelia

Research Campus); *UAS-ETHR RNAi-Sym* and *UAS-ETHR RNAi-IR2* were described previously (Kim et al., 2015); *UAS-rpr,hid* flies, Paul Taghert (Washington University); *OK107-GAL4*, *TH-GAL4*, *Tdc2-GAL4*, *Trh-GAL4*, *OK371-GAL4*, *Orco-GAL4*, *UAS-GCaMP5*, *UAS-RedStinger*, *UAS-Shi$^{ts1}$*, *TubPGAL80$^{ts}$*, tetanus toxin light chain lines (*UAS-TNT$_G$* and *UAS-TNT$_{imp}$*), Bloomington Stock Center. The ETH GeneSwitch line (*EUG8*) was described previously (Cho et al., 2014).

**Quantitative RT-PCR Analysis.** CA were extirpated from 30 flies of each genotype on day 4 posteclosion. All dissections were performed under fluorescent optics; all flies expressed GFP in the CA, which guided clean removal of the CA. Total RNA was isolated from 30 CA of each genotype with Trizol (Ambion) and purified upon RNeasy columns (QIAGEN). cDNA was synthesized using the ProtoScript II First Strand cDNA Synthesis kit (New England Biolabs). Since total RNA yields were low, cDNA was pre-amplified using the SsoAdvanced PreAmp Supermix Kit (Bio-Rad) for unbiased, target-specific pre-amplification of cDNA. Real Time quantitative PCR (qPCR) was performed using the iQ SYBR Green Supermix qPCR kit (Bio-Rad), and Bio-Rad CFX96 Real Time PCR Detection System. Primers were directed to a common region of *ETHR-A* and *ETHR-B* and transcript levels were normalized to actin contained in the same samples. Primers used were as follows:

*ETHR* (sense), 5′-TCCATCGTATATCCGCACAA-3′;

*ETHR* (antisense), 5′-GTTGCGCATATCCTTCGTCT-3′;

*Actin* (sense), 5′-GCGTCGGTCAATTCAATCTT-3′;

*Actin* (antisense), 5′-AAGCTGCAACCTCTTCGTCA-3′.

**Immunohistochemistry and *in vivo* $Ca^{2+}$ Imaging of CA.** For immunohistochemical detection of ETH in adult males, day 4 to 5 males were dissected in ice-chilled PBS. The ventral side of the thorax and abdomen was opened to remove muscle and intestines prior to fixation in 4%

paraformaldehyde overnight at 4°C. After five 10-min washes with PBST (0.5% Triton X-100 in PBS) and 1 hour blocking with 5% NGS (normal goat serum) in PBST at room temperature, samples were incubated with rabbit anti-DmETH1 (1:1,000) for 2 days at 4°C. After six 10 min washes with PBST, samples were incubated with goat anti-rabbit Alexa Fluor 488 (1:500). After five washes with PBST and one wash with PBS, samples were mounted in the mounting media (Aqua Poly/Mount, Polysciences Inc.).

For CA staining, overall CNS and gut of day 4 *JHAMT-GAL4/UAS-mCD8-GFP* males were dissected in ice-chilled PBS. Tissues were fixed in 4 % paraformaldehyde overnight at 4°C. After five 10 min washes with PBST and 1 hour blocking with 5 % NGS in PBST at room temperature, samples were incubated with rabbit anti-JHAMT (1:100) (Niwa et al., 2008) and mouse anti-GFP (1:500) for overnight at 4 °C. Then, samples were incubated with goat anti-rabbit Alexa Fluor 647, and goat anti-mouse Alexa Fluor 488 (1:500 for each). Images were captured with Zeiss LSM510 confocal microscope.

For *in vivo* $Ca^{2+}$ imaging of male CA, anesthetized 4-day old *JHAMT-GAL4/UAS-GCaMP5* or *JHAMT-GAL4/UAS-ETHR RNAi-Sym;UAS-GCaMP5* males were placed on a petri dish with glue dorsal side up following removal of wings and legs. In ice-chilled fly saline, a small part of dorsal thoracic cuticle and flight muscles covering CA were removed. $Ca^{2+}$-mediated responses were visualized with a CCD camera (TILL-Imago) mounted on an Olympus BX51W1 and captured with Live Acquisition software. Excitation (480 nm; 40/1,000 msec excitation/duration) was provided by a Polychrome V monochromator. Following 3 min of pre-application sampling, 15µl of synthetic *Drosophila* ETH1 (34.3 µM) was applied in 500 µl fly saline to achieve a 1.0 µM final.

**Analysis of JH III Levels.** Adult males (4-day posteclosion) were collected on the dry ice and kept at -80°C until extraction. JH III was labelled with a fluorescent tag DBD-COCl (4-(N,N-Dimethylaminosulfonyl)-7-(N-chloroformylmethyl-N-methylamino)-2,1,3-benzoxadiazole), and

analyzed by reverse phase High Performance Liquid Chromatography coupled to a Fluorescence Detector (HPLC-FD) using a Dionex Summit System (Dionex, CA) equipped with a 680 HPLC pump, a TCC oven, a UV detector and an fluorescence detector connected in series. Details of the procedures were described previously (Rivera-Perez et al., 2012).

**Behavioral Assays.** Experimental male pupae were individually sorted into 96-well plates, then housed for 4 days post-eclosion in individual clean glass tubes with fly food to prevent pretest social experiences. For preparation of mated female trainers, 3-4 day old Canton-S virgins were placed with Canton-S males prior to assay the following day. For preparation of immobilized tester females, 4-5 day old Canton-S virgins were anesthetized with $CO_2$ and decapitated with fine scissors immediately before experimentation. Courtship assays were performed in a 14-multi-mating chamber (10 mm diameter, 5 mm depth) (Demir and Dickson, 2005).

For the courtship conditioning, overall experimental procedures followed those described previously, with some modification (Ejima and Griffith, 2011). A single 4-day-old test male was placed in a chamber with a mated female for one hour (training). After a 10-minute post-training isolation period, courtship behavior of the trained male toward a tester (decapitated virgin) female was recorded with a digital camcorder (Sony HDR-XR260). A sham-trained male was kept alone in the courtship chamber for one hour and paired with an immobilized tester female in another chamber for 10 minutes. Training, sham-training, and test sessions were performed under the same conditions. Courtship chambers were washed with 70% ethanol at least 10 min before the experiment to prevent carryover influences from odor artifacts.

Pseudomated females ($\Psi_m$) were *elav-GAL4/UAS-SP* virgins. Although these females have not mated, they reject males due to transgenic expression of sex peptide. Pseudovirgin females ($\Psi_v$) were Canton S females that had been mated with *sex peptide* null ($SP_0$) homozygous males one day

before the courtship conditioning. Although they are receptive to males, aversive pheromone signaling brought about by mating causes male avoidance (Keleman et al., 2012).

Inka cells were selectively ablated using the TARGET system. *ETH-GAL4;TubPGAL80$^{ts}$/UAS-rpr,hid* males were transferred from 19$^{o}$C to 31$^{o}$C within two hours after eclosion, and kept in 31$^{o}$C until courtship conditioning. For drug-dependent conditional gene expression, flies were raised on standard fly food to the pupal stage. Fly food containing 200 μM RU486 (mifepristone, Sigma) or 1.6 % ethanol containing fly food was poured into 96-well plates and stored in 4$^{o}$C. Individually eclosed males in plates transferred into glass tubes with RU486 or ethanol containing fly food. Courtship conditioning was performed 4 days after individual housing. Stage-specific ETHR knockdown using the TARGET system was achieved by transferring flies from 19 $^{o}$C to 31 $^{o}$C (after eclosion) or from 31 $^{o}$C to 19$^{o}$C (before eclosion). Control flies were raised at 19 $^{o}$C (negative) or 31 $^{o}$C (positive) during their entire life until immediately before the courtship assay. Detailed procedures for TARGET and GeneSwitch experiments were previously described (McGuire et al., 2004).

For rescue of JH deficiency phenotypes, (S)-methoprene was applied topically (64 pmol/fly) in acetone to the ventral side of day 0 posteclosion male abdomens following cold anesthesia with a Nanoject II (Drummond) applicator. Vehicle treatment was performed with acetone only. To investigate age-dependent function of JH in adult males, males at different ages were treated on day 0, 1, 2, 3, 4, or 10. Courtship conditioning was performed 4 days after treatment. To investigate the precise methoprene-sensitive time window, we applied methoprene at a dose of either 64 pmol or 322 pmol to *JHAMT-GAL4/UAS-ETHR RNAi-Sym* males on day 0, 1, 2, 3, 4, or 5. Courtship STM was tested 24-hour after treatment.

**Statistical analysis.** Courtship index (CI) is defined as the proportion of time devoted to courtship behavior during a 10-min assay period (e.g., total seconds devoted to courtship behavior over a

total of 600 sec). Courtship memory performance index (MPI) is expressed as ratio of the difference between CI of trained males ($CI_T$) and mean of sham-trained males ($CI_{Sm}$) to $CI_{Sm}$; MPI = ($CI_{Sm}$-$CI_T$)/$CI_{Sm}$. No memory is indicated by 0 MPI, since courtship level of the trained male is equivalent to that of the sham-trained males. Test males that copulated during the training period were excluded from the test session. At least 20 males were tested under equivalent training and test conditions. All indices were scored manually in a blind fashion by two investigators. The Mann-Whitney *U* test was used to test statistical significance between CIs of trained and those of sham-trained males. Permutation tests were used to compare MPIs, with 100,000 permutations of the raw data. Learning performance index (LPI) was determined by comparing mean CI from the first 10-min interval of the 1-h training period ($CI_{Im}$) to the CI of the last 10-min interval ($CI_F$); LPI = ($CI_{Im}$-$CI_F$)/$CI_{Im}$. The Mann-Whitney *U* test was applied to test statistical difference between initial and final CIs. Student's *t* test was used to compare courtship activities of males toward virgin females and toward $\Psi_v$ females.

## Results

**ETH is an obligatory regulator of JH levels in adult male *Drosophila.*** We performed immunohistochemical staining of Inka cell-specific *ETH-GAL4* males bearing a *UAS-RedStinger* reporter to confirm presence of ETH during adulthood. Although Inka cells vary in shape and location on the main tracheal tube, 6 to 9 pairs of cells were co-labeled with RedStinger and ETH-like immunoreactivity in all animals tested (n = 6) (Figure II.1).

To confirm presence of ETHR in male CA, we expressed double-stranded RNA constructs targeting the *ETHR* gene in the CA by using the CA-specific driver, *JHAMT-GAL4* (Figure II.2B) (Wijesekera et al., 2016). *JHAMT* gene encodes JH acid *O*-methyltransferase, which is an enzyme

catalyzing one of the final steps of JH synthesis (Figure II.2A). *JHAMT* is predominantly present in the CA (Niwa et al., 2008). Quantitative PCR measurements showed knockdown of relative transcript number by ~42% in males and ~80% in females (Figure II.3A and 3B).

Silencing of *ETHR* expression specifically in the CA (*JHAMT-GAL4/UAS-ETHR RNAi-Sym*) leads to marked reduction (>70%) in JH levels compared to genetic control males (Figure II.3C), demonstrating an essential role for ETH targeting adult CA for regulation of JH synthesis.

To investigate actions of ETH on CA of adult males, we monitored intracellular calcium levels *in vivo* by preparing a transgenic fly expressing the $Ca^{2+}$ indicator GCaMP5 in CA via the *JHAMT-GAL4* driver (Figure II-4A-a). We observed robust increases in $Ca^{2+}$-associated fluorescence in the CA following exposure to ETH. In contrast, CA of *ETHR*-silenced males exhibit sharply decreased calcium mobilization in response to ETH exposure (Figure II.4A-b and 4A-c). Analysis of $Ca^{2+}$-associated fluorescence traces provides evidence that RNAi silencing of *ETHR* in the CA not only suppresses cytoplasmic $Ca^{2+}$ accumulation, but also delays the response to ETH (Figure II.4B). Together, ETH plays an allatotropic role in adult male *Drosophila* and suppression of ETH signaling in the CA by *ETHR* silencing reduces JH level. This reduction has serious consequences for memory retention, as I demonstrate in subsequent sections of this chapter.

**ETH regulates courtship memory through downstream JH signaling.** We investigated whether reduction of JH levels by RNA knockdown of *ETHR* affects social-context-dependent learning and memory of males using the courtship conditioning paradigm. In this paradigm, males experiencing rejection by unreceptive, mated females reduce courtship activity when paired subsequently with virgin females. We found that courtship index (CI) of *ETHR*-silenced males was not significantly suppressed after the training, while both genetic controls showed significant suppression toward the tester females (Figure II.5A). Memory performance is expressed as memory performance index (MPI) (See the Material and Methods section). Impaired courtship memory performance was

observed using RNAi lines directed at independent sequences in the *ETHR* gene: *UAS-ETHR RNAi-Sym* and *UAS-ETHR RNAi-IR2* (Figure II.5B). These results indicate that ETH signaling in the CA is necessary for short-term courtship memory performance (statistical analyses of each genotype are listed in Table II.1).

We next tested the hypothesis that reduction of JH levels during adulthood affects memory performance by employing rescue experiments with the JH analog methoprene. Methoprene application immediately after eclosion (day 0) rescued memory deficiency of *JHAMT-GAL4/UAS-ETHR RNAi-Sym* males, whereas vehicle-treated *ETHR*-silenced and methoprene-treated genetic control males showed no significant changes in memory performance (Figure II.5C). This provides direct evidence that JH deficiency is of crucial importance for normal memory performance.

Since memory indices presented here (CI, MPI) are based on male locomotory activity directed toward females, changes in basal locomotory activity may influence apparent courtship activity. Influences of JH on behavioral basal activity and courtship activity have been reported, in particular hyperactivity resulting from JH deficiency (Argue et al., 2013; Lin et al., 2016; Liu et al., 2008; Ringo et al., 1992; Wilson et al., 2003). Whether JH levels influence basal activity of males or not, it is notable that the hyperactivity in male locomotion is likely not correlated with courtship avidity (Argue et al., 2013). To clarify, we first tested male locomotion using a negative geotaxis (climbing) assay and found that mean velocity of JH-deficient males was statistically similar to that of genetic controls. We also found no statistical differences from controls in successful copulation rates and courtship behavior (courtship singing) toward mature virgin females. Since we used immobilized virgin females as testers in conditioning trials, courtship indices were analyzed toward a decapitated virgin female. As expected, CI measures of JH-reduced males were not significantly different from control males (Table II.2).

**JH is essential for courtship memory retention.** Decreased memory performance (MPI) may be caused either by loss of learning ability during the training session or by defective retention of memory during the post-training assay period. To assay for learning during the training session, we measured learning performance index (LPI). Upon experiencing continuous rejection during a 1-hour training period with a mated female, both genetic control and *ETHR*-knockdown males exhibit decreased courtship index (CI) during the final 10 min of training as compared to the initial 10 min interval (Figure II.6A). Reduced CI during the training period is considered as memory acquisition or learning ability (Kane et al., 1997). This result thus indicates that marked reduction of MPI in JH-deficient males during the subsequent test period is not attributable to loss of learning ability.

To test whether reduction of JH levels by *ETHR* silencing negatively affects memory retention, we performed a memory decay assay. Following 1-hour training with mated females, males were tested with immobilized, decapitated virgin females at sequential intervals over a total of 10 min. Whereas control males showed no significant loss of memory during this post-training period, *JHAMT-GAL4/UAS-ETHR RNAi-Sym* males exhibited a gradual decline in memory performance over the 10-min interval (Figure II.6B). These data demonstrate that JH deficiency leads to loss of memory retention, even during the short-term post-training interval.

**Although JH-deficient males exhibit olfactory deficits, courtship memory occurs in absence of aversive, mating-associated chemical cues.** Males rejected by mated females are exposed to aversive chemical cues (e.g., cVA) considered to be primary factors contributing to reduction of subsequent courtship intensity, defined as courtship memory (Ejima et al., 2007; Zhou et al., 2012). However, behavioral rejection cues also may contribute to courtship memory. We therefore asked: 1) whether ETH-JH deficiency leads to loss of chemosensory sensitivity to post-mating chemical cues, and 2) whether courtship memory following training with mated females can be demonstrated in the absence of aversive pheromonal cues from the mated trainer female. To address these

questions, we dissociated influences of aversive chemical cues from behavioral rejection cues by pairing males with either pseudovirgin ($\Psi_v$) or pseudomated ($\Psi_m$) females (Keleman et al., 2012).

We prepared $\Psi_v$ females by crossing Canton-S virgin females with sex peptide-null mutant males (*SP0*). Even after mating, $\Psi_v$ females are still receptive to courting males because they did not receive the gift of sex peptide from the prior partner, but nevertheless smell "bad", having been "perfumed" with the aversive male pheromone, cVA (Liu and Kubli, 2003). As expected, GAL4 control males showed reduction in accumulated copulation rates when paired with $\Psi_v$ females compared to pairings with virgin females (Figure II.7A-a). Notably, JH deficient males showed relatively less suppression of copulation rate when paired with $\Psi_v$ females under the same conditions. Likewise, although control males displayed lower courtship indices (CI) toward $\Psi_v$ females, JH-deficient males showed no significant suppression of courting activity toward $\Psi_v$ females compared to virgin females (Figure II.7A-b). These data indicate that JH deficiency indeed reduces sensitivity to aversive chemical cues associated with a mated female, which could account for some measure of elevated CI - defined as loss of courtship memory - in courtship-conditioned, JH-deficient males.

To determine relative importance of behavioral cues vs. aversive chemical cues during training with mated females, we tested memory performance of control males using pseudomated ($\Psi_m$) female (*elav-GAL4/UAS-SP*) trainers, which are virgins that express sex peptide. Since virgin females expressing sex peptide are refractory to male advances without prior mating, we could assay for behavioral cues in the absence of aversive post-mating pheromonal cues. When trained with $\Psi_m$ females, control males exhibited high MPI for suppression of subsequent courtship activities that were indistinguishable to those shown when they were trained with authentic mated females ($F_m$), indicating that rejection in the absence of aversive chemical cues is sufficient for induction of short-term courtship memory (Figure II.7B). We found that MPI exhibited by *ETHR*-

silenced, JH-deficient males was equally low, whether they were trained by mated or $\Psi_m$ females. These data suggest that rejection in the absence of aversive chemical cues (i.e., solely on the basis of behavioral cues) is sufficient to elicit optimum MPI levels in controls and that JH deficiency markedly reduces sexual-deprivation-dependent memory in spite of olfactory deficiencies during training.

**Influences of ETH-JH signaling on memory are specified during the adult period.** Rescue of memory deficits by methoprene (Figure II.5C) suggests essential roles for JH in memory performance during adulthood. Our findings suggest further that ETH plays a critical role in regulating memory performance through its maintenance of JH levels. We were concerned whether this regulation was a residual effect of ETH released at eclosion or whether continued release from Inka cells persists in mature adults.

We therefore investigated the timing of ETH release during adulthood using several genetic approaches. First, Inka cells were ablated by applying the TARGET (temporal and regional gene expression targeting) system (McGuire et al., 2004). Temporal expression of pro-apoptotic genes *reaper* (*rpr*) and *head involution defective* (*hid*) (Grether et al., 1995; White et al., 1994) targeting Inka cells for cell killing resulted in significant memory performance deficit (Figure 2-8A). This was confirmed by applying the ligand-inducible GAL4-based GeneSwitch/UAS system using an Inka cell-specific GeneSwitch line (*ETH-GS, EUG8*) (Cho et al., 2014). As in the TARGET experiment, conditional Inka cell-ablation significantly impaired memory performance. We next performed conditional block of ETH release by expressing tetanus toxin light chain (TeTxLC) via the same GeneSwitch driver (*EUG8*). TeTxLC catalytically inhibits vesicle release once present in the cytosol by cleaving synaptobrevin (Sweeney et al., 1995). Adult-specific expression of active TeTxLC in Inka cells (*UAS-TNT$_G$*) significantly impaired memory performance compared to vehicle-fed males, whereas the inactive, "impaired" TeTxLC expressing males (*UAS-TNT$_{imp}$*)

showed no significant change (Figure II.8A). These data confirm that ETH release from Inka cells, as well as *ETHR* expression in the CA, are essential for normal memory performance through regulation of downstream JH signaling.

We next investigated whether ETH signaling during development is required for proper "wiring" of the CNS through stage-specific *ETHR* silencing in the CA using the TARGET system. *ETHR* silencing in the CA during the pre-adult period led to no deficits in normal memory performance, while post-eclosion (adult period only) *ETHR*-silenced males and positive controls showed significantly impaired memory performance (Figure II.8B). These observations, along with our previous methoprene rescue data, show that ETH signaling-dependent JH levels during adulthood are essential for normal memory performance, and that ETH-induced developmental events do not contribute to the memory deficit phenotypes I describe here.

**ETH-JH signaling is functional during an early adult critical period for memory performance.** During adulthood, JH may play distinctive functional roles in the CNS during different age periods (Argue et al., 2013; Harano et al., 2008; Sasaki et al., 2012). Since age-dependent JH levels are different in males (Figure II.9) and females (Gruntenko et al., 2012), we hypothesized that a critical period for JH action may influence memory performance. We therefore tested age-dependent efficacy of methoprene rescue of memory deficits in JH-deficient males. Interestingly, impaired memory performance of JH deficient males (*JHAMT-GAL4/UAS-ETHR RNAi-Sym*) could be rescued by methoprene only during early adulthood. In the first round of experiments, methoprene was applied topically on the following days posteclosion to separate groups of males: day 0, 1, 2, 3, 4, 10 (courtship conditioning and memory assay were performed 4 days after application in each instance). Methoprene treatment on posteclosion males days 0 and 1 rescued memory performance, while treatment on day 2 showed some degree of MPI improvement that did not reach statistical significance (Figure II.10A and 10B). Later methoprene treatments on days 3, 4, and 10 were

clearly ineffective. Day 2-6 methoprene-treated males show significant courtship suppression (Table II.1), but no significant difference in MPI compared to vehicle-treated animals. Memory performance of GAL4 control males was not affected by methoprene.

GAL4 control males also exhibited gradual loss of memory performance with age; older (day 10) males have low levels of JH (Figure II.9), however age-dependent memory loss is likely JH-independent, since methoprene treatment is ineffective in restoring memory performance after day 6 (Figure II.10B).

To define more precisely the critical period for methoprene-dependent memory recovery, we applied methoprene to progressively older posteclosion *JHAMT-GAL4/UAS-ETHR RNAi-Sym* males and assayed for memory performance 24 hours later. We treated groups of individuals on posteclosion days 0, 1, 2, 3, 4, and 5 with either 64 pmol (1x) or 322 pmol (5x). While the lower dose of methoprene was ineffective, the higher dose clearly rescued memory performance of males treated on posteclosion days 0, 1, and 2 (Figure II.10C). Although the courtship indices of day 0 and 1 *JHAMT-GAL4/UAS-ETHR RNAi-Sym* males are lower than GAL4 control groups, higher dose methoprene treatment likely increases courtship activity of ETHR-silenced males, indicating that JH may also affect sexual maturation in early period (Table II.3). Taken together, our evidence demonstrates that promotion of courtship memory performance by JH is confined to a critical period during the first three days of adulthood.

**JH regulates memory performance by targeting TH-positive neurons.** Although *Drosophila* expresses two JH receptor paralogs (*Met* and *gce*) in the brain (Baumann et al., 2010; Chintapalli et al., 2007; Graveley et al., 2011), their functions in adult behavior remain unclear. Since JH likely plays a role in formation and/or function of the memory circuit, we employed RNA knockdown of both receptors in candidate brain regions thought to be important in memory and behavior. It is well established that mushroom body (MB) neurons are involved in both short-term and long-term

memories (Davis, 1996; McBride et al., 1999), in part through monoamine-based signaling. Glutamate is a key neurotransmitter contributing to cognitive ability and learning and memory in a variety of species. A recent study revealed that subsets of glutamatergic neurons innervating MB neurons operate in the memory retrieval (recall) following short-term conditioning (Bouzaiane et al., 2015). Orco (Or83b), a co-receptor expressed in broad range of olfactory receptor neurons (ORNs), is essential for ORN functions contributing to associative learning and memory (Larsson et al., 2004).

We assessed memory performance following RNAi knockdown of both *Met* and *gce* in a number of different neuronal types, including mushroom body (MB; *OK107-GAL4*), dopaminergic (DA; *TH-GAL4*), octopaminergic (OA; *Tdc2-GAL4*), serotonergic (5-HT; *Trh-GAL4*), glutamatergic (Glut; *OK371-GAL4*), and olfactory receptor (*Orco-GAL4*) neurons. Silencing of *Met*/*gce* in DA neurons significantly impairs memory performance without affecting basal courtship intensity, whereas *Met*/*gce* knockdown in OA, 5-HT, Glut, and olfactory receptor neurons did not cause memory deficiency (Figure II.11A). Notably, although *Met*/*gce* silencing in ORNs (*Orco-GAL4/UAS-gce RNAi;UAS-Met RNAi*) led to significant reduction in courtship of naïve males toward immobilized virgin females (tester) (Figure II.12), they showed normal memory performance following training with a mated female. This is consistent with our prior result (Figure II.7B), showing that behavioral cues associated with rejection (sexual deprivation) are equally or more important than exposure to chemical cues for the memory performance. RNA knockdown of either *Met* or *gce* alone using the *TH-GAL4* driver did not impair memory performance, indicating that the two receptor subtypes are effective in compensating for memory deficits (Figure II.11B). Taken together, our results identify DA neurons as targets for ETH-JH signaling in establishment of short-term courtship memories.

## Discussion

Key findings reported in this study are that ETH signaling is required for maintenance of normal JH levels in adult *Drosophila* males and that JH deficiency brought about by interruption of ETH signaling leads to rapid memory loss. These basic findings, along with important mechanistic details, can be summarized as follows. First, CA cells respond to ETH by mobilizing calcium, while genetic suppression of *ETHR* expression specifically in the CA reduces calcium mobilization leading to a ~70% drop of JH levels during the first week of adult life. Second, JH deficiency produced by interruption of ETH signaling results in impairment of short-term memory (STM) under the courtship conditioning paradigm; this phenotype is rescuable by treatment with methoprene. Third, JH-dependent memory performance relates to memory retention as opposed to acquisition. Fourth, optimal memory performance of trained males toward subsequent encounters with virgin females is induced by behavioral cues provided by mated (pseudomated) trainer females in absence of aversive post-mating chemical cues; ETH-JH deficiency leads to rapid extinction of this memory. Fifth, JH dependence of memory performance occurs during a critical period - the first 2-3 days of adult life. Finally, cellular targets of JH that mediate STM are TH-positive dopaminergic neurons. I propose a model summarizing these findings (Figure II.14). Our results are further discussed below in the context of previous accounts of JH influences on adult behaviors.

**ETH functions as an obligatory allatotropin for courtship memory in adult *Drosophila.*** Neuropeptide allatotropins (AT) known to stimulate JH production in a wide range of insects (Stay, 2000), but these peptides have not been found in *Drosophila*, although some allatotropic actions of neurotransmitters have been reported, including glutamate (Chiang et al., 2002; Gruntenko et al., 2012). We recently reported on peptidergic regulation of JH synthesis in *Drosophila* (Meiselman et al., 2017). In both sexes, ETH-JH signaling is essential for attainment of normal reproductive

potential, including vitellogenesis and egg production in females. However, the functional significance of ETH as an allatotropin with respect to cognitive behavior is not known. Here I provide evidence that ETH signaling is critical for courtship memory performance. Calcium is critical for JH biosynthesis, since CA cells cannot produce JH in calcium-free medium *in vitro* (Richard et al., 1990). A well-known consequence of Gαq-coupled signal transduction is liberation of $IP_3$ (inositol 1,4,5-triphosphate)-dependent intracellular calcium release from stores. We found that ETH mobilizes calcium in CA cells, but much less so following RNAi knockdown of *ETHR* in the CA. These results provide clear evidence that ETH functions as an obligatory allatotropin crucial for STM in adult male *Drosophila*.

**Influences of JH on *Drosophila* courtship behavior and memory performance.** *Drosophila* courtship involves a sophisticated behavioral sequence involving neural circuitry integrating multiple sensory inputs for decision-making (Greenspan and Ferveur, 2000). Roles of JH in male courtship behavior are diverse, depending on the insect species. For example, it is well known that JH influences social interactions through pheromone recognition. In the locust Schistocerca gregaria and the moth *Agrotis ipsilon*, JH plays a critical role in setting male sensitivity to pheromones, which promotes context-specific behavioral responses toward both genders (Anton and Gadenne, 1999; Gadenne and Anton, 2000; Ignell et al., 2001; Jarriault et al., 2009). In male *Drosophila*, JH esterase-binding protein overexpression, which enhances JH esterase function and hence JH degradation, is reported to reduce pheromone production, thus enhancing homosexual tendencies (Liu et al., 2008). A recent study showed the importance of the *JHAMT* gene in male courtship, and that reduced courtship index observed in *JHAMT*-silenced males is likely caused by reduction of JH biosynthesis (Wijesekera et al., 2016). Another study provides a clue for the neural mechanism underlying JH promotion of male courtship. Expression of Methoprene-tolerant (Met) in Or47b neurons enhances male sensitivity to female cuticular hydrocarbons, thereby facilitating

successful courtship (Lin et al., 2016). This account provides compelling evidence supporting a role for JH in regulation of pheromone sensing by male *Drosophila*.

In the present study, we also found that JH may influence pheromone recognition of males. Elevated courtship activity and successful copulation rates of JH-deficient males paired with receptive, pseudo-virgin females ($\Psi_v$ in Figure II.7A) suggest two possible explanations: hypersensitivity to aphrodisiac pheromones (e.g. 9-pentacosene (Siwicki et al., 2005) and palmitoleic acid (Lin et al., 2016)) or insensitivity to anti-aphrodisiac (e.g. cVA) pheromones. Since Met-expressing Or47b neurons promote courtship (Lin et al., 2016), I hypothesize that JH may be also important in recognition of anti-aphrodisiac pheromones (e.g., cVA). This hypothesis is supported by two lines of evidence produced in this study. First, JH-deficient males show no significant increase in courtship toward virgin females (Table II.2), indicating that JH-reduction does not promote hypersensitivity to female pheromones. Second, we found that RNAi knockdown of JH receptors broadly in ORNs suppressed courtship significantly, likely caused by a poor detection/recognition of target females (Figure II.12). Variability in courtship activity of naïve JH receptor-silenced males (*Orco-GAL4*/*UAS-gce RNAi;UAS-Met RNAi*) line could be caused by low expression of Orco (Or83b) in Or47b neurons (Benton et al., 2006).

Our finding that JH deficient, ETHR-silenced males exhibit no change in courtship index differs from results recently reported by Wijesekera et al., who showed that silencing of the *JHAMT* gene in CA suppresses male courtship activity significantly (Wijesekera et al., 2016). Although JH levels were not assessed in this study, it was presumed that JH deficiency resulted from *JHAMT* knockdown, since the phenotype was rescued with methoprene. The apparent discrepancy between the two studies likely arises from differences in courtship assay protocols used. Wijesekera et al. paired males with immature, pheromone deficient females (day 0 posteclosion), whereas we used mature, day 4 decapitated virgin female testers in this study. To clarify the apparent discrepancy

between our study and that of Wijesekera et al., we compared courtship indices of JH-deficient males produced by CA-specific silencing of *JHAMT* paired either with immature females (day 0 posteclosion) or mature 4-day posteclosion females (Figure II.13). As reported by Wijesekera et al., *JHAMT*-silenced males showed significant reduction of courtship activity toward immature females; this reduction is attributable in part to increased latency to courtship initiation (orientation followed by one-wing extension) compared to genetic controls (Figure II.13A). However, when paired with decapitated day-4 females, *JHAMT*-silenced males showed normal courtship indices and courtship latency (Figure II.13B). Silencing of *JHAMT* also caused significant courtship reduction and courtship delay of males when paired with immobilized immature females (Figure II.13C). Indeed, when paired with decapitated immature females, *JHAMT*-silenced males exhibited even more pronounced courtship latency compared to intact, mobile immature females (compare Figure II.13A with II.13C). Increased latency may be attributable to loss of visual inputs provided by mobile, behaving females that are detected by JH-deficient males. I therefore hypothesize that, although JH deficiency in males likely causes reduced sensitivity to aphrodisiac pheromones, normal levels of these pheromones in mature females are sufficient for promotion of normal courtship activity of males. A previous study revealed that pheromone synthesis during female maturation strongly influences courtship latency (Bilen et al., 2013). Although results of this study and those of Lin et al. (Lin et al., 2016) showed that JH receptor expression in olfactory neurons influences detection of mature female pheromones, our findings suggest that JH deficiency caused by silencing of *ETHR* or *JHAMT* in the CA may reduce, but not abolish pheromone sensitivity.

Although JH deficiency may influence male sensitivity to pheromones and hence alter courtship drive, we find that robust courtship memory occurs in the absence of chemical cues such as cVA. In particular, we found that control males show normal MPI following training with pseudo-mated females ($\Psi_m$ in Figure II.7B). Furthermore, JH-deficient males display normal

courtship behavior and learning ability during training (Figure II.6A), but impaired memory performance following pairings with $\Psi_m$ trainer females (Figure II.7B). I therefore conclude that: 1) rejection behavior exhibited by a mated female is the dominant factor driving memory performance, and 2) JH is essential for sexual deprivation-dependent memory retention (Figure II.6B).

Previous studies also suggest that drastic reduction of JH levels increases locomotory activity, which under our paradigm could influence memory performance (Argue et al., 2013; Liu et al., 2008). However, we found that partial JH deficiency (e.g., 70% reduction) caused by *ETHR* knockdown in the CA alters neither climbing nor courtship activities of mature males (Table II.3). In contrast, lower courtship activity of immature animals (day 0-2 post-eclosion), which have minimum JH levels, was partially increased by methoprene (Table II.3), suggesting that absence of JH reduces courtship activity of males. Taken together, it seems possible that the degree of JH deficiency is of crucial importance in determining phenotypic outcomes related to locomotion, courtship, and memory maintenance.

**JH influences dopaminergic neurons and courtship memory during a critical period.** We found that the obligatory role of ETH-JH signaling in courtship memory is limited to early adulthood. In particular, methoprene rescue of courtship memory in JH-deficient males was successful only in day 0-3 post-eclosion males. This critical period for hormonal action on memory may be attributable to neurogenesis and/or completion of CNS circuit assembly in young adult males (Cayre et al., 1997). Notably, we did not observe enhancement of STM by methoprene treatment of control males, confirming that rescue did not involve enhancement of MPI over normal levels. I therefore propose that memory circuit maturation is complete under the influence of normal JH levels.

It is well known that JH promotes brain dopamine levels and learning in male honeybees (Harano et al., 2008; Sasaki et al., 2012). Interestingly, methoprene treatment of young males enhances brain DA levels, with likely consequences for sexual and behavioral maturation. The role of JH in aversive learning of young drones therefore can be understood by this hormone-amine signaling cascade (Maleszka and Helliwell, 2001; McQuillan et al., 2014). I show that JH receptor expression in TH-positive DA neurons is necessary for normal courtship memory performance. In the *Drosophila* brain, approximately 130 TH-positive DA neurons occur as clusters, including protocerebral anterior medial (PAM), protocerebral anterior lateral (PAL), protocerebral posterior medial (PPM), posterior lateral (PPL) subgroups. These neurons innervate diverse central brain regions, including distinct zones of the mushroom body neuropil, which are considered as a memory hub. The *TH-GAL4* line labels most TH-positive DA neurons, with the exception of most PAM subgroups (Mao and Davis, 2009; Waddell, 2010). Since JH-deficient males fail to retain memories, further investigation is required to show JH influences DA neuronal morphologies that contribute to memory maintenance. Although previous studies provided strong evidence for involvement of DA neurons in *Drosophila* behaviors, precise functional roles for dopamine circuits in memory processes is complicated. In particular, recent studies of aversive conditioning demonstrated that distinct populations of dopaminergic neurons contribute to either acquisition or extinction of information (Aso et al., 2012; Berry et al., 2012; Shuai et al., 2015). In courtship conditioning, it has been reported that dopamine plays an important role in the consolidation of short-term memory into long-term memory (Kruttner et al., 2015).

Although suppression of both JH receptors (Met, gce) in TH-positive DA neurons resulted in MPI deficiency, RNA silencing of either *Met* or *gce* alone does not produce the phenotype (Figure II.11B). Previous reports revealed that these receptor types are redundant and compensate the loss

of function in mutant lines, especially during *Drosophila* development (Abdou et al., 2011; Jindra et al., 2015).

In summary, JH signaling is conserved across a wide range of insect species. Functional parallels between JH and the mammalian thyroid hormone signaling have been proposed (Flatt et al., 2006). Beyond metamorphosis and reproductive processes, recent studies suggest involvement of thyroid hormone signaling in cognitive functions, especially learning and memory during a critical period (Willoughby et al., 2013; Yamaguchi et al., 2012). I propose here yet another potential conservation of hormonal function between JH and thyroid hormone signaling: that of social context-dependent neural and behavioral plasticity. Since thyroid hormone also influences persistent memories, further investigations on ETH-JH regulation of long-term memory are underway.

## References


Abdou, M.A., He, Q., Wen, D., Zyaan, O., Wang, J., Xu, J., Baumann, A.A., Joseph, J., Wilson, T.G., Li, S., et al. (2011). Drosophila Met and Gce are partially redundant in transducing juvenile hormone action. Insect Biochem Mol Biol 41, 938-945.

Aigaki, T., Fleischmann, I., Chen, P.S., and Kubli, E. (1991). Ectopic expression of sex peptide alters reproductive behavior of female D. melanogaster. Neuron 7, 557-563.

Anton, S., and Gadenne, C. (1999). Effect of juvenile hormone on the central nervous processing of sex pheromone in an insect. Proc Natl Acad Sci U S A 96, 5764-5767.

Areiza, M., Nouzova, M., Rivera-Perez, C., and Noriega, F.G. (2014). Ecdysis triggering hormone ensures proper timing of juvenile hormone biosynthesis in pharate adult mosquitoes. Insect Biochem Mol Biol 54, 98-105.

Argue, K.J., Yun, A.J., and Neckameyer, W.S. (2013). Early manipulation of juvenile hormone has sexually dimorphic effects on mature adult behavior in Drosophila melanogaster. Horm Behav 64, 589-597.

Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A.B., Ito, K., Scholz, H., and Tanimoto, H. (2012). Three dopamine pathways induce aversive odor memories with different stability. PLoS Genet 8, e1002768.

Baumann, A., Fujiwara, Y., and Wilson, T.G. (2010). Evolutionary divergence of the paralogs Methoprene tolerant (Met) and germ cell expressed (gce) within the genus Drosophila. J Insect Physiol 56, 1445-1455.

Belgacem, Y.H., and Martin, J.R. (2002). Neuroendocrine control of a sexually dimorphic behavior by a few neurons of the pars intercerebralis in Drosophila. Proc Natl Acad Sci U S A 99, 15154-15158.

Benton, R., Sachse, S., Michnick, S.W., and Vosshall, L.B. (2006). Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. PLoS Biol 4, e20.

Berry, J.A., Cervantes-Sandoval, I., Nicholas, E.P., and Davis, R.L. (2012). Dopamine is required for learning and forgetting in Drosophila. Neuron 74, 530-542.

Bilen, J., Atallah, J., Azanchi, R., Levine, J.D., and Riddiford, L.M. (2013). Regulation of onset of female mating and sex pheromone production by juvenile hormone in Drosophila melanogaster. Proc Natl Acad Sci U S A 110, 18321-18326.

Bouzaiane, E., Trannoy, S., Scheunemann, L., Placais, P.Y., and Preat, T. (2015). Two independent mushroom body output circuits retrieve the six discrete components of Drosophila aversive memory. Cell Rep 11, 1280-1292.

Catalan, A., Hutter, S., and Parsch, J. (2012). Population and sex differences in Drosophila melanogaster brain gene expression. BMC Genomics 13, 654.

Cayre, M., Strambi, C., Charpin, P., Augier, R., and Strambi, A. (1997). Specific requirement of putrescine for the mitogenic action of juvenile hormone on adult insect neuroblasts. Proc Natl Acad Sci U S A 94, 8238-8242.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M.F., Smith, H.K., and Partridge, L. (2003). The sex peptide of Drosophila melanogaster: female post-mating responses analyzed by using RNA interference. Proc Natl Acad Sci U S A 100, 9923-9928.

Chiang, A.S., Lin, W.Y., Liu, H.P., Pszczolkowski, M.A., Fu, T.F., Chiu, S.L., and Holbrook, G.L. (2002). Insect NMDA receptors mediate juvenile hormone biosynthesis. Proc Natl Acad Sci U S A 99, 37-42.

Chintapalli, V.R., Wang, J., and Dow, J.A. (2007). Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet 39, 715-720.

Cho, K.H., Daubnerova, I., Park, Y., Zitnan, D., and Adams, M.E. (2014). Secretory competence in a gateway endocrine cell conferred by the nuclear receptor betaFTZ-F1 enables stage-specific ecdysone responses throughout development in Drosophila. Dev Biol 385, 253-262.

Davis, R.L. (1996). Physiology and biochemistry of Drosophila learning mutants. Physiol Rev 76, 299-317.

Demir, E., and Dickson, B.J. (2005). fruitless splicing specifies male courtship behavior in Drosophila. Cell 121, 785-794.

Ejima, A., and Griffith, L.C. (2011). Assay for courtship suppression in Drosophila. Cold Spring Harb Protoc 2011, pdb prot5575.

Ejima, A., Smith, B.P., Lucas, C., van der Goes van Naters, W., Miller, C.J., Carlson, J.R., Levine, J.D., and Griffith, L.C. (2007). Generalization of courtship learning in Drosophila is mediated by cis-vaccenyl acetate. Curr Biol 17, 599-605.

Flatt, T., Moroz, L.L., Tatar, M., and Heyland, A. (2006). Comparing thyroid and insect hormone signaling. Integr Comp Biol 46, 777-794.

Gadenne, C., and Anton, S. (2000). Central processing of sex pheromone stimuli is differentially regulated by juvenile hormone in a male moth. J Insect Physiol 46, 1195-1206.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., et al. (2011). The developmental transcriptome of Drosophila melanogaster. Nature 471, 473-479.

Greenspan, R.J., and Ferveur, J.F. (2000). Courtship in Drosophila. Annu Rev Genet 34, 205-232.

Grether, M.E., Abrams, J.M., Agapite, J., White, K., and Steller, H. (1995). The head involution defective gene of Drosophila melanogaster functions in programmed cell death. Genes Dev 9, 1694-1708.

Gruntenko, N.E., Bogomolova, E.V., Adonyeva, N.V., Karpova, E.K., Menshanov, P.N., Alekseev, A.A., Romanova, I.V., Li, S., and Rauschenbach, I.Y. (2012). Decrease in juvenile hormone level as a result of genetic ablation of the corpus allatum cells affects the synthesis and metabolism of stress related hormones in Drosophila. J Insect Physiol 58, 49-55.

Harano, K., Sasaki, K., Nagao, T., and Sasaki, M. (2008). Influence of age and juvenile hormone on brain dopamine level in male honeybee (Apis mellifera): association with reproductive maturation. J Insect Physiol 54, 848-853.

Ignell, R., Couillaud, F., and Anton, S. (2001). Juvenile-hormone-mediated plasticity of aggregation behaviour and olfactory processing in adult desert locusts. J Exp Biol 204, 249-259.

Ishimoto, H., Sakai, T., and Kitamoto, T. (2009). Ecdysone signaling regulates the formation of long-term courtship memory in adult Drosophila melanogaster. Proc Natl Acad Sci U S A 106, 6381-6386.

Ishimoto, H., Wang, Z., Rao, Y., Wu, C.F., and Kitamoto, T. (2013). A novel role for ecdysone in Drosophila conditioned behavior: linking GPCR-mediated non-canonical steroid action to cAMP signaling in the adult brain. PLoS Genet 9, e1003843.

Jarriault, D., Barrozo, R.B., de Carvalho Pinto, C.J., Greiner, B., Dufour, M.C., Masante-Roca, I., Gramsbergen, J.B., Anton, S., and Gadenne, C. (2009). Age-dependent plasticity of sex pheromone response in the moth, Agrotis ipsilon: combined effects of octopamine and juvenile hormone. Horm Behav 56, 185-191.

Jindra, M., Palli, S.R., and Riddiford, L.M. (2013). The juvenile hormone signaling pathway in insect development. Annu Rev Entomol 58, 181-204.

Jindra, M., Uhlirova, M., Charles, J.P., Smykal, V., and Hill, R.J. (2015). Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. PLoS Genet 11, e1005394.

Kane, N.S., Robichon, A., Dickinson, J.A., and Greenspan, R.J. (1997). Learning without performance in PKC-deficient Drosophila. Neuron 18, 307-314.

Keleman, K., Vrontou, E., Kruttner, S., Yu, J.Y., Kurtovic-Kozaric, A., and Dickson, B.J. (2012). Dopamine neurons modulate pheromone responses in Drosophila courtship learning. Nature 489, 145-149.

Kim, D.H., Han, M.R., Lee, G., Lee, S.S., Kim, Y.J., and Adams, M.E. (2015). Rescheduling behavioral subunits of a fixed action pattern by genetic manipulation of peptidergic signaling. PLoS Genet 11, e1005513.

Kim, Y.J., Zitnan, D., Galizia, C.G., Cho, K.H., and Adams, M.E. (2006). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr Biol 16, 1395-1407.

Krstic, D., Boll, W., and Noll, M. (2009). Sensory integration regulating male courtship behavior in Drosophila. PLoS One 4, e4457.

Kruttner, S., Traunmuller, L., Dag, U., Jandrasits, K., Stepien, B., Iyer, N., Fradkin, L.G., Noordermeer, J.N., Mensh, B.D., and Keleman, K. (2015). Synaptic Orb2A bridges memory acquisition and late memory consolidation in Drosophila. Cell Rep 11, 1953-1965.

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., and Vosshall, L.B. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron 43, 703-714.

Lin, H.H., Cao, D.S., Sethi, S., Zeng, Z., Chin, J.S., Chakraborty, T.S., Shepherd, A.K., Nguyen, C.A., Yew, J.Y., Su, C.Y., et al. (2016). Hormonal modulation of pheromone detection enhances male courtship success. Neuron 90, 1272-1285.

Liu, H., and Kubli, E. (2003). Sex-peptide is the molecular basis of the sperm effect in Drosophila melanogaster. Proc Natl Acad Sci U S A 100, 9929-9933.

Liu, Z., Li, X., Prasifka, J.R., Jurenka, R., and Bonning, B.C. (2008). Overexpression of Drosophila juvenile hormone esterase binding protein results in anti-JH effects and reduced pheromone abundance. Gen Comp Endocrinol 156, 164-172.

Maleszka, R., and Helliwell, P. (2001). Effect of juvenile hormone on short-term olfactory memory in young honeybees (Apis mellifera). Horm Behav 40, 403-408.

Mao, Z., and Davis, R.L. (2009). Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. Front Neural Circuits 3, 5.

McBride, S.M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G., and Siwicki, K.K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron 24, 967-977.

McGuire, S.E., Roman, G., and Davis, R.L. (2004). Gene expression systems in Drosophila: a synthesis of time and space. Trends Genet 20, 384-391.

McQuillan, H.J., Nakagawa, S., and Mercer, A.R. (2014). Juvenile hormone enhances aversive learning performance in 2-day old worker honey bees while reducing their attraction to queen mandibular pheromone. PLoS One 9, e112740.

Meiselman, M., Lee, S.S., Tran, R.T., Dai, H., Ding, Y., Rivera-Perez, C., Wijesekera, T.P., Dauwalder, B., Noriega, F.G., and Adams, M.E. (2017). Endocrine network essential for reproductive success in Drosophila melanogaster. Proc Natl Acad Sci U S A 114, E3849-E3858.

Niwa, R., Niimi, T., Honda, N., Yoshiyama, M., Itoyama, K., Kataoka, H., and Shinoda, T. (2008). Juvenile hormone acid O-methyltransferase in Drosophila melanogaster. Insect Biochem Mol Biol 38, 714-720.

Park, Y., Filippov, V., Gill, S.S., and Adams, M.E. (2002a). Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development 129, 493-503.

Park, Y., Kim, Y.J., and Adams, M.E. (2002b). Identification of G protein-coupled receptors for Drosophila PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. Proc Natl Acad Sci U S A 99, 11423-11428.

Park, Y., Kim, Y.J., Dupriez, V., and Adams, M.E. (2003). Two subtypes of ecdysis-triggering hormone receptor in Drosophila melanogaster. J Biol Chem 278, 17710-17715.

Park, Y., Zitnan, D., Gill, S.S., and Adams, M.E. (1999). Molecular cloning and biological activity of ecdysis-triggering hormones in Drosophila melanogaster. FEBS Lett 463, 133-138.

Postlethwait, J.H., and Weiser, K. (1973). Vitellogenesis induced by juvenile hormone in the female sterile mutant apterous-four in Drosophila melanogaster. Nat New Biol 244, 284-285.

Richard, D.S., Applebaum, S.W., and Gilbert, L.I. (1990). Allatostatic regulation of juvenile hormone production in vitro by the ring gland of Drosophila melanogaster. Mol Cell Endocrinol 68, 153-161.

Ringo, J., Werczberger, R., Altaratz, M., and Segal, D. (1991). Female sexual receptivity is defective in juvenile hormone-deficient mutants of the apterous gene of Drosophila melanogaster. Behav Genet 21, 453-469.

Ringo, J., Werczberger, R., and Segal, D. (1992). Male sexual signaling is defective in mutants of the apterous gene of Drosophila melanogaster. Behav Genet 22, 469-487.

Rivera-Perez, C., Nouzova, M., and Noriega, F.G. (2012). A quantitative assay for the juvenile hormones and their precursors using fluorescent tags. PLoS One 7, e43784.

Sasaki, K., Akasaka, S., Mezawa, R., Shimada, K., and Maekawa, K. (2012). Regulation of the brain dopaminergic system by juvenile hormone in honey bee males (Apis mellifera L.). Insect Mol Biol 21, 502-509.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci 23, 10495-10502.

Shuai, Y., Hirokawa, A., Ai, Y., Zhang, M., Li, W., and Zhong, Y. (2015). Dissecting neural pathways for forgetting in Drosophila olfactory aversive memory. Proc Natl Acad Sci U S A 112, E6663-6672.

Siegel, R.W., and Hall, J.C. (1979). Conditioned responses in courtship behavior of normal and mutant Drosophila. Proc Natl Acad Sci U S A 76, 3430-3434.

Sitaraman, D., Zars, M., Laferriere, H., Chen, Y.C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G.E., and Zars, T. (2008). Serotonin is necessary for place memory in Drosophila. Proc Natl Acad Sci U S A 105, 5579-5584.

Siwicki, K.K., Riccio, P., Ladewski, L., Marcillac, F., Dartevelle, L., Cross, S.A., and Ferveur, J.F. (2005). The role of cuticular pheromones in courtship conditioning of Drosophila males. Learn Mem 12, 636-645.

Sroka, P., and Gilbert, L.I. (1974). The timing of juvenile hormone release for ovarian maturation in Manduca sexta. J Insect Physiol 20, 1173-1180.

Stay, B. (2000). A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem Mol Biol 30, 653-662.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C.J. (1995). Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14, 341-351.

Teal, P.E., Gomez-Simuta, Y., and Proveaux, A.T. (2000). Mating experience and juvenile hormone enhance sexual signaling and mating in male Caribbean fruit flies. Proc Natl Acad Sci U S A 97, 3708-3712.

Waddell, S. (2010). Dopamine reveals neural circuit mechanisms of fly memory. Trends Neurosci 33, 457-464.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. Science 264, 677-683.

Wijesekera, T.P., Saurabh, S., and Dauwalder, B. (2016). Juvenile hormone is required in adult males for Drosophila courtship. PLoS One 11, e0151912.

Willoughby, K.A., McAndrews, M.P., and Rovet, J. (2013). Effects of early thyroid hormone deficiency on children's autobiographical memory performance. J Int Neuropsychol Soc 19, 419-429.

Wilson, T.G., DeMoor, S., and Lei, J. (2003). Juvenile hormone involvement in Drosophila melanogaster male reproduction as suggested by the Methoprene-tolerant(27) mutant phenotype. Insect Biochem Mol Biol 33, 1167-1175.

Wu, C.L., Shih, M.F., Lee, P.T., and Chiang, A.S. (2013). An octopamine-mushroom body circuit modulates the formation of anesthesia-resistant memory in Drosophila. Curr Biol 23, 2346-2354.

Yamaguchi, S., Aoki, N., Kitajima, T., Iikubo, E., Katagiri, S., Matsushima, T., and Homma, K.J. (2012). Thyroid hormone determines the start of the sensitive period of imprinting and primes later learning. Nat Commun 3, 1081.

Zhou, C., Huang, H., Kim, S.M., Lin, H., Meng, X., Han, K.A., Chiang, A.S., Wang, J.W., Jiao, R., and Rao, Y. (2012). Molecular genetic analysis of sexual rejection: roles of octopamine and its receptor OAMB in Drosophila courtship conditioning. J Neurosci 32, 14281-14287.

Zitnan, D., Kingan, T.G., Hermesman, J.L., and Adams, M.E. (1996). Identification of ecdysis-triggering hormone from an epitracheal endocrine system. Science 271, 88-91.

Zitnan, D., Zitnanova, I., Spalovska, I., Takac, P., Park, Y., and Adams, M.E. (2003). Conservation of ecdysis-triggering hormone signalling in insects. J Exp Biol 206, 1275-1289.

**Table II.1. Statistical analysis summary for courtship memory tests (Mann-Whitney *U* Test)**

(a) Figure II.5B and 5C

| Experiment | Genotype : Condition | P-value |
|---|---|---|
| ETHR silencing in the CA | *JHAMT/+* | <0.001 |
| | *+/ETHR RNAi-Sym* | <0.001 |
| | *JHAMT/ETHR RNAi-Sym* | 0.155 (ns) |
| | *+/ETHR RNAi-IR2* | <0.001 |
| | *JHAMT/ETHR RNAi-IR2* | 0.245 (ns) |
| Methoprene rescue | *JHAMT/+* : acetone day 0-4 | <0.001 |
| | *JHAMT/+* : methoprene day 0-4 | <0.001 |
| | *JHAMT/ETHR RNAi-Sym* : acetone day 0-4 | 0.495 (ns) |
| | *JHAMT/ETHR RNAi-Sym* : methoprene day 0-4 | 0.013 |

(b) Figure II.6B

| Experiment | Genotype : Condition | P-value |
|---|---|---|
| Memory decay assay | *JHAMT/+* (2 min interval) | <0.0001 |
| | *JHAMT/+* (4 min interval) | <0.0001 |
| | *JHAMT/+* (6 min interval) | <0.001 |
| | *JHAMT/+* (8 min interval) | <0.001 |
| | *+/ETHR RNAi-Sym* (2 min interval) | <0.001 |
| | *+/ETHR RNAi-Sym* (4 min interval) | <0.0001 |
| | *+/ETHR RNAi-Sym* (6 min interval) | <0.001 |
| | *+/ETHR RNAi-Sym* (8 min interval) | 0.003 |
| | *JHAMT/ETHR RNAi-Sym* (2 min interval) | <0.001 |
| | *JHAMT/ETHR RNAi-Sym* (4 min interval) | 0.022 |
| | *JHAMT/ETHR RNAi-Sym* (6 min interval) | 0.096 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (8min interval) | 0.399 (ns) |

(c) Figure II.7B

| Experiment | Genotype : Condition | P-value |
|---|---|---|
| Dissociation assay (trained by $\Psi_m$ female) | *JHAMT/+* | <0.001 |
| | *JHAMT/ETHR RNAi-Sym* | 0.345 (ns) |

(d) Figure II.8A and 8B

| Experiment | Genotype : Condition | P-value |
|---|---|---|
| Conditional Inka cell ablation (TARGET) | *ETH-GAL4;TubPG80$^{ts}$/+* (31°C) | <0.001 |
| | *+/rpr,hid* (31°C) | <0.001 |
| | *ETH-GAL4;TubPG80$^{ts}$/rpr,hid* (31°C) | 0.092 (ns) |
| Conditional Inka cell ablation (Geneswitch) | *+/rpr,hid* (- RU486) | <0.001 |
| | *+/rpr,hid* (+ RU486) | <0.001 |
| | *EUG8/rpr,hid* (- RU486) | <0.001 |
| | *EUG8/rpr,hid* (+ RU486) | 0.084 (ns) |
| Blocking vesicle release from Inka cells | *EUG8/TNT$_{imp}$* (-RU486) | <0.001 |
| | *EUG8/TNT$_{imp}$* (+RU486) | <0.001 |
| | *EUG8/TNT$_{G}$* (-RU486) | <0.001 |
| | *EUG8/TNT$_{G}$* (+RU486) | 0.016 |
| Conditional *ETHR* KD in CA (TARGET) | *JHAMT/ETHR RNAi-Sym;TubPG80$^{ts}$* (X) | <0.001 |
| | *JHAMT/ETHR RNAi-Sym;TubPG80$^{ts}$* (pre/post) | 0.103 (ns) |
| | *JHAMT/ETHR RNAi-Sym;TubPG80$^{ts}$* (pre) | <0.001 |
| | *JHAMT/ETHR RNAi-Sym;TubPG80$^{ts}$* (post) | 0.386 (ns) |
| | *JHAMT/+* (post) | <0.001 |
| | *+/ETHR RNAi-Sym;TubPG80$^{ts}$* (post) | 0.001 |

(e) Figure II.10

| Experiment | Genotype : Condition | P-value |
|---|---|---|
| Periodic methoprene rescue (Fig. II.10A and 10B, 4 days) | *JHAMT*/+ (acetone day 1-5) | <0.001 |
| | *JHAMT*/+ (methoprene day 1-5) | <0.001 |
| | *JHAMT*/+ (acetone day 2-6) | <0.001 |
| | *JHAMT*/+ (methoprene day 2-6) | 0.001 |
| | *JHAMT*/+ (acetone day 3-7) | <0.001 |
| | *JHAMT*/+ (methoprene day 3-7) | 0.001 |
| | *JHAMT*/+ (acetone day 4-8) | 0.001 |
| | *JHAMT*/+ (methoprene day 4-8) | 0.016 |
| | *JHAMT*/+ (acetone day 10-14) | 0.009 |
| | *JHAMT*/+ (methoprene day 10-14) | 0.004 |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 1-5) | 0.118 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (methoprene day 1-5) | 0.001 |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 2-6) | 0.197 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (methoprene day 2-6) | 0.004 |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 3-7) | 0.111 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (methoprene day 3-7) | 0.083 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 4-8) | 0.187 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (methoprene day 4-8) | 0.151 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 10-14) | 0.156 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (methoprene day 10-14) | 0.205 (ns) |
| Periodic methoprene rescue (Fig. II.10C, 24-hour) | *JHAMT/ETHR RNAi-Sym* (acetone day 0-1) | 0.221 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 1x, day 0-1) | 0.071 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 5x, day 0-1) | <0.001 |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 1-2) | 0.449 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 1x, day 1-2) | 0.098 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 5x, day 1-2) | <0.001 |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 2-3) | 0.272 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 1x, day 2-3) | 0.164 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 5x, day 2-3) | 0.021 |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 3-4) | 0.212 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 1x, day 3-4) | 0.136 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 5x, day 3-4) | 0.071 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 4-5) | 0.196 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 1x, day 4-5) | 0.225 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 5x, day 4-5) | 0.099 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (acetone day 5-6) | 0.123 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 1x, day 5-6) | 0.361 (ns) |
| | *JHAMT/ETHR RNAi-Sym* (met 5x, day 5-6) | 0.195 (ns) |

(f) Figure II.11

| Experiment | Genotype : Condition | P-value |
|---|---|---|
| JH receptor KD (*Met* & *gce*) | *+/gce RNAi;Met RNAi* | <0.001 |
| | *OK107/+* | <0.001 |
| | *OK107/gce RNAi;Met RNAi* | <0.001 |
| | *TH/+* | <0.001 |
| | *TH/gce RNAi;Met RNAi* | 0.041 |
| | *Tdc2/+* | <0.001 |
| | *Tdc2/gce RNAi;Met RNAi* | <0.001 |
| | *Trh/+* | <0.001 |
| | *Trh/gce RNAi;Met RNAi* | <0.001 |
| | *OK371/+* | <0.001 |
| | *OK371/gce RNAi;Met RNAi* | <0.001 |
| | *Orco/+* | <0.001 |
| | *Orco/gce RNAi;Met RNAi* | 0.005 |
| JH receptor KD (*Met* or *gce*) | *+/Met RNAi* | <0.001 |
| | *TH/Met RNAi* | <0.001 |
| | *+/gce RNAi* | <0.001 |
| | *TH/gce RNAi* | <0.001 |

**Table II.2. Locomotion test and heterosexual activity assay**

| Genotype | Neg. Geotaxis[a] | Heterosexual Activities | | |
|---|---|---|---|---|
| | Velocity | WEI (%)[b] | Cop. Rate (%)[b] | CI (%)[c] |
| *JHAMT/+* | 20.6 ± 1.0 (30) | 20.7 ± 1.2 (20) | 85 (17/20) | 89.3 ± 2.0 (22) |
| *+/ETHR RNAi-Sym* | 18.9 ± 1.4 (31) | 16.9 ± 2.5 (20) | 80 (16/20) | 84.1 ± 2.0 (20) |
| *JHAMT/ETHR RNAi-Sym* | 20.0 ± 0.8 (30) | 19.1 ± 1.3 (20) | 85 (17/20) | 84.9 ± 1.8 (27) |

Animals of the indicated genotypes were single-raised day 4-day old males (see Material and Methods).

a. The modified geotaxis assay was tested with six five-day old males by measuring the speed of climbing 6.2 cm.

b. An indicator of courtship activity, Wing Extension Index (WEI; one wing extension or courtship singing), was determined as total time spent in wing extension posture up to the time of copulation with a virgin Canton-S female (day 4-5). Copulation rate was computed by counting the number of males that copulated successfully with virgin females during a 10-minute interval.

c. Courtship indices (CI) were registered as total time spent courting activities an unreceptive immobilized virgin female during a 10-minute interval.

**Table II.3. Methoprene effects on courting activity of different aged flies.**

| Genotype | methoprene application (24-hour): mean CIs (%) of vehicle$^{+}$ / 322 pmol met$^{+}$ | | | | | |
|---|---|---|---|---|---|---|
| | **0 - 1** | **1 - 2** | **2 - 3** | **3 - 4** | **4 - 5** | **5 - 6** |
| *JHAMT/+* | 40.2 / 49.1 | 68.2 / 78.1 | 82.5 / 82.1 | 85.3 / 82.7 | 79.7 / 80.9 | 84.6 / 82.2 |
| *JHAMT/ETHR RNAi-Sym* | 33.6 / 48.8 | 65.7 / 79.6 | 84.6 / 85.5 | 84.4 / 80.1 | 82.8 / 84.5 | 83.3 / 84.8 |

Courtship indices were determined as time spent by males in courting activities toward an unreceptive immobilized virgin female during a 10-minute interval.

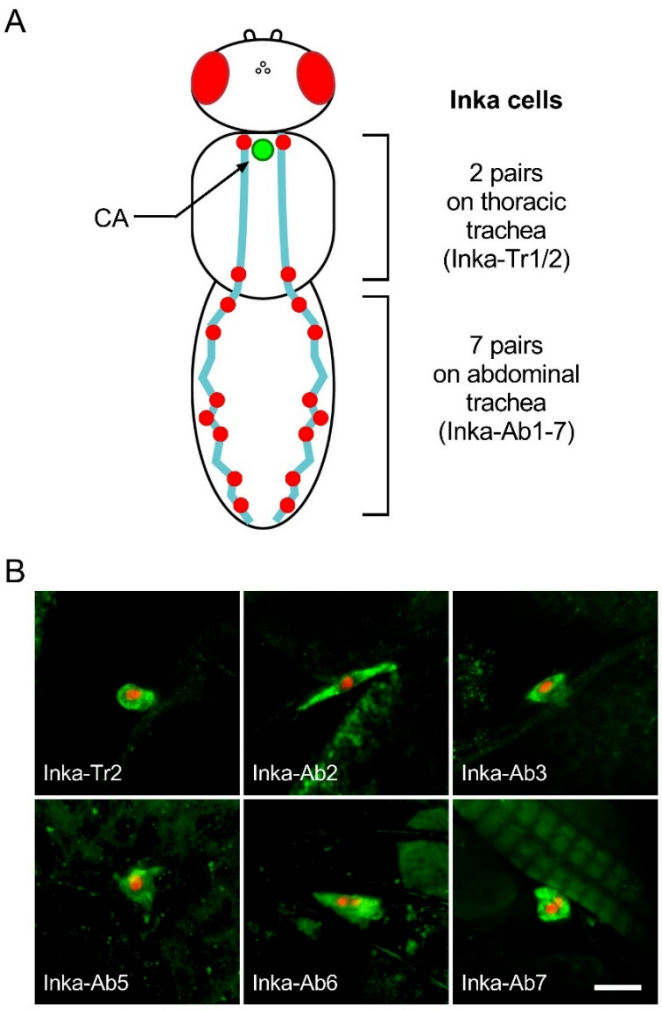



**Figure II.1. Inka cells and ETH peptides persist in adult male *Drosophila*.** (A) Two pairs of Inka cells are located on the thoracic trachea (Tr1 and Tr2) and seven pairs are detected on the abdominal trachea (Ab1 to Ab7). (B) Presence of Inka cells and ETH peptides in adult male (Day 4 or 5 after eclosion) shown by ETH1immunohistochemistry (green) and a nuclear marker RedStinger expression (red) in the *ETH-GAL4* transgenic line. Scale bar: 10 μm.

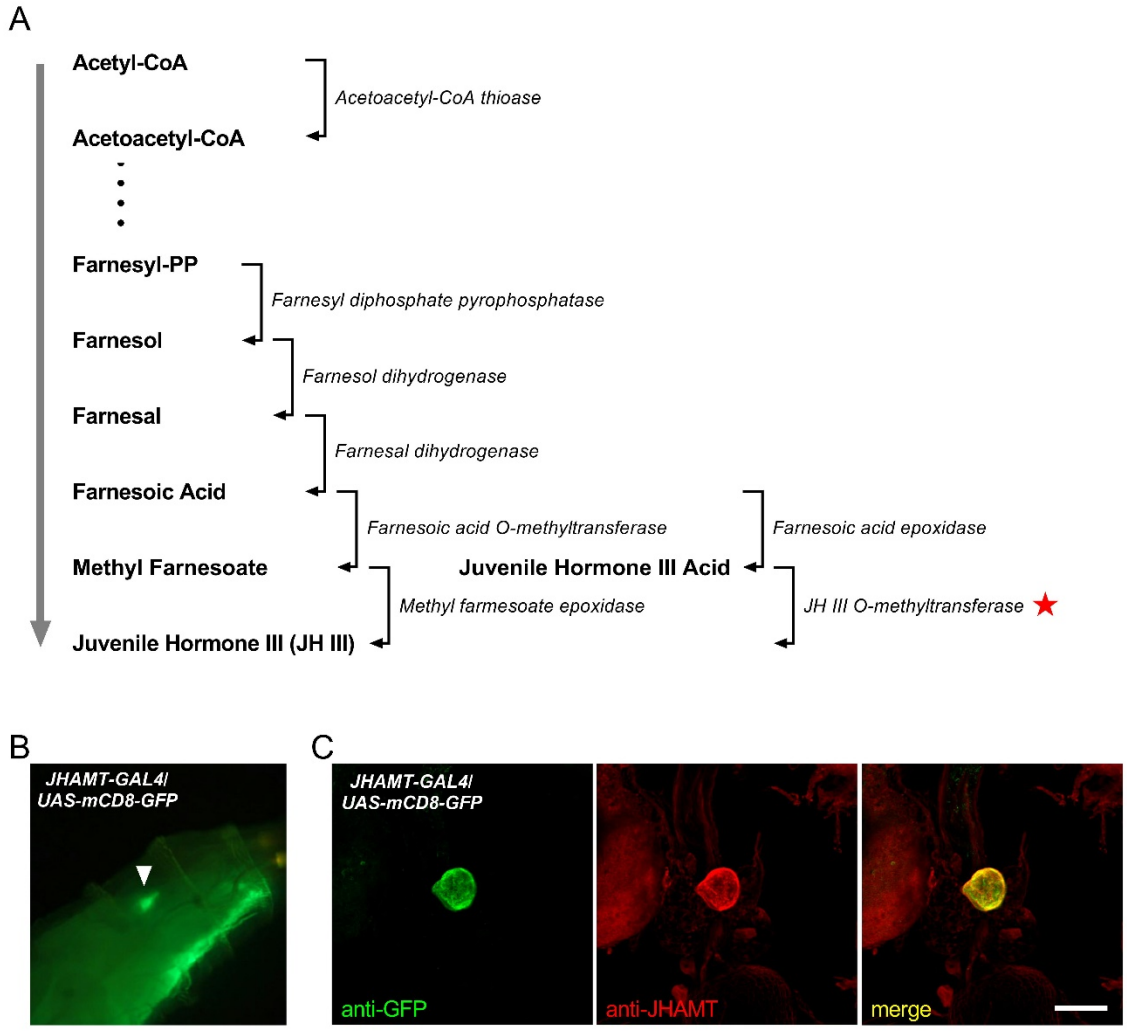



**Figure II.2. *JHAMT-GAL4* labels the CA specifically.** (A) *JHAMT* gene encodes JH acid *O*-methyltransferase, which is important for the final step of JH synthesis. (B) *JHAMT-GAL4/UAS-mCD8-GFP* drives expression in the ring gland of third instar larvae (arrowhead). (C) CA of *JHAMT-GAL4/UAS-mCD8-GFP* males were stained with anti-GFP (left, green) and anti-JHAMT (middle, red) antibodies; superimposed images merged in the right panel. Scale bar: 50 μm.

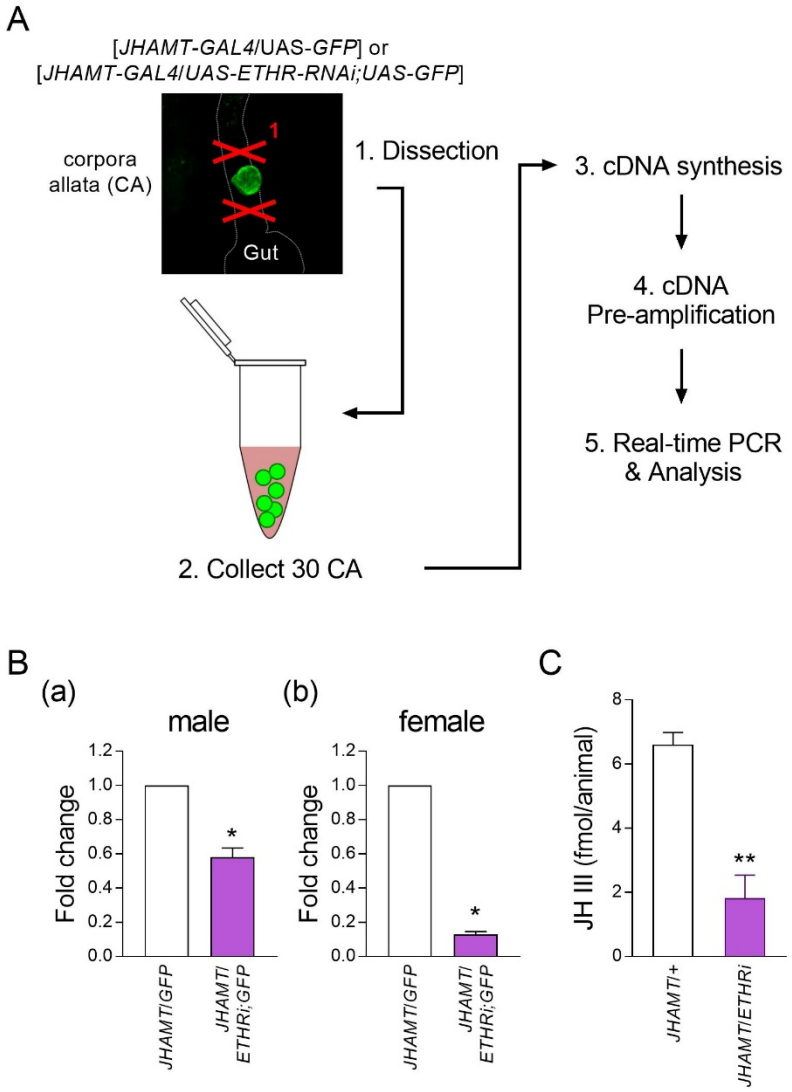



**Figure II.3. ETHR in the CA regulates JH synthesis of adult male *Drosophila*.** (A) Experimental procedure for Real-Time qRT-PCR from adult CA. (B) Relative *ETHR* transcript abundance in CA of control (*JHAMT-GAL4/UAS-mCD8-GFP*; white bar) and *ETHR*-silenced (*JHAMT-GAL4/UAS-ETHR RNAi-Sym;UAS-mCD8-GFP*; purple bar) males (a) and females (b) measured by qPCR. Error bar represents s.e.m (*t*-test, $*P < 0.001$). (C) Silencing of *ETHR* in CA reduces JH levels in day 4 adult males. JH III levels are represented as mean ± s.e.m. of 4 independent replicate groups (total numbers of animals tested: 264 *JHAMT-GAL4/+*; 268 *JHAMT-GAL4/UAS-ETHR RNAi-Sym* (*t*-test, $**P < 0.01$)).

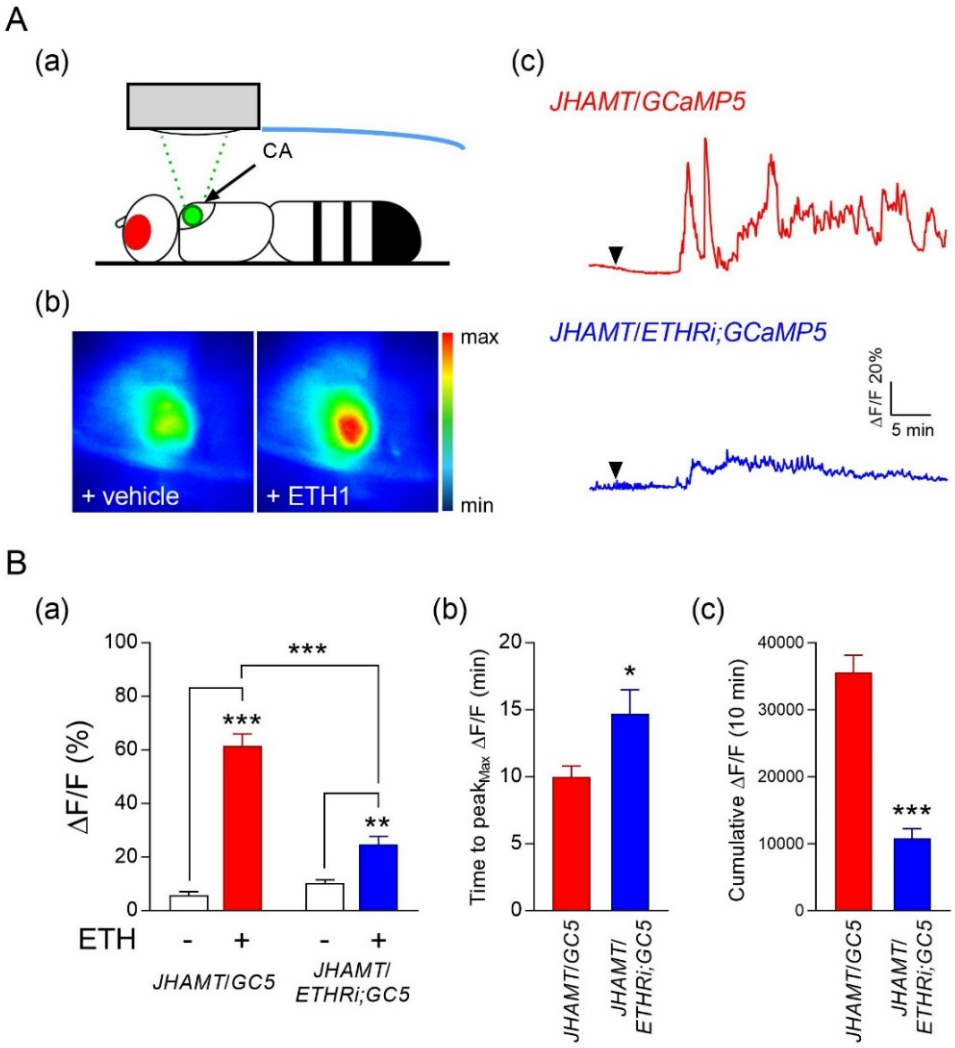

A
(a)
CA
(b)
max
min
+ vehicle
+ ETH1
(c)
JHAMT/GCaMP5
JHAMT/ETHRi;GCaMP5
ΔF/F 20%
5 min
B
(a)
ΔF/F (%)
100
80
60
40
20
0
***
***
**
ETH
-
+
-
+
JHAMT/GC5
JHAMT/ ETHRi;GC5
(b)
Time to peak$_{Max}$ ΔF/F (min)
20
15
10
5
0
*
JHAMT/GC5
JHAMT/ ETHRi;GC5
(c)
Cumulative ΔF/F (10 min)
40000
30000
20000
10000
0
***
JHAMT/GC5
JHAMT/ ETHRi;GC5

**Figure II.4. ETH signaling promotes activity of the CA.** (A) *In vivo* $Ca^{2+}$-induced fluorescence in CA of a day 4 male CA expressing GCaMP5 after application of ETH1 (1 μM). (a) Diagram of the experimental setup for *in vivo* CA $Ca^{2+}$ imaging. (b) Representative $Ca^{2+}$-mediated fluorescence in CA of a vehicle- or ETH1-treated *JHAMT-GAL4/UAS-GCaMP5* male. (c) Representative traces of the ΔF/F of the CA following ETH1 (1 μM) application. Upper trace (red) represents $Ca^{2+}$ elevation in CA of a *JHAMT-GAL4/UAS-GCaMP5* male, while the trace below (blue) shows $Ca^{2+}$ elevation in CA of an *ETHR*-silenced male (*JHAMT-GAL4/UAS-ETHR RNAi-Sym;UAS-GCaMP5*) in response to 1 μM ETH1 application. (B) Analysis of $Ca^{2+}$ dynamics at the CA responding to ETH application. (a) Mean maximum fluorescence responses of male CA exposed to fly saline (-, white bar) or 1 μM ETH1 (+, red bar for *JHAMT-GAL4/UAS-GCaMP5*; blue bar for *JHAMT-GAL4/UAS-ETHR RNAi-Sym;UAS-GCaMP5*). (b) Latency to maximum fluorescence amplitude following ETH application. (c) Cumulative fluorescence changes (area under the curve) over a 10 min interval starting from onset of the response. Error bar represents s.e.m. (n = 6-9, t-test, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

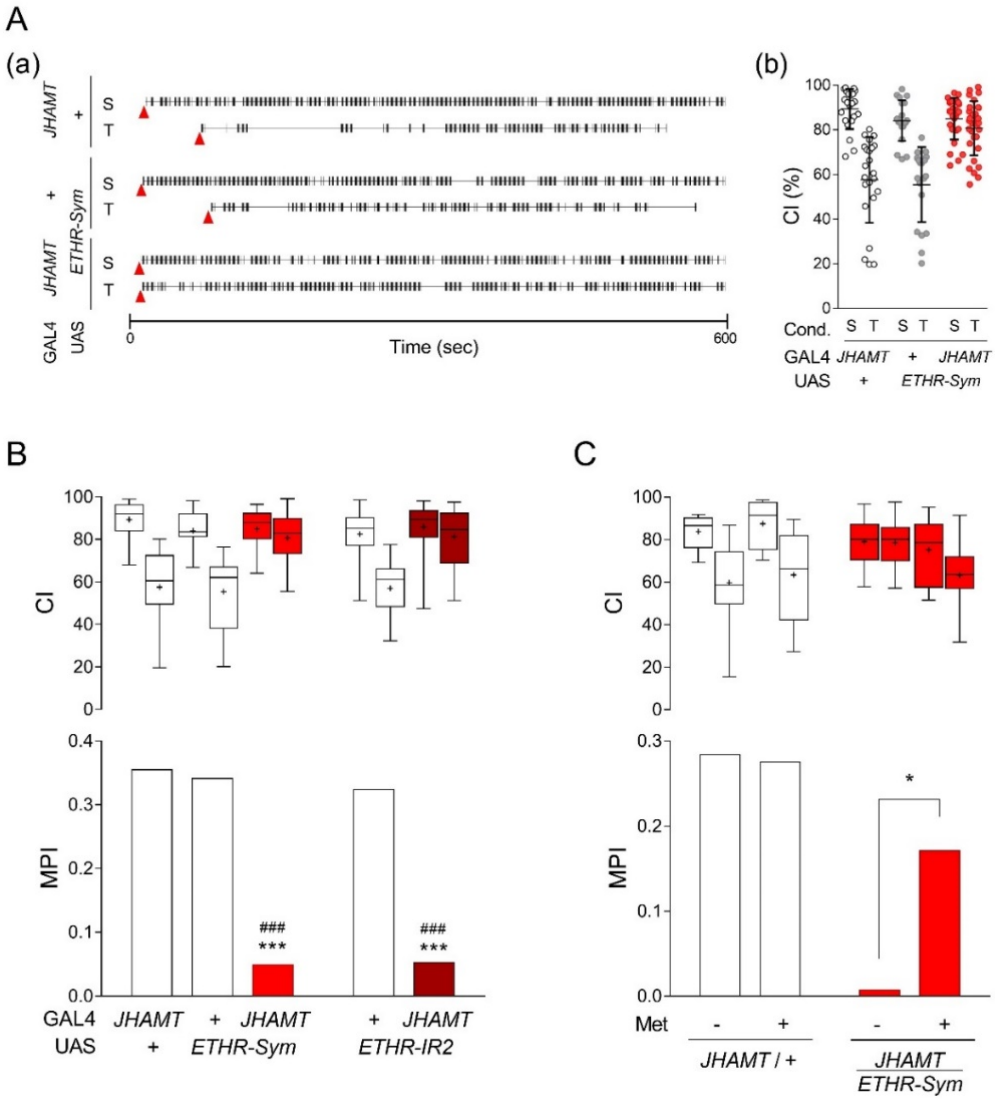

A
(a)
JHAMT
+
S
T
+
ETHR-Sym
S
T
JHAMT
ETHR-Sym
S
T
GAL4
UAS
0
600
Time (sec)
(b)
CI (%)
100
80
60
40
20
0
Cond.
S T S T S T
GAL4 JHAMT + JHAMT
UAS + ETHR-Sym
B
CI
MPI
0.4
0.3
0.2
0.1
0.0
###
***
GAL4 JHAMT + JHAMT + JHAMT
UAS + ETHR-Sym ETHR-IR2
C
CI
MPI
0.3
0.2
0.1
0.0
*
Met - + - +
JHAMT / +
JHAMT
ETHR-Sym

**Figure II.5. JH deficiency creates short-term memory deficit in courtship conditioning.** (A-a) Courtship bouts of sham-trained (S) or trained (T) JH-reduced males toward immobilized (decapitated) virgin female testers. Red arrowheads indicate the latency to courting of males toward female tester. (A-b) Individual courtship distribution of sham-trained and trained males. Lines indicate mean ± STD. (B) Short-term memory performances of JH-deficient males subjected to courtship conditioning; males were tested 10 min after completion of 1 hour training with a mated female. Upper plot compares CI of sham-trained (left) and trained (right) males following each treatment. Genetic controls are shown with white bars; test males using two independent RNAi constructs are shown in either red or brown. Bottom plot shows memory performance indices (MPI) of genetic control and test males. "*" represents significant difference between MPI of GAL4 control and test males (***$P < 0.001$), and "#" indicates the significant difference between MPI of UAS control and test males ($^{\#\#\#}P < 0.001$) (n = 40-57). (C) CI distributions and MPI of methoprene-treated *JHAMT-GAL4/UAS-ETHR RNAi-Sym* males. Acetone was applied as a vehicle; "*" denotes the significant difference between MPI of vehicle-treated and that of methoprene-treated males (*$P < 0.01$) (n = 40-46).

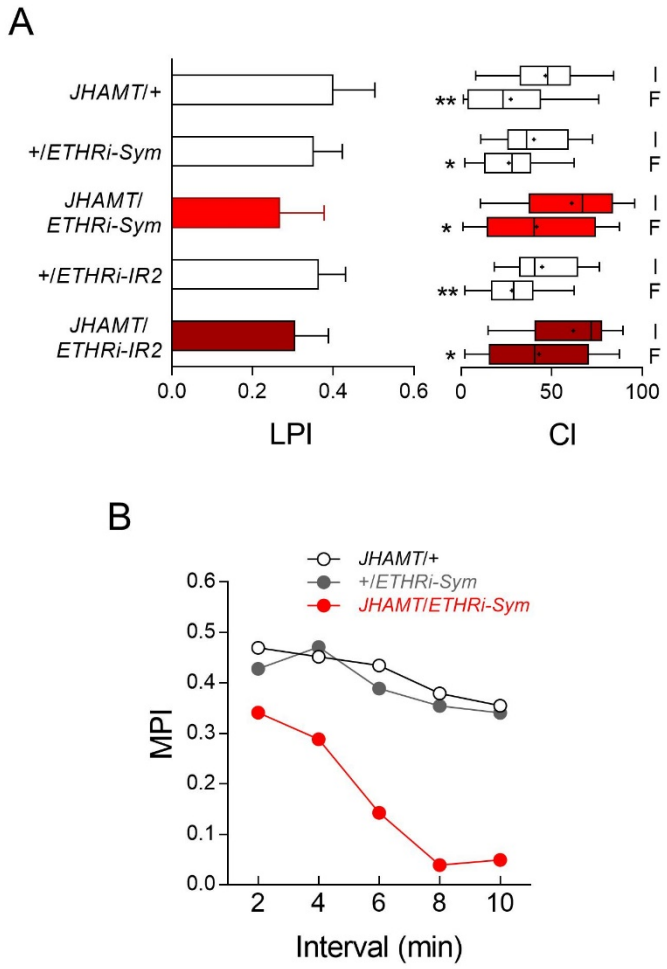



**Figure II.6. JH deficiency causes the impairment of memory retention but not acquisition.** (A) During the hour-long training period, both control and JH-deficient males exhibit learning through reduction of CI and LPI. (JH-deficient fly genotype: *JHAMT-GAL4/UAS-ETHR RNAi-Sym* and *JHAMT-GAL4/UAS-ETHR RNAi-IR2*). Asterisks indicate significant differences between CI during the initial 10 min interval (I) of the 1 h pairing period and CI during the final (F) 10 min interval of the pairing period (Mann-Whitney *U* test, $^{**}P < 0.01$, $^{*}P < 0.05$) (n = 40-52). (B) Memory decay assay following 1-hour exposure to mated females (n = 48-56).

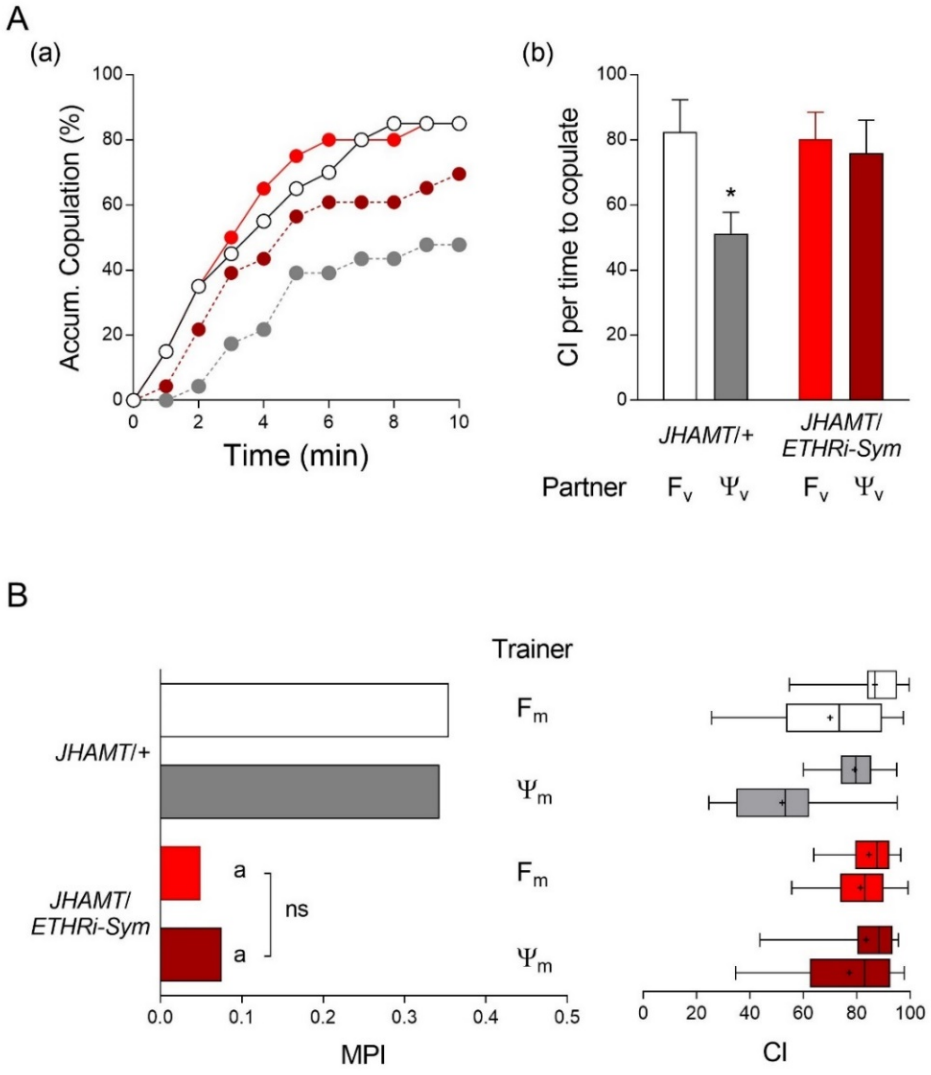

A
(a)
Accum. Copulation (%)
Time (min)
(b)
CI per time to copulate
JHAMT/+
JHAMT/ ETHRi-Sym
Partner
F_v
Ψ_v
B
Trainer
JHAMT/+
JHAMT/ ETHRi-Sym
F_m
Ψ_m
a
ns
MPI
CI

**Figure II.7. JH-deficient males exhibit olfactory deficits, but robust courtship memory occurs in absence of aversive, mating-associated chemical cues.** (A) (a) Accumulated time to copulation of JH-deficient and GAL4 control males paired with mature virgin ($F_v$) or pseudovirgin ($\Psi_v$) females (n = 20-23). (open circle: *JHAMT-GAL4/+* paired with $F_v$; filled circle (red): *JHAMT-GAL4/UAS-ETHR RNAi-Sym* paired with $F_v$; filled circle (gray): *JHAMT-GAL4/+* paired with $\Psi_v$; filled circle (brown): *JHAMT-GAL4/UAS-ETHR RNAi-Sym* paired with $\Psi_v$). (b) CI of those males toward $F_v$ or $\Psi_v$ females until copulation (Student's *t* test, $*P<0.05$). (B) CI and MPI of JH-deficient and GAL4 control males trained with either mated ($F_m$) or pseudomated ($\Psi_m$) females. "a" represents significant difference between MPI of GAL4 control and test males trained with equivalent trainer type (*JHAMT-GAL4/+* vs. *JHAMT-GAL4/UAS-ETHR RNAi-Sym* trained with $F_m$, or trained with $\Psi_m$ ($^aP < 0.01$) (n = 44-57).

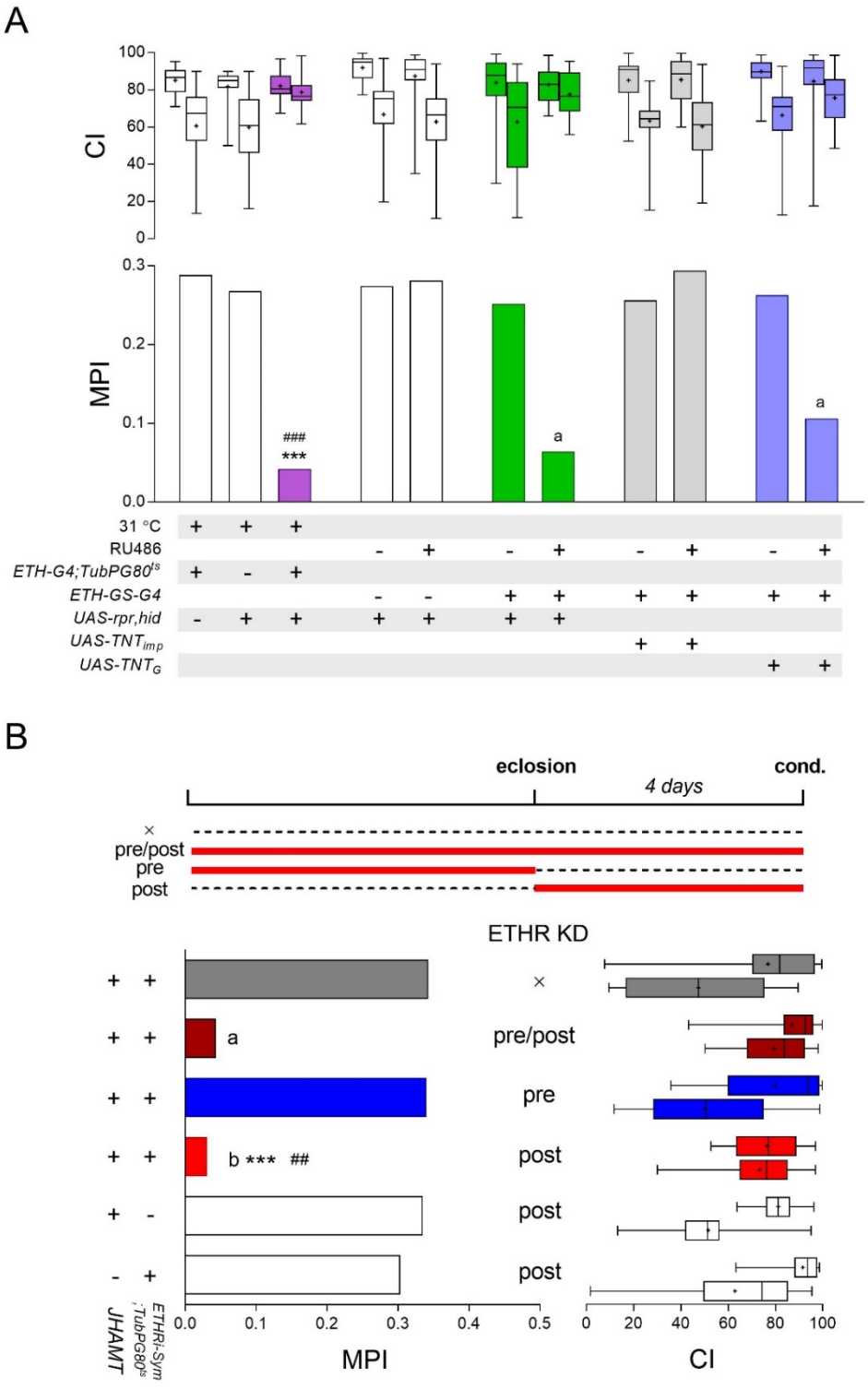

A
CI
MPI
###
***
a
a
31 °C
RU486
ETH-G4;TubPG80ts
ETH-GS-G4
UAS-rpr,hid
UAS-TNTimp
UAS-TNTG
B
eclosion
cond.
4 days
×
pre/post
pre
post
ETHR KD
a
b *** ##
JHAMT
ETHRi-Sym ;TubPG80ts
MPI
CI

**Figure II.8. ETH-driven JH functions in memory performance during the adult period.** (A) CI and MPI following temporal ablation of Inka cells or suppression of ETH release using the TARGET system (*ETH-GAL4;TubPG80$^{ts}$*) or GeneSwitch (*ETH-GS-GAL4*) for conditional expression of pro-apoptotic cell death genes *reaper* (*rpr*) and *head involution defective* (*hid*) or tetanus toxin active ($TNT_G$) or impaired ($TNT_{imp}$) light chain in Inka cells. In GeneSwitch experiments, "a" denotes significant difference between MPI of vehicle-treated and RU486-treated animals ($^aP < 0.01$) (n = 48-64). (B) Upper schematic diagram shows conditional *ETHR* knockdown in the CA. *JHAMT-GAL4/UAS-ETHR RNAi-Sym;TubPGAL80$^{ts}$* males were kept for entire life at 19°C (X), at 31°C (pre/post), pre-adult stage at 31°C (pre) or adult stage at 31°C (post). CI distributions and MPI of conditional knockdown males. Significant differences: "*"- GAL4 control and test males (***$P < 0.001$); "#"- UAS control and test males ($^{\#\#}P < 0.01$); "a"- negative control (X) and that of positive control (pre/post) males ($^aP < 0.01$); "b"- negative control (X) and that of test (post) males ($^bP < 0.001$) (n = 40-56).

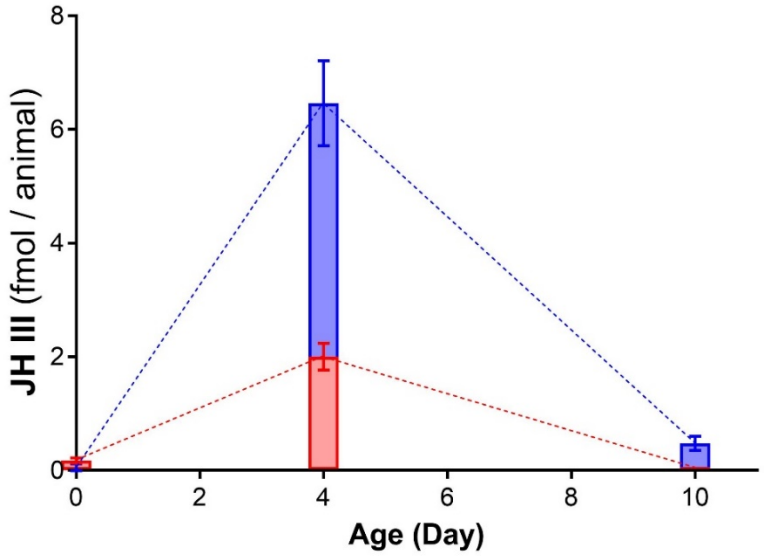



**Figure II.9. JH level is changed by aging of adult males.** The JH III titre in different aged CA-specific ETHR knockdown male plotted per animal. Each data point is mean of two independent replicates of sample groups (mean ± s.e.m). *JHAMT-GAL4/+* (n = day 0: 180, day 4: 162, day 10:176); *JHAMT-GAL4/UAS-ETHR RNAi-Sym* (Day 0: 174, Day 4: 162, Day 10: 190).

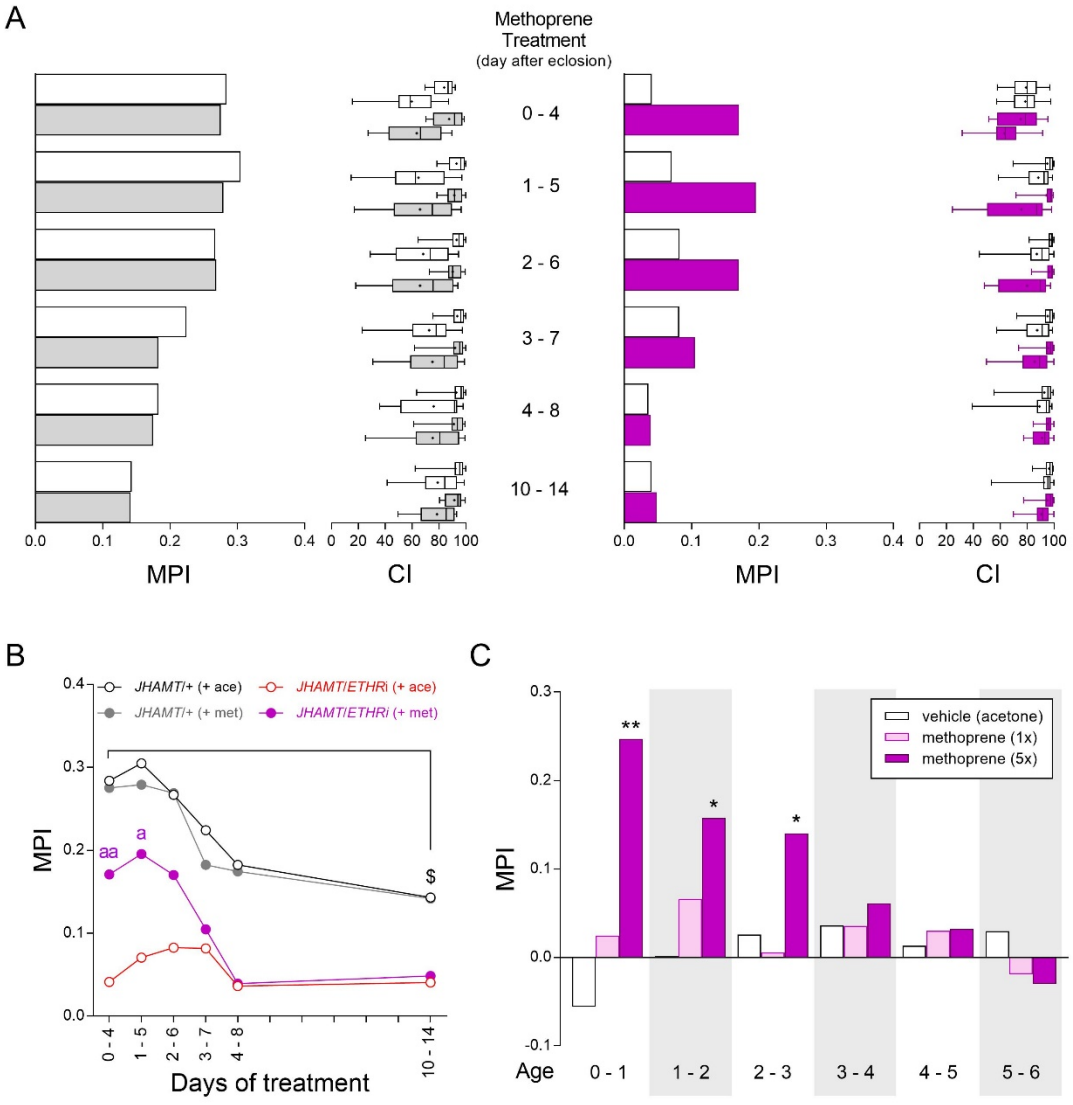

A
Methoprene
Treatment
(day after eclosion)
0 - 4
1 - 5
2 - 6
3 - 7
4 - 8
10 - 14
MPI
CI
B
JHAMT/+ (+ ace)
JHAMT/+ (+ met)
JHAMT/ETHRi (+ ace)
JHAMT/ETHRi (+ met)
MPI
a
aa
$
Days of treatment
C
vehicle (acetone)
methoprene (1x)
methoprene (5x)
MPI
**
*
*
Age
0 - 1
1 - 2
2 - 3
3 - 4
4 - 5
5 - 6

**Figure II.10. JH action on memory performance operates during a critical period in the first week of adulthood.** Memory deficits following ETHR-silencing were rescued by topical application of methoprene to different aged flies. Acetone was used as a vehicle. (A) CI distributions and MPI of GAL4 control (left) and JH-deficient (*JHAMT-GAL4/UAS-ETHR RNAi-Sym*) males. Empty bars indicate MPI of vehicle-treated, and purple bars represent MPI of methoprene-treated males (n = 40-58). (B) Dynamics of MPI of aged control and test males. "$" denotes significant difference between MPI of day 0-4 and day 10-14 vehicle or methoprene-treated males ($^{\$}P < 0.01$). "a" indicates the significant difference between MPI of vehicle-treated and that of methoprene-treated animals ($^{aa}P < 0.01$, $^{a}P < 0.05$). (C) Precise methoprene sensitive period of *JHAMT-GAL4/UAS-ETHR RNAi-Sym* males. Two doses of methoprene (1x, 64 pmol; 5x, 322 pmol) were applied to individuals of each age and courtship conditioning was performed 24-hour later (**$P < 0.01$, *$P < 0.05$).

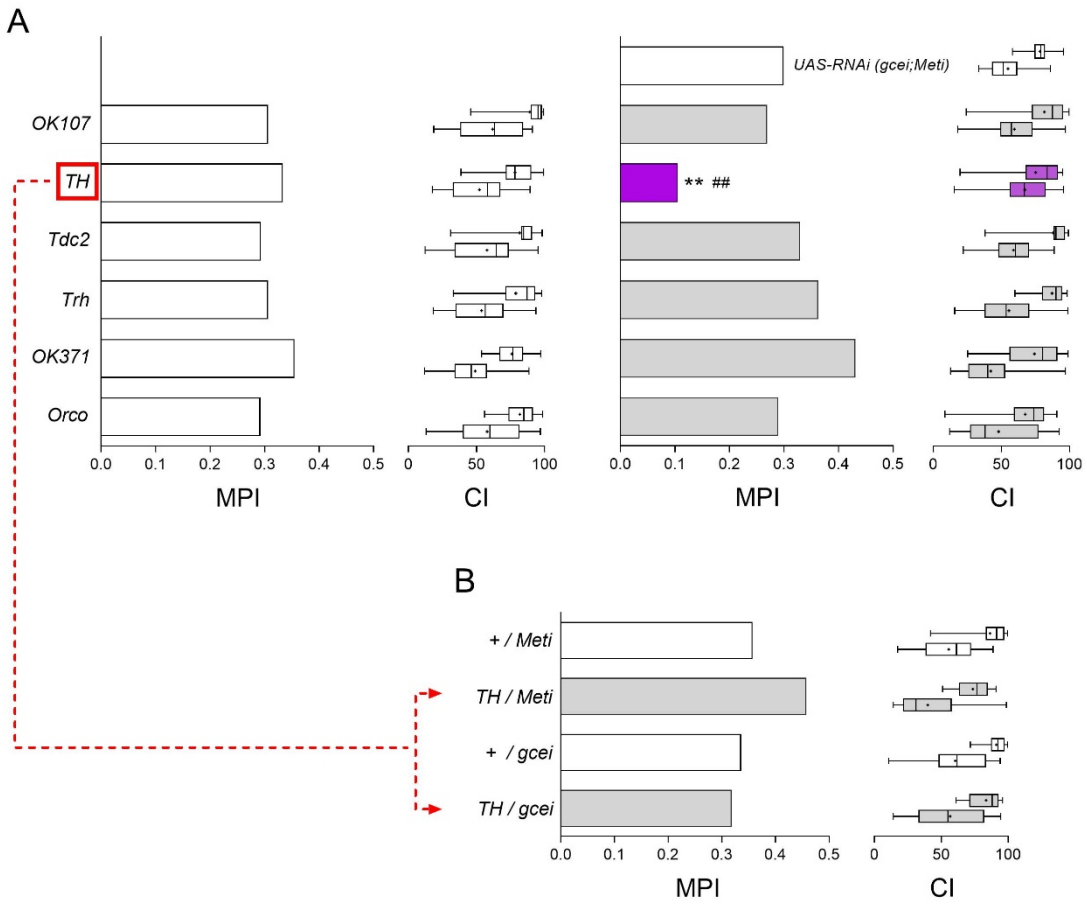



**Figure II.11. TH-positive dopaminergic (DA) neurons are functional targets of JH.** (A) Knockdown of *Met* and *gce* was accomplished through use of diverse GAL4 drivers for introduction of dsRNAs directed against *Met* and *gce* sequences. CI distribution and MPI of GAL4 controls (left), and those of UAS control and test males (right) (n = 44-52). Drivers: *OK107-GAL4*, whole mushroom body; *TH-GAL4*, tyrosine hydroxylase (DA); *Tdc2-GAL4*, tyrosine decarboxylase 2 (neuronal OA); *Trh-GAL4*, tryptophan hydroxylase (5-HT); *OK371-GAL4*, glutamatergic neurons; *Orco-GAL4*, broad odorant receptor neurons (co-receptor Or83b). ($^{**/\#\#}P < 0.01$). (B) Suppression of *Met* or *gce* expression in TH-positive DA neurons was performed by preparing *TH-GAL4/UAS-Met RNAi* and *TH-GAL4/UAS-gce RNAi* lines (n = 49-54).

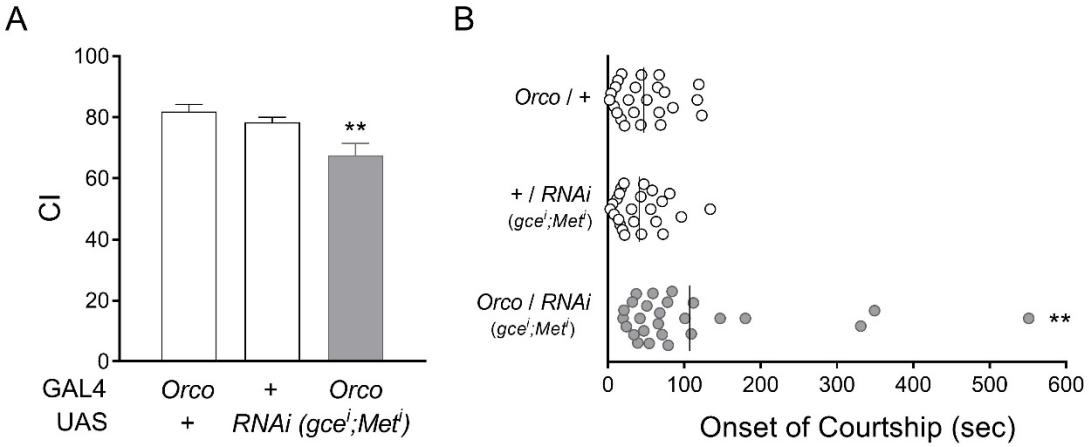



**Figure II.12. JH receptors in ORNs play a role in courtship behavior of naïve males.** In the courtship conditioning, courtship activities of sham-trained animals toward decapitated virgin females were analyzed. (A) Overall courtship behaviors of test and genetic control males (mean ± s.e.m, One-way ANOVA, $^{**}P < 0.01$, n = 24-26). (B) Onset of courtship behavior of individual animals in plot (A). Lines indicate mean time of courtship onset (Kruskal-Wallis nonparametric test, $^{**}P < 0.01$).

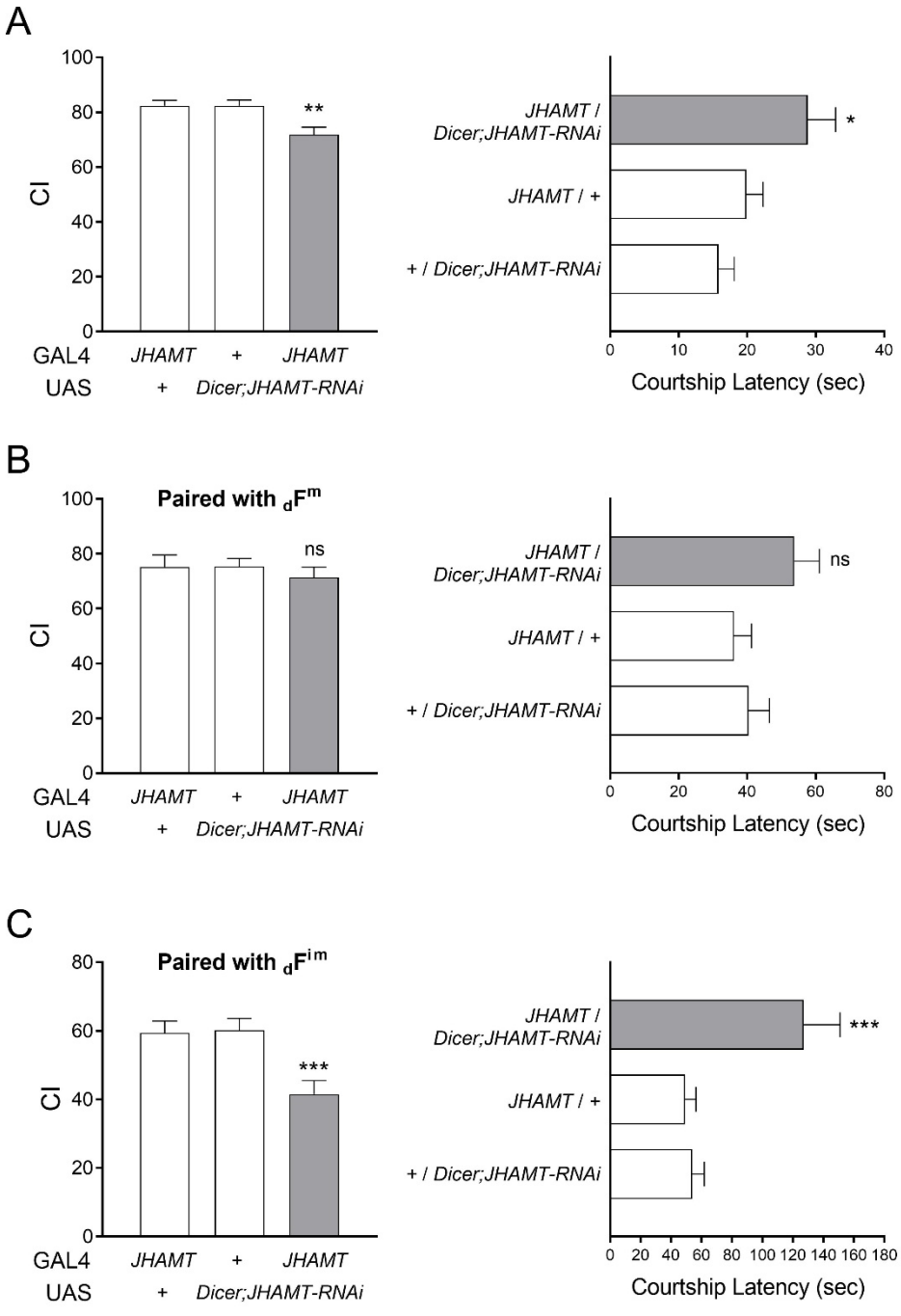

A
100
80
60
40
20
0
CI
**
GAL4 JHAMT + JHAMT
UAS + Dicer;JHAMT-RNAi
JHAMT / Dicer;JHAMT-RNAi
JHAMT / +
+ / Dicer;JHAMT-RNAi
*
0 10 20 30 40
Courtship Latency (sec)
B
Paired with dFm
ns
GAL4 JHAMT + JHAMT
UAS + Dicer;JHAMT-RNAi
0 20 40 60 80
Courtship Latency (sec)
C
Paired with dFim
***
0 20 40 60 80 100 120 140 160 180
Courtship Latency (sec)

**Figure II.13. *JHAMT* silencing in the CA reduces male courtship specifically toward an immature female.** (A) Overall courtship behaviors (left) and courtship latencies (right) of test and genetic control males toward an immature female (n = 25-27). (B) Overall courtship behaviors (left) and courtship latencies (right) of test and genetic control males toward an immobilized (decapitated) mature (day-4 post-eclosion) female (n = 23). (B) Overall courtship behaviors (left) and courtship latencies (right) of test and genetic control males toward an immobilized (decapitated) immature (in 2-hour post-eclosion) female (n = 22). mean ± s.e.m, One-way ANOVA for CIs and , Kruskal-Wallis nonparametric test for courtship latencies. *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001; ns, no significance.

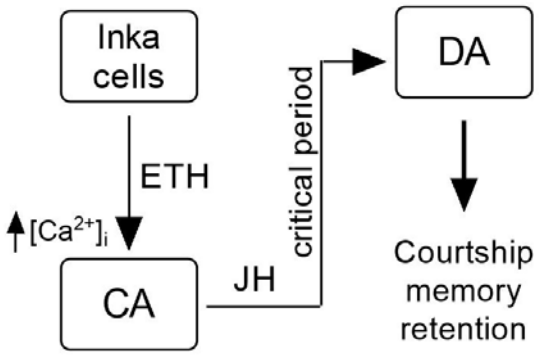



**Figure II.14. A model for the hormonal cascade in regulating *Drosophila* short-term courtship memory.** Proposed model as described in the text depicting the function of ETH-JH signaling in regulating male courtship memory retention.

# CHAPTER III

## Endocrine Convergence in Regulation of Long-term Courtship Memory Circuits of *Drosophila*


### Abstract

Hormonal state is an important determinant of learning and memory in animals. Here I rshow that the endocrine peptide ecdysis triggering hormone (ETH) and the sesquiterpenoid juvenile hormone (JH) converge on the mushroom body (MB) for regulation of long term courtship memory (LTM) in *Drosophila melanogaster*. Deficits of either ETH or JH abolish LTM, as does knockdown of the ETH receptor (ETHR) or the JH receptor Met specifically in the MB γ-lobe. In addition, ETH signaling in DAL neurons is required for LTM. Promotion of ETH release during courtship conditioning both improves memory performance and reduces the training period required for LTM formation through *de novo* protein synthesis. Exposure of the CNS to ETH results in calcium mobilization in MB γ-lobe neurons and DAL neurons. These findings identify endocrine-enabled circuitry in the brain crucial to promotion and homeostatic regulation of behavioral changes resulting from aversive social experience.


### Introduction

As observed in a broad range of animal species, social experiences have a profound influence on fruit fly *Drosophila* internal states and thereby modify subsequent behavioral patterns (Chabaud et al., 2009; Shohat-Ophir et al., 2012). An altered behavior affects subsequent social interactions in fly community, and may be critical for survival and propagation. An example of social context-dependent behavioral plasticity of *Drosophila* is courtship memory, in which a male has been

exposed to a recently mated female suppresses further mating avidities toward other female partners (McBride et al., 1999; Siegel and Hall, 1979). A considerable number of studies have reported structural and molecular components of this memory process, which are shared with olfactory-induced memories. In particular, both types of memory are triggered by chemosensory inputs, chief among these being pheromones. However, courtship memory cannot be fully explained by olfactory influences, since a male exposed to a mated female gains multiple aversive sensory inputs including not only olfactory but also behavioral (rejection by the female) during training (Lee et al., 2017). Also, distinct neural pathways involved in memory recall depend on the reproductive state (mated vs. virgin) of target females during the post-training test session (Ejima et al., 2007; Keleman et al., 2012). Furthermore, this type of associative memory may require additional internal components, including endocrine states. Although the steroid ecdysone promotes short-term and long-lasting courtship memories through distinct pathways (Ishimoto et al., 2009; Ishimoto et al., 2013), few studies have focused on hormonal influences on regulation of learning and memory processes.

One of the most critical innate behaviors performed during insect development is ecdysis, which is required for shedding of old cuticle and advancement to the next developmental stage (Zitnan et al., 2003). This fixed action pattern is regulated by circulating ecdysis triggering hormones (ETHs). ETHs, released by epitracheal gland Inka cells, orchestrate downstream peptidergic neuronal ensembles leading to sequential motor controls (Kim et al., 2006; Zitnan et al., 1996). Identification of ETH receptor genes (*ETHR-A* and *ETHR-B*) and cellular activity assays suggest that ETH signaling promotes G protein-mediated calcium elevation in target neurons (Kim et al., 2015; Park et al., 2003). Although Inka cells and associated transcripts of ETH and ETHRs persist into the adult stage *Drosophila* (Graveley et al., 2011; Park et al., 2002), the role of ETH signaling in adult behavior remains largely unknown.

We recently reported that ETH has an allatotropic function (promotion of juvenile hormone (JH) biosynthesis) in adult male and female *Drosophila* (Meiselman et al., 2017). ETHRs expressed in the JH secreting gland corpora allata (CA) regulate intracellular calcium levels in the response to ETH. In females, ETH-driven JH synthesis is essential for reproductive fitness. In males, this hormonal cascade also affects the male reproduction, possibly through the regulation of the accessory gland protein synthesis.

We also reported that the adult ETH-JH cascade is essential for *Drosophila* short-term memory (STM) retention of male flies in courtship conditioning (Lee et al., 2017, Chapter II). JH targets dopaminergic neurons (DA) for maintenance of STM.

In this study, I provide evidence that ETH and JH also are essential for long-term courtship memory (LTM). ETH promotes LTM indirectly through regulation of JH production. ETH and JH converge through direct actions on mushroom body (MB) γ-neurons to regulate protein synthesis-dependent LTM formation. My results not only reveal the importance of hormonal states in homeostatic neural functions, but also provide evidence that the endocrine system in *Drosophila* acts as a molecular promotor for social context-dependent behavioral plasticity.

## Materials and Methods

**Fly stocks.** *Drosophila melanogaster* stocks were maintained at 25℃ on standard cornmeal-agar media under a 12 hr light/dark (LD) regimen, except for certain experimental manipulations. *Canton-S* flies were used as wild-type. To reduce variation from genetic background, all lines in this study were outcrossed to a wild-type line, *wCS10* at least five generations. *JHAMT-GAL4* was obtained from B. Dauwalder (University of Houston) (Wijesekera et al., 2016). *ETH-GAL4* was obtained from D. Anderson (California Institute of Technology). *UAS-hid,rpr* was provided by P.

Taghert (Washington University). An *elav-GS-GAL4* driver *GSG301* was provided by R. Davis (The Scripps Research Institute Florida) (Osterwalder et al., 2001). *ETHR-GAL4* lines were provided by B. White (National Institute of Mental Health) (Diao et al., 2016). *4.59* line was provided from U. Heberlein (Janelia Research Campus). *UAS-ETHR RNAi-Sym* and *UAS-ETHR RNAi-IR2* lines are described previously (Kim et al., 2015). *UAS-Met RNAi* and *UAS-gce RNAi* lines were obtained from the Vienna Drosophila Research Center. The following Split GAL4 lines were obtained from Janelia Research Campus: *MB009B*, *MB011B*, *MB093C*, *MB131B*, *MB399B*. The following lines were obtained from the Bloomington Stock Center: *OK107*, *201Y*, *c739*, *1471*, *TH-GAL4*, *Orco-GAL4*, *Dilp2-GAL4*, *G0431*, *UAS-dTrpA1*, *UAS-mCD8-GFP*, *UAS-Shi$^{ts1}$*, *UAS-GCaMP5*, *UAS-RICIN$^{CS}$*.

**Courtship Conditioning and Statistical Analysis.** Animals were prepared for courtship conditioning as previously described (Lee et al., 2017). In short, males of appropriate strain were collected during the pupal stage and individually housed in clean glass tubes containing fresh fly food for 4 days posteclosion to prevent pre-test social interaction. To prevent anesthetic effects, I used a mouth aspirator transfer or collect animals. Trainer females were prepared by housing day 3-5 virgin *Canton-S* females with *Canton-S* males in food-containing glass tubes overnight. Immobilized tester females were prepared by decapitating day 4-5 virgin *Canton-S* females with fine scissors under $CO_2$ anesthesia. Protocols for long-term courtship conditioning are described previously (Ishimoto et al., 2009; Keleman et al., 2007), and I followed the same general strategies with some modifications. Briefly, a 4-day old test male was paired with a mated *Canton-S* female for 5-hours in the food chamber (2.0 ml tube). Trained (or sham-trained) males were individually housed in a fresh food tube for 24 hours prior to testing. For testing, courtship activity of a trained (or sham-trained) male toward an immobilized (decapitated) tester female was recorded for 10 min using a high-frame digital camcorder (SONY HDR-XR260V). All assays were manually scored

for courtship index, blind to the genotype and, as far as possible, the experimental condition. Positive contributions to courtship index (CI) included all courtship activities such as orienting, tapping, singing, licking, and attempting to copulate. Courtship memory is quantified through computation of a memory performance index (MPI), which is the relative reduction of CI of each trained male ($CI_T$) from the mean CI of sham-trained males ($^mCI_S$): MPI = ($^mCI_S$ – $CI_T$) / $^mCI_S$. Statistical analyses were carried out using the Mann-Whitney *U* test for the comparison between CI of trained and sham-trained males, and a custom R script to perform 100,000 random permutation test for comparison between individual MPI of each control group and test group (Keleman et al., 2007). Asterisks (*) indicate statistical difference between MPI of test males and that of each control group, except in cases indicated in figure legends. *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001; ns, not significant.

**Thermogenetic Controls for Gene Expression, Cell Excitability, and Protein Synthesis.** For the TARGET experiment shown in Figure III.2C, flies were raised at 19℃ during the juvenile period and individually housed at 19℃ for 6 days following eclosion. Flies were then moved to 31℃ until the courtship conditioning, which was performed at room temperature. In Figure III.10D and III.14A, flies were raised at 19℃ during the juvenile period. Immediately after the eclosion, they were transferred and housed at 31℃ for 4 days until training. After training at room temperature for 5-hours, animals were kept at 31℃ for 24 hours until testing, which was performed at room temperature.

For the shibire experiments (Figure III.1C), flies were raised at 19℃ during the juvenile period and housed at 31℃ for 4 days following eclosion. Animals were then kept at 31℃ during the training session and 24-hour post-training isolation. Testing was performed at room temperature.
For dTrpA1 experiments (Figure III.3 and III.4), male flies were raised at 19℃ during the first 4-days of adulthood, then housed at 31℃ or 21℃ with a mated female for the indicated period (5-,

3-, and 1-hour training). After a 24-hour isolation period at 19℃, memory performance was tested at room temperature.

For conditional RICIN expression (Figure III.16), males were raised at 19℃ until courtship conditioning, then trained at 20℃ for 5 hours. Trained or sham-trained animals were then transferred to 30℃ and kept for 24 hours until testing, which were performed at room temperature (Chen et al., 2012).

**Drug Treatment.** Drug treatment associated with GeneSwitch experiments was described previously (McGuire et al., 2004). Briefly, flies were fed with 200 μM mifepristone (RU486, Sigma) with normal fly food immediately after eclosion for four days (Figure III.1B), and from day 4 to 7 (Figure III.5) until courtship conditioning. Ethanol (1.6 %) was used as vehicle. Methoprene (64.4 pmol; 1x or 644 pmol; 10x) was applied topically in acetone to the ventral side of the abdomen of day 0 posteclosion males held under cold anesthesia (1–2℃) using a Nanoject II (Drummond). Cycloheximide (CXM) treatment was modified from a previous protocol (Tully *et al.*, 1994). Flies were fed with 35 mM cycloheximide (Sigma) in 5% glucose dissolved in 3% ethanol at 19℃ for 12 hours before training or sham-training and for 24 hours after the training until testing.

**Immunohistochemistry.** Brains dissected from adults 4-5 days after eclosion were fixed in 4% paraformaldehyde overnight at 4℃, washed with PBS and with PBST (0.5% Triton X-100 in PBS), then blocked in 3% normal goat serum (NGS) for 1 hour at room temperature. Brains were incubated with primary antibodies in PBST with 3% NGS at 4℃ overnight, followed by incubation in fluorophore-conjugated secondary antibodies overnight at 4℃. Brains were mounted in Aqua Poly/Mount on slides for imaging. Primary antibodies used here are rabbit anti-GFP IgG (1:500, Invitrogen) and mouse anti-Bruchpilot (nc82) (1:50, DSHB). Fluorophore-conjugated secondary antibodies used are Alexa Fluor 488 goat anti-rabbit (1:200, Invitrogen) and Alexa Fluor 568 goat anti-mouse (1:200, Invitrogen). MB structure was detected using the mouse anti-Fas II (1:10,

DSHB), and visualized by Alexa Fluor 488 goat anti-mouse (1:200, Invitrogen) or Alexa Fluor goat anti-mouse (1:200, Invitrogen). Confocal images were acquired with a Zeiss LSM 510 microscope. To obtain clearer expression patterns for some GAL4 lines (Figure III.14B), confocal image colors were inverted using raster graphics software (Adobe Photoshop CC).

**Functional Brain $Ca^{2+}$ imaging.** Calcium dynamics were monitored via functional imaging utilizing genetically encoded calcium indicator GCaMP5 (Akerboom et al., 2012). GCaMP is expressed in specific neuronal populations with the GAL4/UAS system. For the functional imaging, brains were dissected, maintained in an ice-chilled fly saline while the recording. Calcium-mediated fluorescence responses were visualized with a CCD camera (TILL-Imago) mounted on an Olympus BX51W1 and captured with Live Acquisition software. Excitation (488 nm; 50/1,000 msec excitation/duration) was provided by a Polychrome V monochrmomator. Following 2 min of pre-application sampling, *Drosophila* ETH1 was applied.

## Results

**ETH signaling is required for LTM in courtship conditioning.** Mature virgin *Drosophila* females are generally receptive to male courtship advances, whereas recently mated females reject mating attempts. Males experiencing rejection or exposure to male-specific anti-aphrodisiac pheromones from mated females suppress subsequent courtship attempts when encountering females, even fully receptive virgins. Depending on the extent of sexual deprivation during the pairing with a mated female, male courtship suppression toward a new partner can be shorter (~30 min following 1-hour training) or longer (at least 24 hours after 5 to 7-hour training). These are considered as courtship STM and LTM of male Drosophila (McBride et al., 1999).

In the previous chapter, I showed that disruption of the ETH-JH signaling cascade impairs courtship STM retention, whereby a normal male suppresses courting activities by ~30% toward an immobilized virgin female 10 minutes after a 1-hour training interval with a mated female (Lee et al., 2017). To investigate whether disruption of ETH signaling also affects courtship LTM, I applied a long-term courtship conditioning protocol depicted in Figure III.1A. I first tested whether ETH deficiency affects LTM by conditionally ablating Inka cells posteclosion in order to avoid lethal ecdysis deficiencies during development (Park et al., 2002). This was accomplished by preparing transgenic flies expressing apoptotic genes (*hid* and *rpr*) specifically in Inka cells using the drug-inducible GeneSwitch driver (*ETH-GS-GAL4* line, *EUG8*) (Cho et al., 2014). I found that adult-specific Inka cell ablation by feeding males the GAL4 activator RU486 causes memory loss; RU486 feeding had no effect on memory performance of a genetic control group (Figure III.1B).

I next used an alternative strategy for creating ETH deficiency by expressing the dominant-negative, temperature-sensitive dynamin mutant shibire (*UAS-Shi$^{ts}$*) using an Inka cell-specific driver (*ETH-GAL4*). Perturbing vesicle release at the restrictive temperature during both maturation and courtship conditioning periods suppressed LTM (Figure III.1C). This indicates that vesicular release from Inka cells is essential for LTM formation.

**ETH-JH cascade regulates courtship LTM.** I previously showed that ETH signaling is required for STM retention through promotion of JH biosynthesis (Lee et al., 2017, Chapter II). To investigate whether this hormonal cascade also regulates LTM, I subjected JH-deficient males (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym*) to long-term courtship conditioning and found impaired LTM (Figure. III.2A).

To confirm the necessity of JH in LTM performance, I employed two experimental strategies. First, I performed applied the JH analog methoprene to JH-deficient males and measured partial, but significant rescue of LTM memory deficiency. Application of a higher methoprene dose (10x,

644 pmol) likely improved the rescue, but this treatment failed to reach statistical significance compared to treatment with the lower dose (1x) of methoprene. Methoprene application had no effect on courtship LTM of the control group (Figure III.2B).

I next confined ETHR knockdown in the CA to the adult period using temperature-sensitive GAL80$^{ts}$ (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym;TubPGAL80$^{ts}$*). I found that adult-specific ETHR knockdown also significantly impaired LTM, whereas both genetic control groups under the identical experimental condition showed normal memory performance (Figure III.2C). Thus, formation of courtship LTM requires the ETH-JH signaling cascade during the adult stage.

**Augmented Inka cell activity enhances LTM in a protein synthesis-dependent manner.** I have shown that ablation of Inka cells or block of exocytotic release during adulthood suppresses LTM (Figure. III.1). To test whether augmented ETH release from Inka cells during courtship conditioning affects memory, I thermogenetically enhanced release through expression of the temperature-sensitive cation channel, *Drosophila* TrpA1 (dTrpA1) using the *ETH-GAL4* driver. Induction of dTrpA1 during the normal long-term training period (5-hour exposure to a mated female) at 31℃ likely improved memory performance of test males compared to GAL4 and UAS genetic control groups under the same conditions. MPI of test males trained at 21℃ showed no difference from genetic controls (Figure. III.3A-a).

Using the long-term courtship conditioning protocol described in the Materials and Methods section, male flies require a minimum of 5-hour training with a mated female. I asked whether augmented Inka cell activity through dTrpA1 activation reduces this training period and found that the training period required for significant LTM could be reduced not just to 3 hr, but to 1 hr. When paired with a mated female for either a 3-hour or 1-hour at 31℃, males showed significant 24 hr LTM (Figure III.3A-b and -c). These shorter training periods were not sufficient for the LTM formation in any of the genetic control groups.

In a broad range of previous studies on different animal paradigms, formation of LTM requires de novo protein synthesis. I therefore tested whether improved LTM memory performance gained through augmented Inka cell activity is protein synthesis-dependent by feeding *ETH-GAL4*/*UAS-dTrpA1* males the protein synthesis inhibitor, cycloheximide, which resulted in abolition of 24 hr MPI measured intest groups trained for 5-, 3-, or 1-hour (Figure III.A).

I next tested whether augmented Inka cell activity affects maintenance of LTM. Although *Drosophila* long-term courtship memory following 5-hour training with a mated female lasts at least a week, the level of memory performance decays gradually over a period of days (McBride et al., 1999). I found that improved memory performance by augmented Inka cell activity during the 5-hour training is extended an additional 24 hours compared to both genetic control groups, but is not significant by day 4 (Figure III.3B).

Since I have demonstrated that the ETH-JH signaling cascade is required for LTM and that augmented Inka cells activity enhances LTM, I asked whether augmented JH levels through expression of dTrpA1 in the CA would enhance LTM. I expressed dTrpA1 in the CA using the *JHAMT-GAL4* driver and found that, unlike enhancement obtained by augmenting Inka cell activity, training *JHAMT-GAL4*/*UAS-dTrpA1* at 31°C did not alter memory performance (Figure III.4). This finding, together with the result that methoprene does not affect LTM level in control groups (Figure III.2B), suggests that enhancement of LTM by augmented Inka cell activity does not occur through elevation of JH levels.

**Courtship LTM requires ETHR and Methoprene-tolerant (Met) expression in adult neurons.** Genes encoding ETHR and two JH-receptor paralogs (Methoprene-tolerant (Met) and germ cell-expressed (gce)) are expressed in the adult male brain (Baumann et al., 2010; Crocker et al., 2016). I asked whether cell-specific RNAi knockdown of these receptor genes affects courtship LTM. Since ETHRs and JH receptors in the CNS are required during development, Iemployed adult-

specific RNAi knockdown of the *ETHR* gene using the pan-neuronal, conditional Geneswitch driver *elav-GS-GAL4* (*GSG-301*) conditional driver by treatment with RU486. Employing ETHR knockdown with two independent RNAi constructs, (*UAS-ETHR RNAi-Sym* and *UAS-ETHR RNAi-IR2*) targeting distinct regions of the *ETHR* sequence (Kim et al., 2015), I obtained complete suppression of LTM performance (Figure III.5A).

I next performed conditional RNA knockdown of JH receptors with the same pan-neuronal driver. Since functional redundancy and compensation between the two receptor types have been reported (Abdou et al., 2011; Baumann et al., 2010), I expressed RNAi constructs for both Met and gce using *elav-GS-GAL4* (*UAS-gce RNAi;UAS-Met RNAi*). RU486 treatment of transgenic animals significantly impaired LTM. Subsequent experiments in which each JH receptor gene was silenced individually demonstrated that Met silencing alone is sufficient for LTM impairment. Silencing of gce alone results in no significant impairment of LTM (Figure III.5B).

I previously demonstrated that JH receptor expression in TH (tyrosine hydroxylase)-positive dopaminergic (DA) neurons is required for the courtship STM performance (Lee et al., 2017, Chapter II). I therefore asked whether DA neurons are necessary for LTM by silencing expression of both Met and gce using the *TH-GAL4* driver. Interestingly, transgenic animals showed no LTM impairment (Figure III.6). This indicates that, although JH targets DA neurons for regulation of STM, they are not required for LTM performance.

**ETH targets MB γ neurons to regulate courtship LTM.** I observed *ETHR* gene expression in broad areas of the mature male brain (Figure III.7A) and observed that many neurons express both subtypes of the alternatively spliced *ETHR* gene (*ETHR-A* and *ETHR-B*) (Figure III.7B). The expression pattern in antennal lobe (Figure III.8) is discussed in the Discussion section. Although expression of ETHR in Kenyon cells and MB neuropils is low (Crocker et al., 2016), I observed a small population of cells (approximately 2 μm diameter) present near the calyx (Figure III.9). Since

Kenyon cells and MB neuropils are considered as a main center for memory processes, I used the MB GAL4 driver *OK107* to test whether ETHR knockdown affects LTM (Figure III.10A). While ETHR knockdown in the entire MB had no effect on courtship STM (Figure III.10B), LTM performance was strongly suppressed using two independent dsRNA constructs (*UAS-ETHR RNAi-Sym* and *UAS-ETHR-IR2*) (Figure III.10C).

To confirm the requirement of ETHR expression in the MB for LTM, I applied the TARGET system to confine ETHR knockdown to the adult stage, again using the *OK107* driver. I found that ETHR knockdown impaired courtship LTM severely (Figure III.10D). ETHR knockdown did not affect the size or gross morphology of MB lobes (Figure III.11). These findings demonstrate that ETH regulates courtship LTM specifically through direct action on ETHR-expressing MB neurons.

The MB consists of three major classes of neuropil: the αβ, α′β′, and γ (Figure III.12A). To identify specific functional targets of ETH, I performed ETHR knockdown with a set of GAL4 drivers specific for the following neuropilar areas: αβ and γ (*201Y*); αβ (*c739*), α′β′ (*4.59*); γ (*1471*) (Figure III.12B). ETHR knockdown using the *201Y* and *1471* drivers impaired LTM significantly, whereas LTM performance was unaffected by ETHR silencing in the αβ lobe (*c739*) (Figure III.12C). Although I observed slight suppression of memory formation by ETHR knockdown in α′β′, reduction of MPI did not reach statistical significance. *Ex vivo* functional imaging of the male MB (*OK107*/*UAS-GCaMP5*) shows gradual elevation of $Ca^{2+}$-mediated fluorescence mainly in the γ-lobes of lateral neuropils of the MB in response to ETH application (Figure III.13). Taken together, mt results demonstrate that ETH signaling targets MB γ neurons to regulate courtship LTM.

**ETH regulates courtship LTM by targeting multiple memory-related neurons.** The ETHR expression pattern (Figure III.7) and a recent study (Crocker et al., 2016) suggest that ETH could regulate memory formation by targeting multiple neuronal circuits. Based on this, I applied the

TARGET system for conditional knockdown of ETHR in diverse sets of neurons (Figure III.14A): *Orco-GAL4* for olfactory receptor neurons (ORNs), *TH-GAL4* for most DA neurons, which include important input circuits for memory processes (Kaun and Rothenfluh, 2017), and *Dilp2-GAL4* for clusters of insulin like peptide (ILP)-producing cells in the pars intercerebralis. Recent studies reported that misregulation of insulin signaling in the MB causes defects in olfactory and courtship memories (Chambers et al., 2015; Monyak et al., 2016).

In addition, I employed Split GAL4 lines *MB009B* and *MB131B* targeting MB γ neurons exclusively (Masek et al., 2015), *MB093C*, *MB011B*, and *MB399B* for MB output neurons (MBONs) (Aso et al., 2014), and the dorsal anterior lateral (DAL) neuronal driver, *G0431*. A previous study provided evidence that a single pair of DAL neurons belonging to the set of dorsal lateral neurons (LNd) are essential for memory consolidation in olfactory conditioning (Chen et al., 2012). Consistent with the previous result, posteclosion ETHR silencing in MB γ neurons suppressed LTM. Furthermore, ETHR silencing in DAL neurons also impaired LTM significantly. However, ETHR knockdown in ORNs, DA neurons, and ILP secretory cells resulted in no significant change in memory performance. Although a MBON driver shows weak memory performance following ETHR silencing (*MB399B*), all MBON driver lines I tested show no statistical difference in memory performance.

I next tested sensitivity of MB γ neurons and DAL neurons to ETH using $Ca^{2+}$ imaging by crossing *MB131B* and *G0431* GAL4 lines associated with the strongest memory phenotypes (Figure III.15A and III.15B) with a *UAS-GCaMP5* line. ETH application led $Ca^{2+}$ mobilization in both MB γ neurons (Figure III.15A) and DAL neurons (Figure III.15B).

Since enhancement of LTM by augmented Inka cell activity during courtship conditioning is protein synthesis-dependent (Figure III.3A), I tested whether protein synthesis in MB γ lobe and/or DAL neurons is required for courtship LTM by conditionally expressing the ribosomal inactivator

RICIN specifically in these neurons during the post-training interval period (24-hour). RICIN expression in DAL neurons appears to suppress LTM ($P = 0.073$), whereas expression in MB γ-neurons had no significant effect. (Figure III.16).

**Convergence of ETH and JH regulation of LTM circuits.** A recent study of JH receptor expression patterns in *Drosophila* (Baumann et al., 2017) demonstrated strong expression of Met in MB lobes and lateral dorsal neurons (LNd), which include DAL neurons. I therefore tested whether Met knockdown in these neurons compromises LTM. RNA silencing of Met using the *OK107-GAL4* driver resulted in strong suppression of LTM performance. Similar to results obtained by silencing ETHR expression, Met knockdown in MB γ lobe-containing enhancer trap lines showed significant memory impairment (Figure III.17). However, silencing Met expression in *c739* (αβ) or *4.59* (α′β′) line had no effect on LTM performance. Since these enhancer trap lines also target other areas of the brain, I performed Met knockdown using the more specific MB γ lobe-directed Split GAL4 lines *MB009B* and *MB131B*. These experiments confirm that Met expression in the MB γ lobe plays a critical role in LTM function. Although Met silencing in DAL neurons likely affects memory performance ($P = 0.095$), there was less statistical significance in memory performance. These results provide evidence that JH and ETH signaling converges on MB γ neurons to regulate courtship LTM performance.

## Discussion

I have demonstrated that hormonal state has a profound influence on formation of courtship LTM in *Drosophila*. ETH signaling operates both directly and indirectly to promote memories: (i) direct actions target the MB γ-lobe and memory consolidation DAL neurons; (ii) indirect actions involve promotion of JH production as an allatotropin. ETH and JH converge on MB γ-neurons essential

for LTM formation. Augmentation of ETH levels during training enhances memory and reduces the minimum training interval for LTM from 5 hr to 1 hr. I find ETH promotion of LTM is protein synthesis-dependent and that the signaling pathway is independent of the STM pathway described previously (Lee et al., 2017, Chapter II). I propose a model that incorporates an endocrine cascade in LTM formation following stressful, aversive social experiences (Figure III.18).

**A direct hormonal pathway for courtship memory modulation: ETH targeting of MB and DAL neurons.** Disruption of epitracheal Inka cell function in mature adult males impairs courtship memory performance, demonstrating that ETH signaling is required for memory formation. We reported that the steroid hormone ecdysone (20E) up-regulates biosynthesis of ETH in juveniles (Cho et al., 2014) and adults (Meiselman et al., 2017), and that decline of 20E confers secretory competence and release of ETH from Inka cells. Ishimoto *et al.* reported that long-term courtship conditioning leads to increased ecdysone levels in male flies (Ishimoto et al., 2009). I therefore propose that unsuccessful or aversive sexual experiences of males may induce ETH production through elevation of ecdysone.

Direct actions of ETH on two distinct brain centers - MB γ-neurons and consolidation DAL neurons - were identified by targeted RNA silencing of ETHRs. Not only is LTM dependent on ETH signaling, but reinforced Inka cell activity during courtship conditioning augments courtship suppression, which is interpreted as memory enhancement. Cycloheximide treatment eliminates enhanced LTM, indicating that improved memory by ETH release during courtship training is *de novo* protein synthesis-dependent (Figure III.3).

ETHR silencing in candidate memory circuit neurons revealed that ETH signaling affects the function of the MB γ lobe and the DAL neurons, which have important roles in post-training memory processes such as consolidation, storage, and retrieval of information (Chen et al., 2012; Dubnau et al., 2001; Qin et al., 2012). In particular, expression of Orb2, a cytoplasmic

polyadenylation element-binding (CPEB) protein in the MB γ lobe is essential for *Drosophila* courtship memory (Keleman et al., 2007). CPEB is considered as an important regulator for local protein synthesis in neurons thereby affecting synaptic strength. However, conditional RICIN activation in MB γ neurons during the consolidation period does not affect memory performance by olfactory conditioning (Chen et al., 2012) and our courtship conditioning (Figure III.16). I hypothesize that the MB γ lobe may contain anti-aphrodisiac pheromone (e.g., cVA)-specific memory circuits, and *de novo* protein synthesis in these neurons have important functions when trained males encounter cVA associated with mated female testers (Keleman et al., 2012). In contrast to this conditioning protocol, I employed mature virgin females as tester. My results indicate that different sets of neurons in γ lobe may be involved in our courtship memory formation with protein synthesis-independent mechanism. Moreover, using aversive olfactory conditioning, Chen *et al*. also showed that protein synthesis in DAL neurons contributes to memory consolidation after the spaced training (Chen et al., 2012). Since silencing of ETHR in these neurons impaired LTM and blocking synthesis in these neurons suppressed the memory performance in our courtship conditioning, I argue that the ETH signaling-mediated protein synthesis in DAL neurons may play a general role for consolidation of aversive memory.

A clock gene, *period* (*per*) is essential for retrieval of courtship memory (Sakai et al., 2004), and its expression in DAL neurons is involved in olfactory memory consolidation (Chen et al., 2012). However, PER expression in DAL neurons is not required for the courtship LTM (Sakai et al., 2012). I hypothesize that ETHR-mediated downstream signaling molecules in DAL neurons may be involved in the homeostasis of protein synthesis. Even though PER expression in these neurons is not critical role for courtship memory, the ETHR signaling pathway may involve additional steps critical for regulation of memory-forming effectors such as the cAMP-responsive element binding (CREB) protein (Yin et al., 1995).

In line with previous observations of ETHR-expressing peptidergic neurons of juvenile animals (Diao et al., 2016; Kim et al., 2006) ETH exposure mobilizes $Ca^{2+}$ in central neurons of the mature male (Figure III.15). Thus, I hypothesize that ETHR-mediated intracellular $Ca^{2+}$ signaling may be a key sequential modulator for LTM formation. In a broad range of animal species, $Ca^{2+}$ plays a critical role in memory formation. For example, $Ca^{2+}$ entry through glutamate receptors in mammalian cortical neurons promotes phosphorylation of eEF2, a GTPase that mediates ribosomal translocation along mRNA following peptide bond formation, and therefore affects long-lasting memory (Belelovsky et al., 2005). The significance of cytoplasmic or nuclear $Ca^{2+}$ levels in memory formation also has been suggested by several lines of evidence in both vertebrate and invertebrate models (Meneses, 2007; Perisse et al., 2009; Weislogel et al., 2013).

In addition to targeting memory circuits, ETH may also affect pheromone sensitivity, which can influence behavior during the courtship conditioning. As illustrated in Figure III.7 and III.8, ETHRs are likely present in primary olfactory neurons projecting to specific olfactory glomeruli such as DA1 (Or67d), VA6 (Or67d and/or Or82a), and DA2 (Or33a and/or Or56a). In particular, Or67d neuron senses a male-specific pheromone cVA (*cis*-vaccenyl acetate) (Kurtovic et al., 2007), which is sensed by males courting recently mated females (Ejima et al., 2007). Although previous studies showed the importance of sensing cVA in learning and memory induced by courtship conditioning, lines of evidence suggest that possible ETHR expression in Or67d neuron may not affect the memory formation of males in this study. First, although Keleman and colleagues reported that Or67d serves an essential input for courtship learning, they focused on aversive olfactory learning and retrieval processes using mated females as both trainer and tester (Keleman et al., 2012). Since I used mature virgin females as testers, I hypothesize that the possible expression of ETHR in Or67d neurons is less effective in our conditioning. Second, a study by Ejima et al. showed that, under experimental condition similar to ours, the cVA-sensing olfactory neuron Or65a

rather than Or67d plays a significant role in memory formation (Ejima et al., 2007). From the ETHR expression pattern in mature male brain, glomeruli innervated by Or65a (DL3) were not detected. Although Or65a is less sensitive to cVA than Or67d (van der Goes van Naters and Carlson, 2007), it has a critical function in chronic male aggression distinct from that of Or67d (Liu et al., 2011), indicating multimodal neural circuits for pheromone sensing. Based on this information, I conclude that ETH targeting of olfactory neurons, particularly Or67d, does not affect memory performance. This is confirmed by the fact that global silencing of ETHR in ORNs has no effect on LTM (Figure III.14A).

**Promotion of LTM by convergence of ETH and JH actions in the MB γ-lobe and DAL neurons.** ETH plays another important role in promoting LTM by maintaining JH at normal levels (Lee et al., 2017; Meiselman et al., 2017). Suppression of JH production by ETHR silencing in the corpora allata impairs male courtship memory. This indicates that JH is essential for normal memory performance. However, two observations in this study suggest that elevation of JH beyond normal levels do not improve memory performance. First, although methoprene treatment rescues LTM JH-deficient males, high doses (10X) do not improve memory performance in either JH-deficient or control males (Figure III.2B). Second, unlike the memory enhancement obtained following Inka cell activation, reinforced corpora allata function induced by TrpA1 channel activity during courtship conditioning does not improve LTM (Figure III.4). I thus hypothesize that JH in is necessary for maintenance of homeostasis in memory circuits.

I also found that the JH receptor Met is essential for courtship LTM (Figure III.5B). Interestingly, knockdown of Met expression in either the MB γ lobe or DAL neurons, both targets of ETH, diminishes LTM. Based on this finding, we propose a hormonal convergence model for memory formation induced by social experience.

In mammals, interactions of neuromodulatory influences within strategic brain areas play essential roles in emotional memory consolidation. Stress-induced release of glucocorticoids (cortisol in human) affect memory consolidation via both direct and indirect pathways that converge in the amygdala (McGaugh, 2004). In particular, secreted glucocorticoids promote activity of brain stem nuclei (nucleus of the solitary tract, NTS) that connect noradrenergic projections to the basolateral amygdala (BLA), thereby leading to memory formation. Glucocorticoid receptors present in the BLA also facilitate memory consolidation by potentiating noradrenergic signaling through the interactions with G protein-mediated cascades.

It remains unclear how convergence of ETH and JH signaling in key brain areas contributes to memory circuit function. As already discussed, G protein-mediated $Ca^{2+}$ signaling is critical for memory formation through diverse downstream mechanisms in many animals. Previous studies reported that JH induces protein phosphorylation in moth and mosquito, and provided possible collaboration between GPCR signaling and Met function for diverse cellular mechanisms (Arif et al., 2002; Liu et al., 2015). We thus hypothesize that ETH-driven calcium signaling may interact with the action of the JH receptor to enhance the presynaptic protein synthesis. It will be important to investigate the detailed mechanisms underlying ETH- and JH-mediated signaling at the molecular level.

It is widely accepted that LTM is formed through consolidation of STM, but in the circuit level, recent studies in *Drosophila* conditioning assays report parallel processes in STM and LTM (Blum et al., 2009; Trannoy et al., 2011). I previously reported that expression of JH receptors in TH-positive neurons is important for STM performance induced by courtship conditioning (Lee *et al.*, 2017, Chapter II). The function of JH in DA neurons likely contributes to maintenance of information, even during the short-term interval following courtship conditioning. However, I found in the current study that silencing of Met and gce in DA neurons does not affect LTM

performance (Figure III.6). Instead, Met expression in the MB γ lobe and DAL neurons is critical for LTM (Figure III.5B and III.17). Thus, my findings argue for parallel pathways in regulation in courtship STM and LTM through distinct neuronal targets of endocrine signals ETH and JH.

Expression patterns of JH receptors in the adult Drosophila CNS (Baumann et al., 2017) suggest that JH may also play roles in diverse behaviors such as locomotion, sleep, feeding, and courtship. In our recent study, we showed that disrupting expression of JH receptors negatively affects female detection by males (Lee et al., 2017, Chapter II). Lin et al. revealed that endocrine state as related to JH levels determines female pheromone sensitivity of young males. Met expression in Or47b neurons sensing female-enriched cuticular hydrocarbons (e.g., palmitoleic acid) is required for appropriate detection of a target female by the male (Lin et al., 2016). Therefore, establishing functions for the ETH-JH endocrine cascade in regulation of diverse adult behaviors provide numerous opportunities for further study.

In summary, I have identified novel hormonal pathways in regulation of *Drosophila* LTM. ETH modulates memory via direct and indirect actions: through $Ca^{2+}$-mediated mechanisms in MB lobe and memory consolidating DAL neurons, and through regulation of JH production, respectively. Influences of ETH and JH converge in specific areas of the brain critical for LTM formation. Thus, evidence generated in this study lead to a testable model (Figure III.18) for the essential involvement of hormonal state in formation of context-dependent social memories.

## References


Abdou, M.A., He, Q., Wen, D., Zyaan, O., Wang, J., Xu, J., Baumann, A.A., Joseph, J., Wilson, T.G., Li, S., et al. (2011). Drosophila Met and Gce are partially redundant in transducing juvenile hormone action. Insect Biochem Mol Biol 41, 938-945.

Akerboom, J., Chen, T.W., Wardill, T.J., Tian, L., Marvin, J.S., Mutlu, S., Calderon, N.C., Esposti, F., Borghuis, B.G., Sun, X.R., et al. (2012). Optimization of a GCaMP calcium indicator for neural activity imaging. J Neurosci 32, 13819-13840.

Arif, A., Shanavas, A., Murthy Ch, R., and Dutta-Gupta, A. (2002). Juvenile hormone stimulated tyrosine kinase-mediated protein phosphorylation in the CNS of the silk worm, Bombyx mori. Arch Insect Biochem Physiol 50, 139-146.

Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K.R., Vogt, K., Belliart-Guerin, G., Placais, P.Y., Robie, A.A., Yamagata, N., Schnaitmann, C., et al. (2014). Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. Elife 3, e04580.

Baumann, A., Barry, J., Wang, S., Fujiwara, Y., and Wilson, T.G. (2010). Paralogous genes involved in juvenile hormone action in Drosophila melanogaster. Genetics 185, 1327-1336.

Baumann, A.A., Texada, M.J., Chen, H.M., Etheredge, J.N., Miller, D.L., Picard, S., Warner, R., Truman, J.W., and Riddiford, L.M. (2017). Genetic tools to study juvenile hormone action in Drosophila. Sci Rep 7, 2132.

Belelovsky, K., Elkobi, A., Kaphzan, H., Nairn, A.C., and Rosenblum, K. (2005). A molecular switch for translational control in taste memory consolidation. Eur J Neurosci 22, 2560-2568.

Blum, A.L., Li, W., Cressy, M., and Dubnau, J. (2009). Short- and long-term memory in Drosophila require cAMP signaling in distinct neuron types. Curr Biol 19, 1341-1350.

Chabaud, M.A., Isabel, G., Kaiser, L., and Preat, T. (2009). Social facilitation of long-lasting memory retrieval in Drosophila. Curr Biol 19, 1654-1659.

Chambers, D.B., Androschuk, A., Rosenfelt, C., Langer, S., Harding, M., and Bolduc, F.V. (2015). Insulin signaling is acutely required for long-term memory in Drosophila. Front Neural Circuits 9, 8.

Chen, C.C., Wu, J.K., Lin, H.W., Pai, T.P., Fu, T.F., Wu, C.L., Tully, T., and Chiang, A.S. (2012). Visualizing long-term memory formation in two neurons of the Drosophila brain. Science 335, 678-685.

Cho, K.H., Daubnerova, I., Park, Y., Zitnan, D., and Adams, M.E. (2014). Secretory competence in a gateway endocrine cell conferred by the nuclear receptor betaFTZ-F1 enables stage-specific ecdysone responses throughout development in Drosophila. Dev Biol 385, 253-262.

Crocker, A., Guan, X.J., Murphy, C.T., and Murthy, M. (2016). Cell-Type-Specific Transcriptome Analysis in the Drosophila Mushroom Body Reveals Memory-Related Changes in Gene Expression. Cell Rep 15, 1580-1596.

Diao, F., Mena, W., Shi, J., Park, D., Diao, F., Taghert, P., Ewer, J., and White, B.H. (2016). The Splice Isoforms of the Drosophila Ecdysis Triggering Hormone Receptor Have Developmentally Distinct Roles. Genetics 202, 175-189.

Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. Nature 411, 476-480.

Ejima, A., Smith, B.P., Lucas, C., van der Goes van Naters, W., Miller, C.J., Carlson, J.R., Levine, J.D., and Griffith, L.C. (2007). Generalization of courtship learning in Drosophila is mediated by cis-vaccenyl acetate. Curr Biol 17, 599-605.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., et al. (2011). The developmental transcriptome of Drosophila melanogaster. Nature 471, 473-479.

Ishimoto, H., Sakai, T., and Kitamoto, T. (2009). Ecdysone signaling regulates the formation of long-term courtship memory in adult Drosophila melanogaster. Proc Natl Acad Sci U S A 106, 6381-6386.

Ishimoto, H., Wang, Z., Rao, Y., Wu, C.F., and Kitamoto, T. (2013). A novel role for ecdysone in Drosophila conditioned behavior: linking GPCR-mediated non-canonical steroid action to cAMP signaling in the adult brain. PLoS Genet 9, e1003843.

Kaun, K.R., and Rothenfluh, A. (2017). Dopaminergic rules of engagement for memory in Drosophila. Curr Opin Neurobiol 43, 56-62.

Keleman, K., Kruttner, S., Alenius, M., and Dickson, B.J. (2007). Function of the Drosophila CPEB protein Orb2 in long-term courtship memory. Nat Neurosci 10, 1587-1593.

Keleman, K., Vrontou, E., Kruttner, S., Yu, J.Y., Kurtovic-Kozaric, A., and Dickson, B.J. (2012). Dopamine neurons modulate pheromone responses in Drosophila courtship learning. Nature 489, 145-149.

Kim, D.H., Han, M.R., Lee, G., Lee, S.S., Kim, Y.J., and Adams, M.E. (2015). Rescheduling Behavioral Subunits of a Fixed Action Pattern by Genetic Manipulation of Peptidergic Signaling. PLoS Genet 11, e1005513.

Kim, Y.J., Zitnan, D., Galizia, C.G., Cho, K.H., and Adams, M.E. (2006). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr Biol 16, 1395-1407.

Kurtovic, A., Widmer, A., and Dickson, B.J. (2007). A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature 446, 542-546.

Lee, S.S., Ding, Y., Karapetians, N., Rivera-Perez, C., Noriega, F.G., and Adams, M.E. (2017). Hormonal Signaling Cascade during an Early-Adult Critical Period Required for Courtship Memory Retention in Drosophila. Current Biology.

Lin, H.H., Cao, D.S., Sethi, S., Zeng, Z., Chin, J.S., Chakraborty, T.S., Shepherd, A.K., Nguyen, C.A., Yew, J.Y., Su, C.Y., et al. (2016). Hormonal Modulation of Pheromone Detection Enhances Male Courtship Success. Neuron 90, 1272-1285.

Liu, P., Peng, H.J., and Zhu, J. (2015). Juvenile hormone-activated phospholipase C pathway enhances transcriptional activation by the methoprene-tolerant protein. Proc Natl Acad Sci U S A 112, E1871-1879.

Liu, W., Liang, X., Gong, J., Yang, Z., Zhang, Y.H., Zhang, J.X., and Rao, Y. (2011). Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in Drosophila. Nat Neurosci 14, 896-902.

Masek, P., Worden, K., Aso, Y., Rubin, G.M., and Keene, A.C. (2015). A dopamine-modulated neural circuit regulating aversive taste memory in Drosophila. Curr Biol 25, 1535-1541.

McBride, S.M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G., and Siwicki, K.K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron 24, 967-977.

McGaugh, J.L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci 27, 1-28.

McGuire, S.E., Roman, G., and Davis, R.L. (2004). Gene expression systems in Drosophila: a synthesis of time and space. Trends Genet 20, 384-391.

Meiselman, M., Lee, S.S., Tran, R.T., Dai, H., Ding, Y., Rivera-Perez, C., Wijesekera, T.P., Dauwalder, B., Noriega, F.G., and Adams, M.E. (2017). Endocrine network essential for reproductive success in Drosophila melanogaster. Proc Natl Acad Sci U S A 114, E3849-E3858.

Meneses, A. (2007). Stimulation of 5-HT1A, 5-HT1B, 5-HT2A/2C, 5-HT3 and 5-HT4 receptors or 5-HT uptake inhibition: short- and long-term memory. Behav Brain Res 184, 81-90.

Monyak, R.E., Emerson, D., Schoenfeld, B.P., Zheng, X., Chambers, D.B., Rosenfelt, C., Langer, S., Hinchey, P., Choi, C.H., McDonald, T.V., et al. (2016). Insulin signaling misregulation underlies circadian and cognitive deficits in a Drosophila fragile X model. Mol Psychiatry.

Osterwalder, T., Yoon, K.S., White, B.H., and Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. Proc Natl Acad Sci U S A 98, 12596-12601.

Park, Y., Filippov, V., Gill, S.S., and Adams, M.E. (2002). Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development 129, 493-503.

Park, Y., Kim, Y.J., Dupriez, V., and Adams, M.E. (2003). Two subtypes of ecdysis-triggering hormone receptor in Drosophila melanogaster. J Biol Chem 278, 17710-17715.

Perisse, E., Raymond-Delpech, V., Neant, I., Matsumoto, Y., Leclerc, C., Moreau, M., and Sandoz, J.C. (2009). Early calcium increase triggers the formation of olfactory long-term memory in honeybees. BMC Biol 7, 30.

Qin, H., Cressy, M., Li, W., Coravos, J.S., Izzi, S.A., and Dubnau, J. (2012). Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in Drosophila. Curr Biol 22, 608-614.

Sakai, T., Inami, S., Sato, S., and Kitamoto, T. (2012). Fan-shaped body neurons are involved in period-dependent regulation of long-term courtship memory in Drosophila. Learn Mem 19, 571-574.

Sakai, T., Tamura, T., Kitamoto, T., and Kidokoro, Y. (2004). A clock gene, period, plays a key role in long-term memory formation in Drosophila. Proc Natl Acad Sci U S A 101, 16058-16063.

Shohat-Ophir, G., Kaun, K.R., Azanchi, R., Mohammed, H., and Heberlein, U. (2012). Sexual deprivation increases ethanol intake in Drosophila. Science 335, 1351-1355.

Siegel, R.W., and Hall, J.C. (1979). Conditioned responses in courtship behavior of normal and mutant Drosophila. Proc Natl Acad Sci U S A 76, 3430-3434.

Trannoy, S., Redt-Clouet, C., Dura, J.M., and Preat, T. (2011). Parallel processing of appetitive short- and long-term memories in Drosophila. Curr Biol 21, 1647-1653.

Tully, T., Preat, T., Boynton, S.C., and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in Drosophila. Cell 79, 35-47.

van der Goes van Naters, W., and Carlson, J.R. (2007). Receptors and neurons for fly odors in Drosophila. Curr Biol 17, 606-612.

Weislogel, J.M., Bengtson, C.P., Muller, M.K., Hortzsch, J.N., Bujard, M., Schuster, C.M., and Bading, H. (2013). Requirement for nuclear calcium signaling in Drosophila long-term memory. Sci Signal 6, ra33.

Wijesekera, T.P., Saurabh, S., and Dauwalder, B. (2016). Juvenile Hormone Is Required in Adult Males for Drosophila Courtship. PLoS One 11, e0151912.

Yin, J.C., Del Vecchio, M., Zhou, H., and Tully, T. (1995). CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in Drosophila. Cell 81, 107-115.

Zitnan, D., Kingan, T.G., Hermesman, J.L., and Adams, M.E. (1996). Identification of ecdysis-triggering hormone from an epitracheal endocrine system. Science 271, 88-91.

Zitnan, D., Zitnanova, I., Spalovska, I., Takac, P., Park, Y., and Adams, M.E. (2003). Conservation of ecdysis-triggering hormone signalling in insects. J Exp Biol 206, 1275-1289.

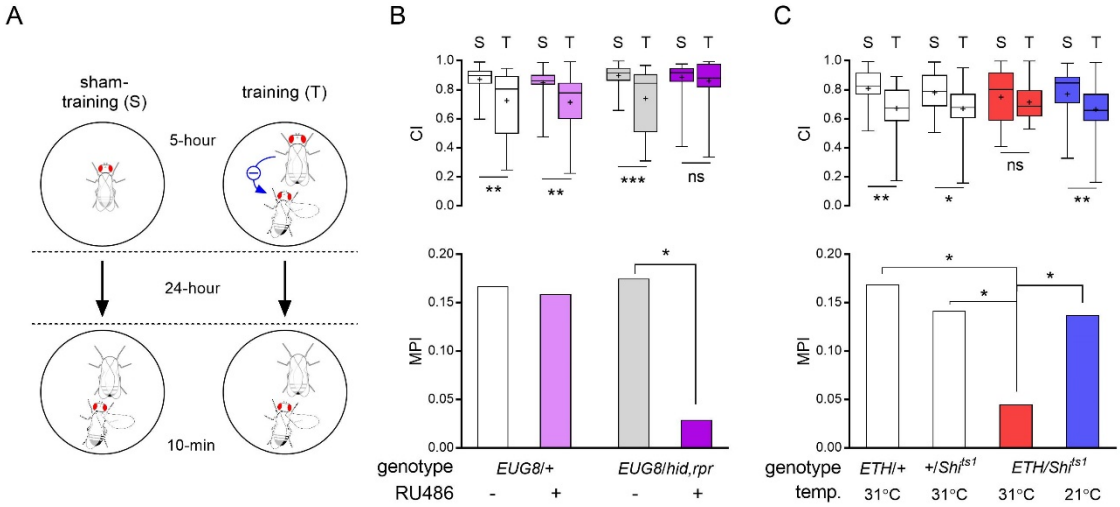



**Figure III.1. ETH signaling is essential for *Drosophila* courtship LTM.** (A) Schematic of long-term courtship conditioning procedure. (B) The role of adult ETH in the LTM was investigated by ectopically expressing apoptotic genes in Inka cells. Transgenic animals showed significantly less courtship (2.9%) when they were applied with the GAL4 activator (RU486+). (C) Conditional blocking of vesicle release from Inka cells was achieved by expressing shibire in Inka cells. Courtship conditioning except testing (at room temp.) was performed at the restrictive temperature. Transgenic flies less suppressed courtship activities by the training (4.5%) than those of genetic control. The permissive temperature (21℃) did not induce the memory impairment of transgenic males (n = 48-52). Statistical significance was tested using Mann-Whitney *U* test (CI) and random permutation test (MPI). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

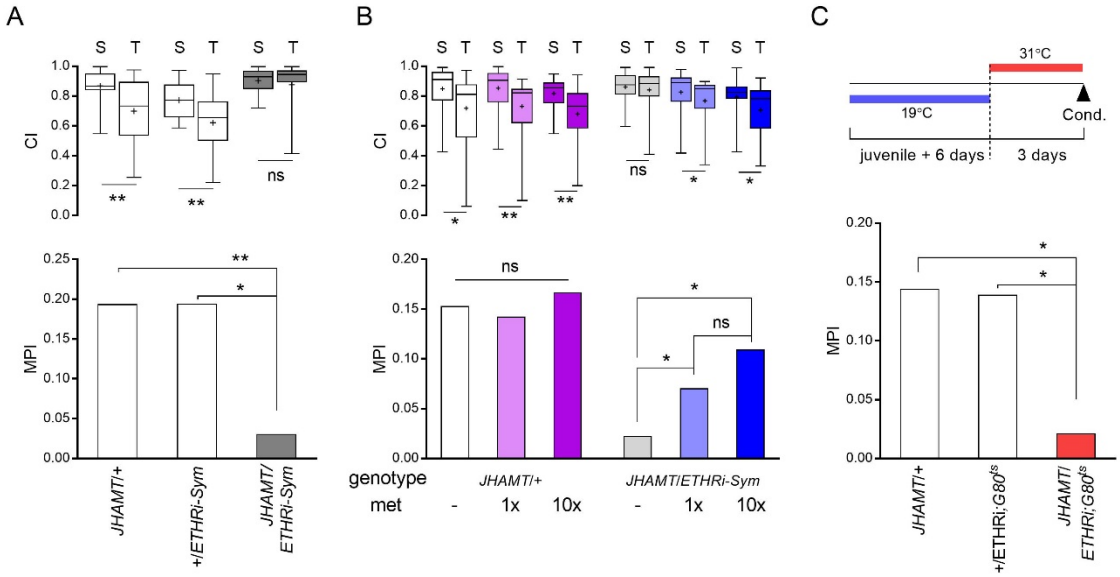



**Figure III.2. ETHR-mediated ETH-JH hormonal cascade is required for courtship LTM.** (A) The role of ETH signaling-driven JH synthesis in LTM was studied by ETHR knockdown in the CA. CA-specific RNA silencing caused memory defect (3.1% courtship suppression), whereas both genetic control groups are normal (n = 50-64). (B) JH analog methoprene (met) was topically applied on the ETHR-silenced males to investigate the role of JH in adult male's LTM performance (n = 48-62). "-", vehicle (acetone); "met", methoprene (1x, 64.4 pmol; 10x, 644 pmol per animal). Vehicle-treated *JHAMT-GAL4*/*UAS-ETHR RNAi* males suppressed 2.3% courtship, and 1x met-treated males showed 7.0% of courtship suppression by training. The MPI of 10x met-treated males is 10.9%. (C) Conditional ETHR silencing in the CA was performed utilizing the TARGET system Adult-specific ETHR-silenced males showed 2.1% memory by the prior training. (n = 58-66).

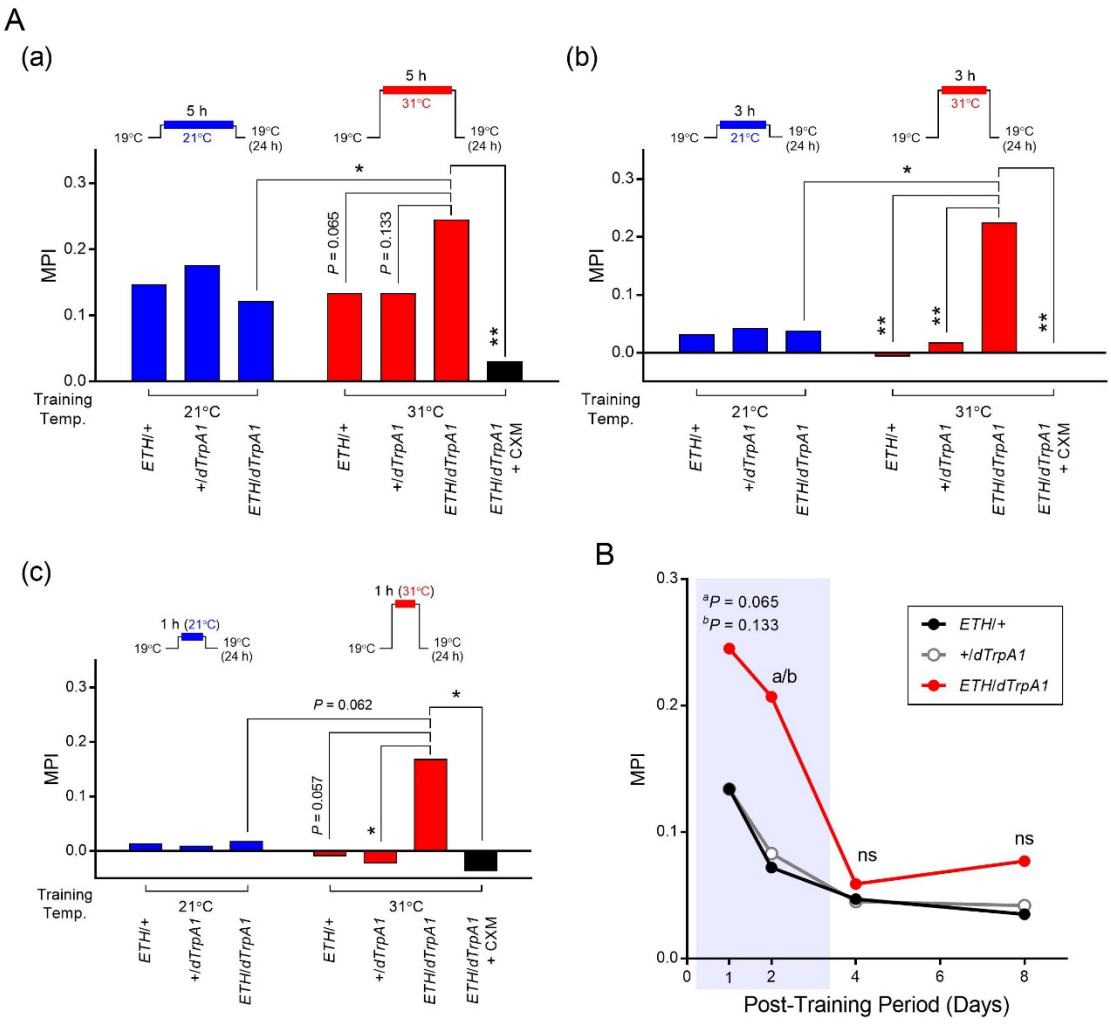

A
(a)
5 h
21°C
19°C
19°C (24 h)
5 h
31°C
19°C
19°C (24 h)
MPI
0.3
0.2
0.1
0.0
*
P = 0.065
P = 0.133
**
Training Temp.
21°C
31°C
ETH/+
+/dTrpA1
ETH/dTrpA1
ETH/dTrpA1 + CXM
(b)
3 h
21°C
3 h
31°C
(c)
1 h (21°C)
1 h (31°C)
P = 0.062
P = 0.057
B
aP = 0.065
bP = 0.133
a/b
ns
ns
ETH/+
+/dTrpA1
ETH/dTrpA1
0
1
2
4
6
8
Post-Training Period (Days)

**Figure III.3. Activity of Inka cells during the training improves LTM via protein synthesis.** Adult Inka cells were specifically, and temporally activated during 5- (A-a), 3- (A-b), and 1-hour (A-c) training sessions with a mated female at 31℃ utilizing dTrpA1 overexpression in Inka cells. (A-a) *ETH-GAL4*/*UAS-dTrpA1* suppressed 24.5% courtship activity by 5-hour training at 31℃, whereas GAL4 and UAS genetic control groups suppressed 13.4% for both by the same condition. Test males trained at 21℃ suppressed 12.2% courtship activity toward tester virgin females. ETH-GAL4/UAS-dTrpA1 showed 22.5% (A-b) and 16.9% (A-c) MPI by 3- or 1-hour training, respectively. 35 mM Cycloheximide (CXM) was applied to transgenic animals for 12 hours before the training and for 24 hours between the training and testing sessions. Random-permutation test, $*P < 0.05$, $**P < 0.01$ (n = 60-80). (B) Effect of Inka cell activation during 5-hour training on memory persistence. Training and sham-training of all animals were performed at 31℃. “a” and “b” represent statistical differences between data from test animals and GAL4 and UAS genetic control groups, respectively. $^{a/b}P < 0.05$ (n = 64-78).

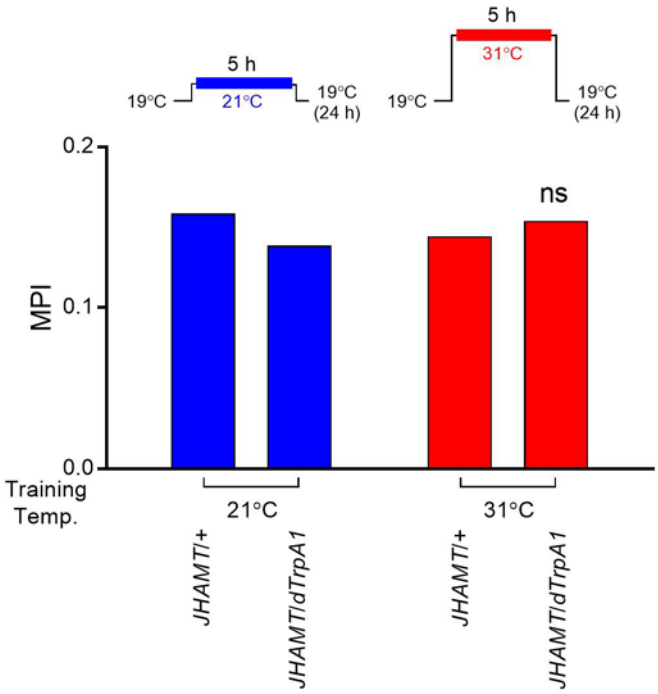



**Figure III.4. Reinforced CA activity during the training does not affect memory performance.** Adult CA was temporally activated during the 5-hour training with a mated female using dTrpA1 overexpression. Reinforced TrpA1 channel activity in CA cells during 5-hour training period did not change courtship LTM performance (n = 64-66).

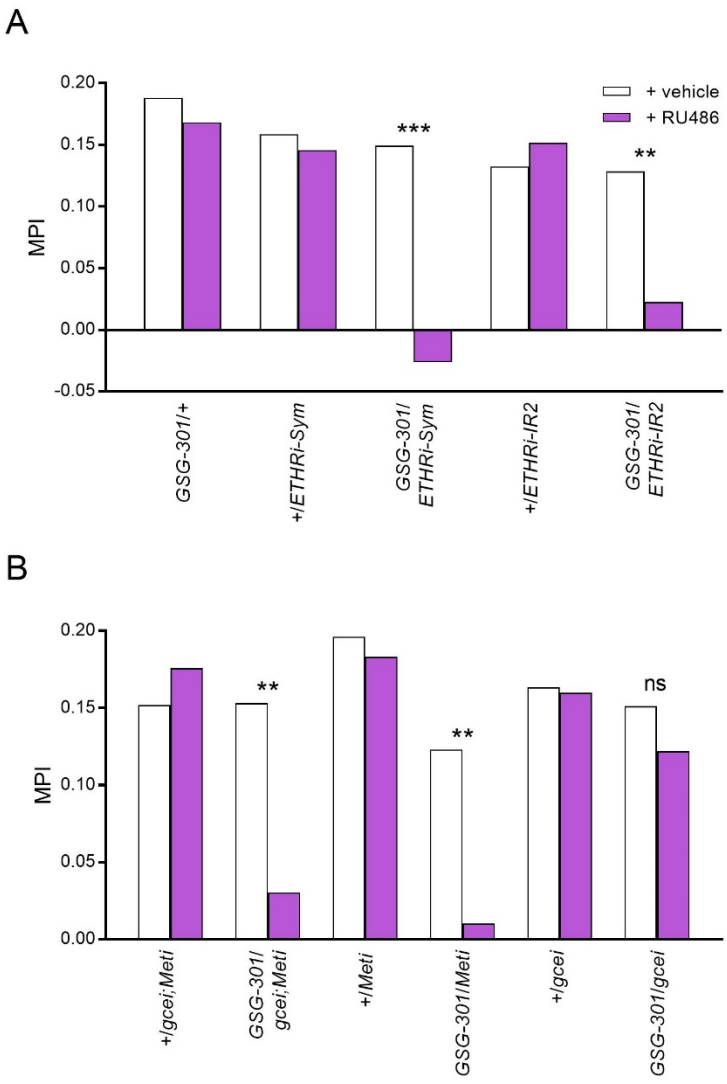

A
+ vehicle
+ RU486
MPI
0.20
0.15
0.10
0.05
0.00
-0.05
***
**
GSG-301/+
+/ETHRi-Sym
GSG-301/ ETHRi-Sym
+/ETHRi-IR2
GSG-301/ ETHRi-IR2
B
MPI
0.20
0.15
0.10
0.05
0.00
**
**
ns
+/gcei;Meti
GSG-301/ gcei;Meti
+/Meti
GSG-301/Meti
+/gcei
GSG-301/gcei

**Figure III.5. Expression of ETHR and Met in adult nervous system is required for courtship LTM formation.** Conditional knockdown of ETHR- (A) and JH receptor-encoding genes (B) was performed using a drug-inducible pan-neuronal driver *elav-GS-GAL4* (*GSG-301*) by feeding RU486 for 3 days to day-4 males. (A) LTM is lost in both *GSG-301*/*UAS-ETHR RNAi-Sym* males and *GSG-301*/*UAS-ETHR RNAi-IR2* males. (B) Loss of LTM in *GSG-301*/*UAS-gce RNAi;UAS-Met RNAi* and *GSG-301*/*UAS-Met RNAi* males, whereas *GSG-301*/*UAS-gce RNAi* males showed 12.2% MPI. Vehicle treatment did not affect memory phenotype and genetic control groups did not show significant change in LTM level following drug treatment. Random permutation test, $^{**}P < 0.01$, $^{***}P < 0.001$ (n = 60-68).

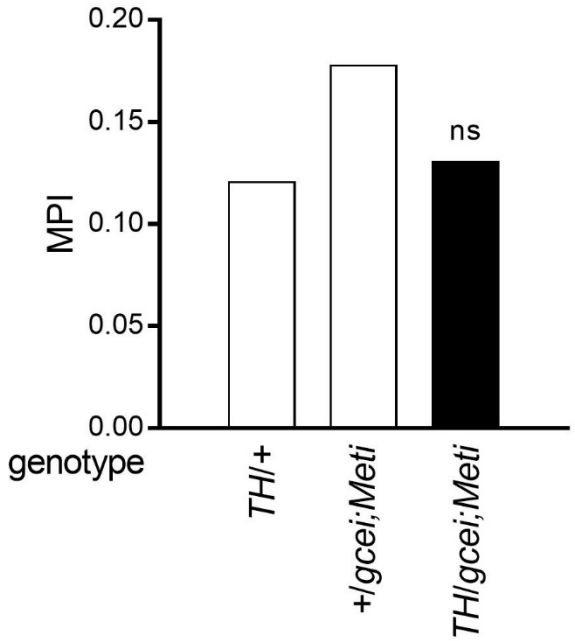



**Figure III.6. TH-positive dopaminergic neurons are not targets of JH signaling to regulate courtship LTM.** Expression of both JH receptors *Met* and *gce* in a majority of dopaminergic neurons was suppressed using *TH-GAL4* driver (n = 54-58).

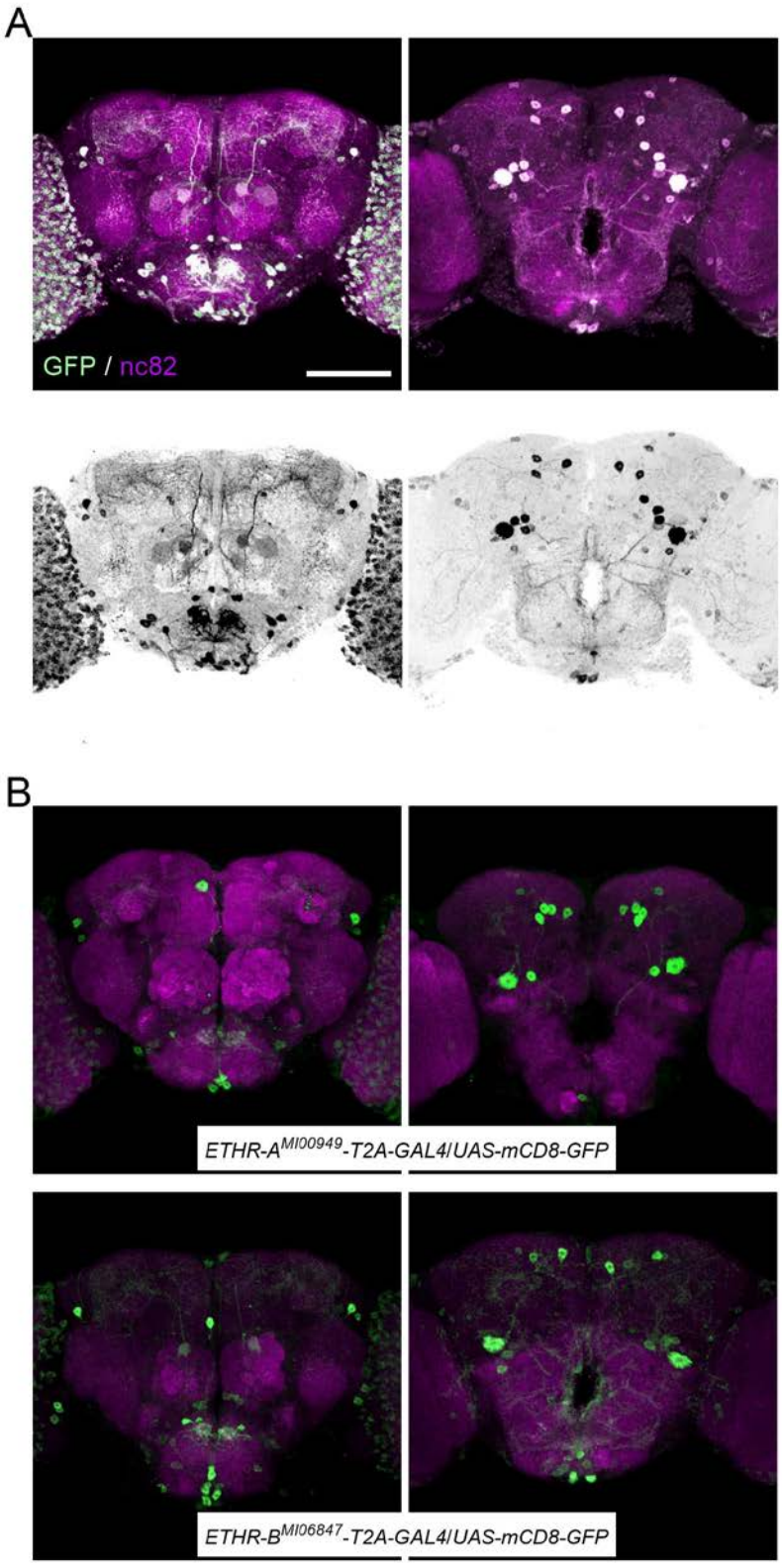

A
GFP / nc82
B
ETHR-A^MI00949-T2A-GAL4/UAS-mCD8-GFP
ETHR-B^MI06847-T2A-GAL4/UAS-mCD8-GFP

**Figure III.7. ETHR is expressed in broad areas of adult male brain.** (A) Day-4 male brain of a *ETHR-GAL4* line, MH1504 expressing GFP (*UAS-mCD8-GFP*). This line drives to cells expressing both *ETHR-A* and *ETHR-B* subtypes. Anterior (left) and posterior (right). Scale bar represent 100 μm. (B) Expression of *ETHR* subtypes in day-4 male brain. *ETHR-A* (above) and *ETHR-B* (bottom) expression in anterior (left) and posterior (right) brain sides.

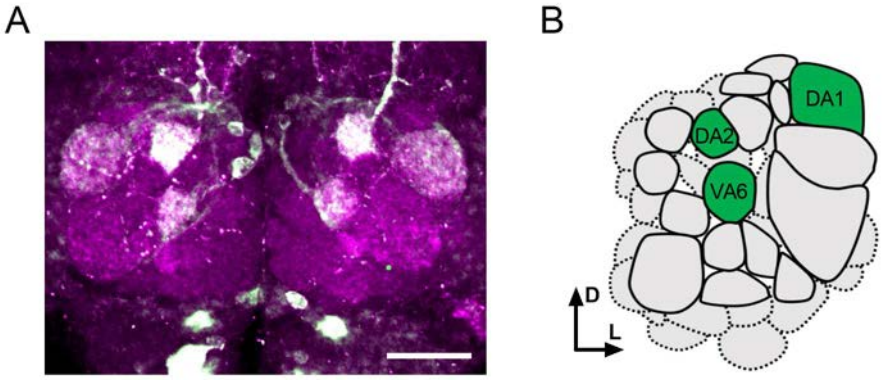



**Figure III.8. *ETHR-GAL4* expression pattern in antennal lobe (AL) glomeruli of mature male *Drosophila*.** A. The expansion of AL area in the confocal image Figure III-7A. Scale bar indicates 20 μm. DA1, DA2, and VA6 AL glomeruli were putatively detected. B. A map of AL glomeruli projected by designated olfactory neurons. Green areas indicate $ETHR^{+}$-putative glomeruli. D, dorsal; L, lateral.

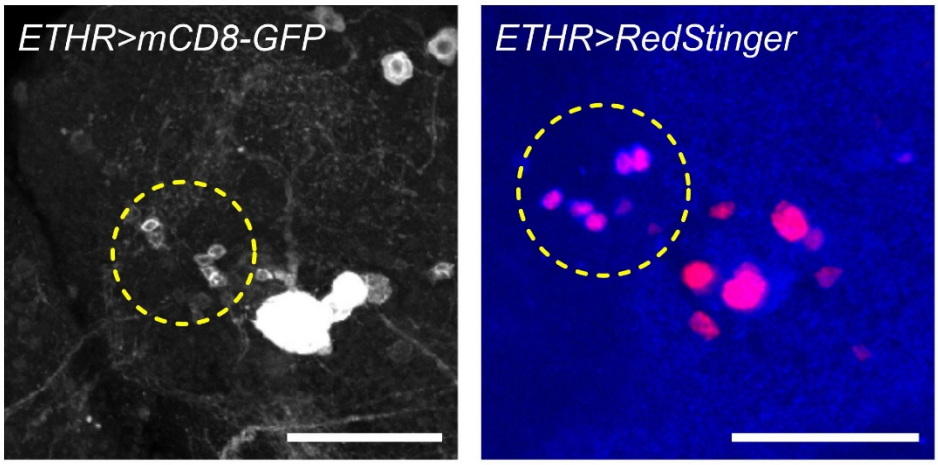



**Figure III.9. *ETHR-GAL4* drives gene expression to Kenyon cell-like neurons.** Confocal images for posterior view of a *ETHR-GAL4* line MH1504 visualizes the gene expression of this driver to Kenyon cell-like neurons (yellow circles) nearby the calyx area. Left: *MH1504/UAS-mCD8-GFP*, Right: *MH1504/UAS-RedStinger*. Scale bars represent 20 μm.

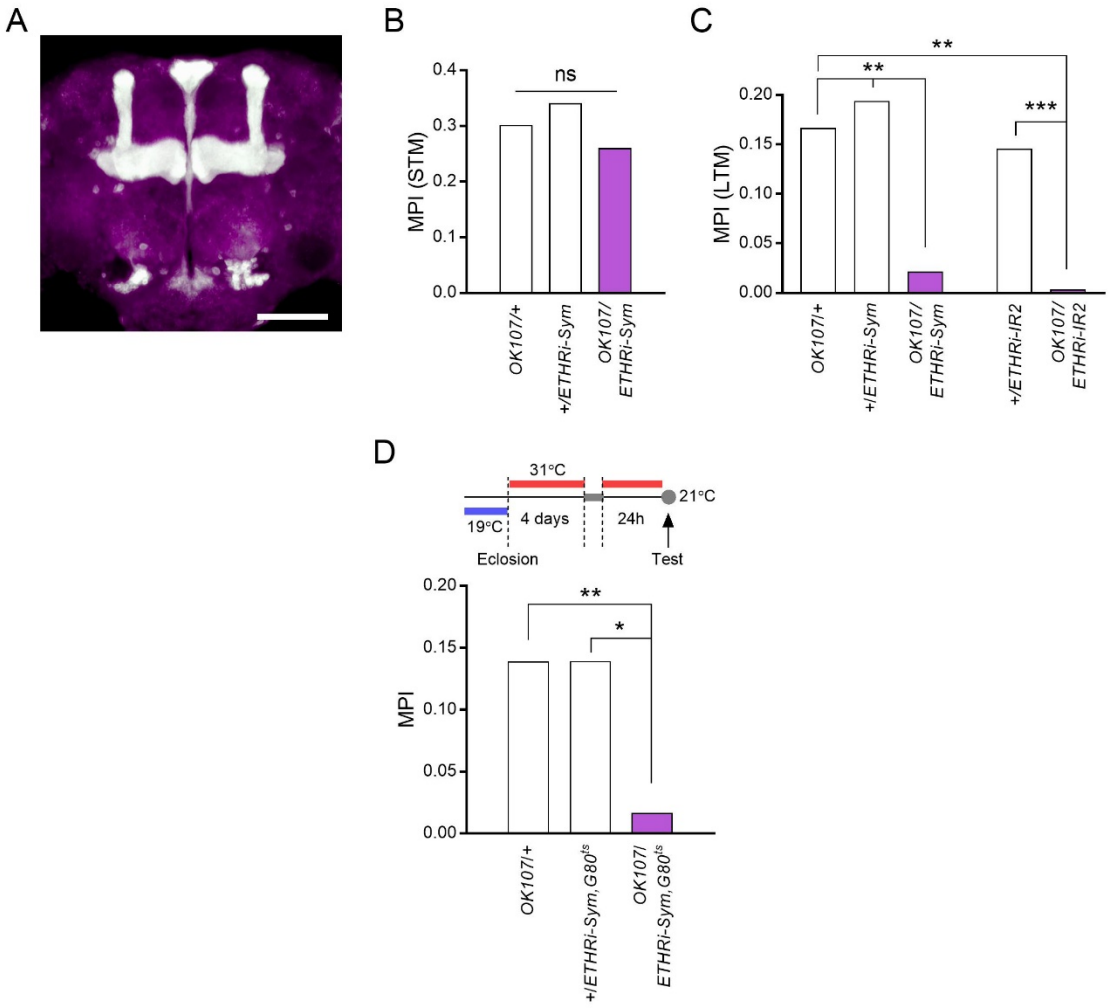



**Figure III.10. *ETHR* expression in mushroom body neurons is essential for the courtship LTM.** (A) *OK107-GAL4* line drives to whole mushroom body structure. Day-4 male brain, scale bar, 50 μm. (B) A role of ETHR expression in mushroom body for STM was tested by expressing dsRNA targeting common *ETHR* sequence using *OK107-GAL4* driver. A male was paired with a mated female for 1 hour. With 10 min interval, STM performance was tested by pairing with an immobilized virgin female (n = 50-54). (C) *ETHR* knockdown in mushroom body neurons suppresses 24-hour courtship memory of males. ***P*<0.01, ****P*<0.001 (n = 60-66). (D) Conditional *ETHR* silencing in adult stage results in courtship LTM deficiency. **P*<0.05, ***P*<0.01 (n = 64-72).

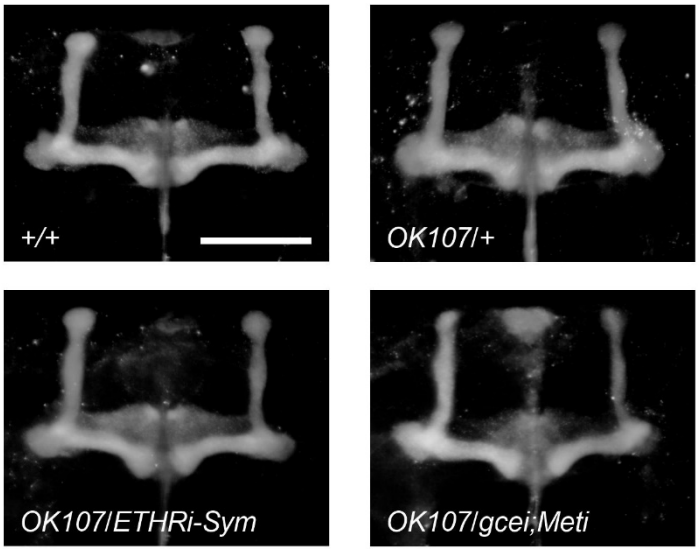



**Figure III.11. RNAi silencing of *ETHR* and JH receptors *Met* and *gce* in the mushroom body does not induce gross morphological change of mushroom body structure.** Mushroom body neuropil structure is detected by utilizing a mushroom body neuropil marker, mouse anti-Fasciclin-II (Fas-II) then visualized using anti-mouse Alexa Fluor 488. Scale bar represents 50 μm.

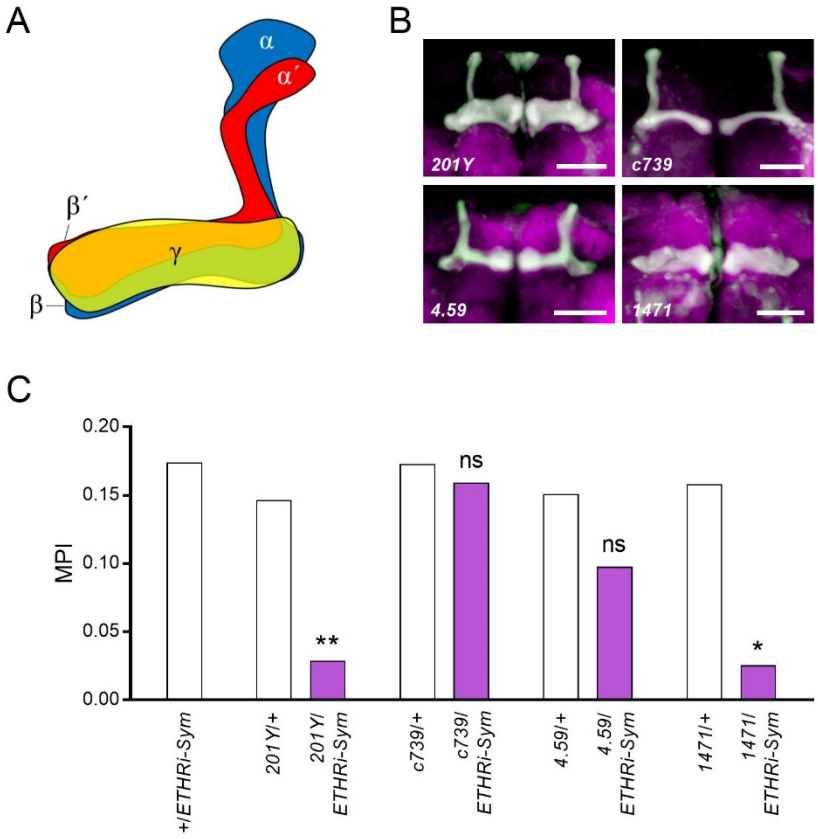



**Figure III.12. ETH targets mushroom body γ neurons to regulate courtship LTM.** (A) Schematic of mushroom body neuropil subdivisions. (B) GAL4 lines utilized in this study: *201Y* (αβ and γ), *c739* (αβ), *4.59* (α´β´), and *1471* (γ). Expression patterns were confirmed by expressing *UAS-mCD8-GFP*. Scale bars represent 50 μm. (C) *ETHR* knockdown in mushroom body subdivisions. Suppressing *ETHR* expression in γ neuron-containing mushroom body neuropils impaired the courtship LTM. **P*<0.05, ***P*<0.01 (n =64-70).

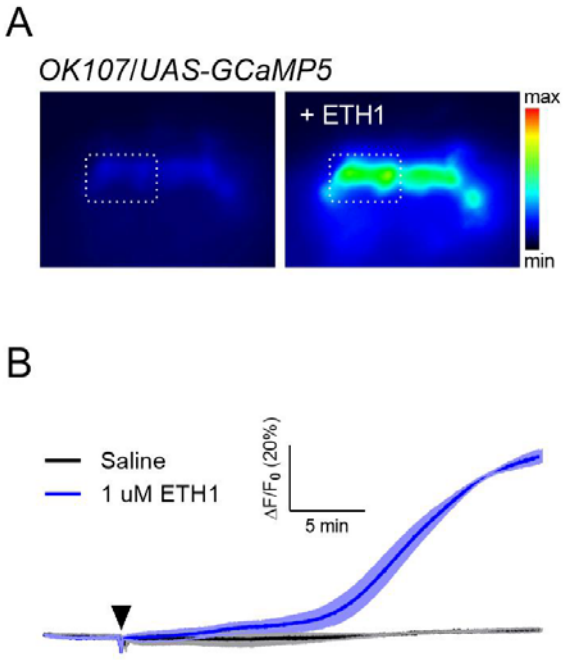



**Figure III.13. ETH increases calcium level in mushroom body lateral neuropils.** (A) Calcium elevation of mushroom body lateral neuropils of *OK107/UAS-GCaMP5* male brain in response to 1 μM ETH1 in the absence (left) and presence of ETH (right) are shown. (B) Averaged traces of percent $\Delta F/F_0$ by saline (black) and 1 μM ETH1 (blue) treatment. An arrowhead represents saline or ETH application. Darker lines represent means, and the light envelopes represent SEM (n = 3 for each).

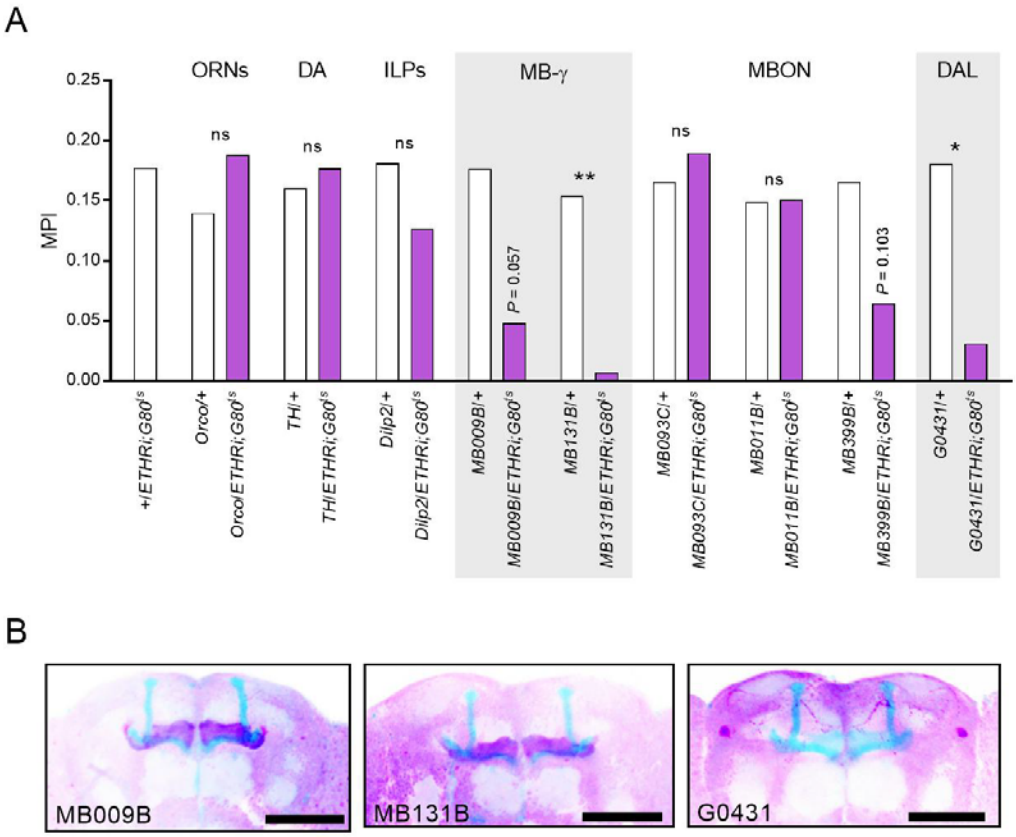



**Figure III.14. ETH targets specific memory circuit neurons to regulate memory.** (A) Conditional ETHR-silencing in candidate neuronal drivers was performed by housing individual males at 31℃ in adult stage except the training and testing sessions. **P*<0.05, ***P*<0.01 (n =56-68). (B) Expression patterns of GAL4 drivers which shows memory defects by suppressing ETHR expression. For clear recognition of neural structure, the color was inverted: purple, GFP (GAL4 driving); blue, Fas-II (mushroom body). Scale bars represent 50 μm.

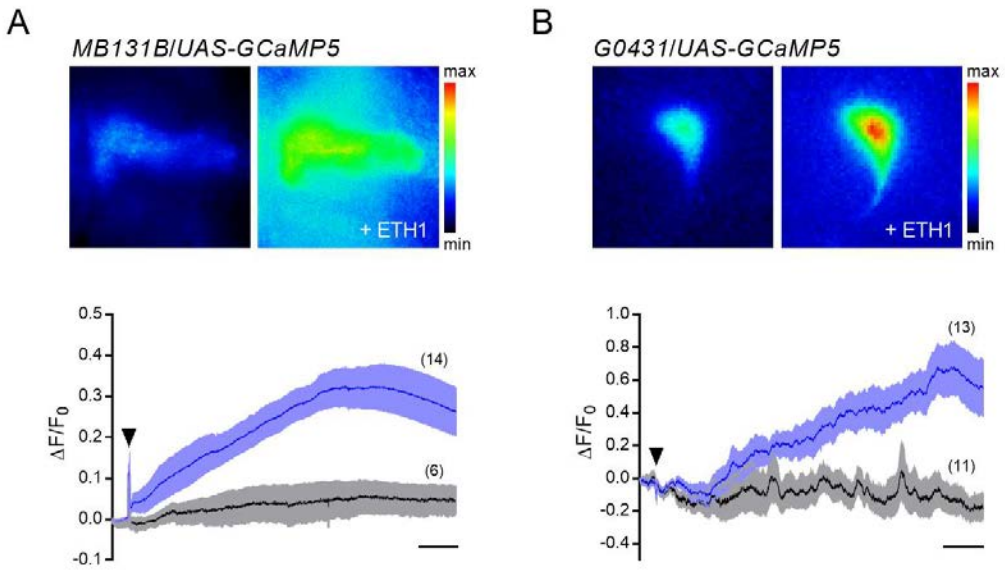



**Figure III.15. ETH elevates calcium levels in the mushroom body γ lobe and DAL neuron.** Upper representative images show before (left) and after (right) treatment of 1 μM ETH1. Bottom: averaged traces of $\Delta F/F_0$ by saline (black) and 1 μM ETH1 (blue) treatment. An arrowhead represents saline or ETH application. Darker lines represent means, and the light envelopes represent SEM. Parentheses indicate the number of recordings. Scale bars represent 5 min interval.

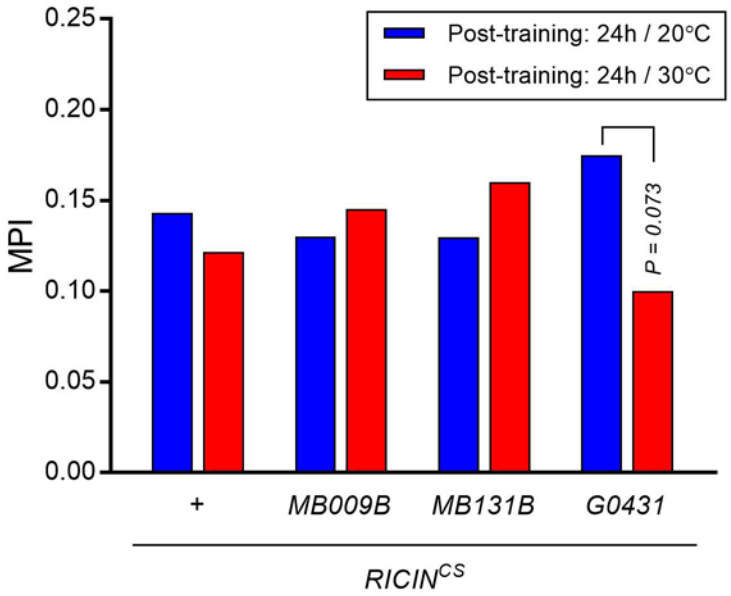



**Figure III.16. Blocking the protein synthesis in ETH-targeted DAL neurons during the memory consolidation period negatively affects the LTM performance.** Cold-sensitive RICIN ($RICIN^{CS}$) was conditionally activated at 30℃ for 24 hours after the 5-hour training at 20℃. Tests were performed at the room temperature (21-22℃). I tested GAL4 lines which were selected by the ETHR knockdown screening (Figure III-14). n = 60-64.

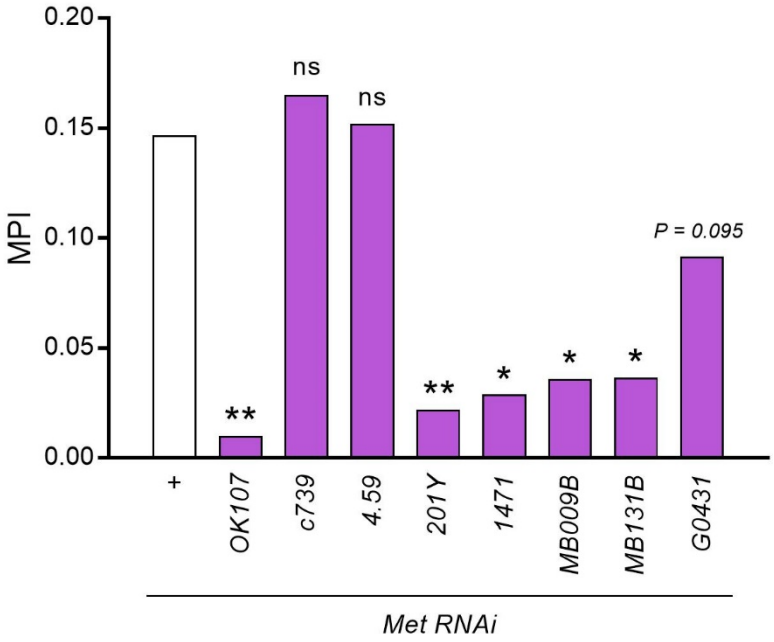



**Figure III.17. RNA silencing of Met in ETH-targeting neurons:** mushroom body enhancer trap GAL4 lines (*OK107*, *c739*, *4.59*, *201Y*, and *1471*), γ lobe-specific Split GAL4 lines (*MB009B* and *MB131B*), and DAL neuron-specific line (*G0431*). **P*<0.05, ***P*<0.01 (n =60-62).

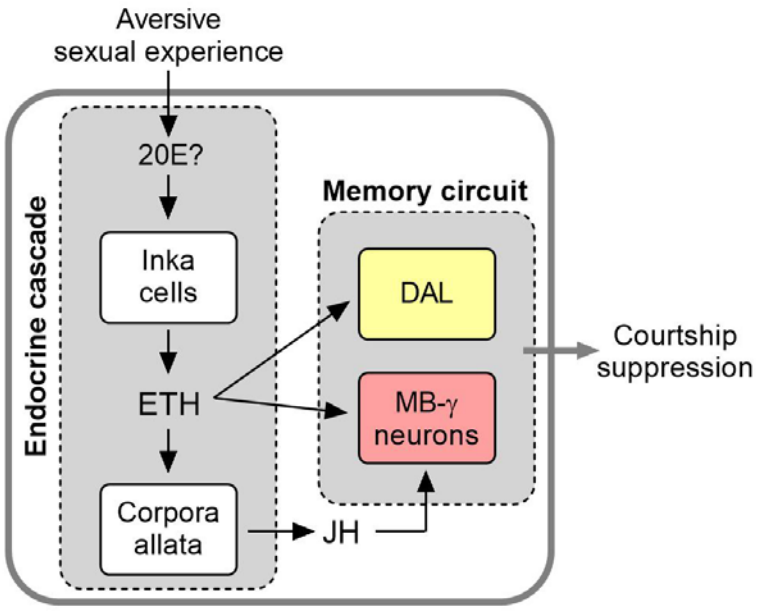



**Figure III.18. A model for the endocrine system function in modulating long-term memory circuits.** Proposed model as described in the text depicting the direct and indirect pathways of ETH signaling and the hormonal convergence in regulation of *Drosophila* courtship suppression by prior aversive experience.

# CHAPTER IV

## ETH Receptor Signal Transduction Regulates Pupal Ecdysis through TRPC-type Channels


### Abstract

G protein-coupled receptor (GPCR)-mediated intracellular signal transduction plays fundamental roles in a broad range of cellular events, providing essential modulating influences, either through amplification or inhibition, of neural excitability, transmitter release, and gene expression. However, downstream molecular interactions that couple GPCR activation with electrical activity in neurons remain unclear. In this study, I identified molecular components of the Gαq signaling cascade coupling GPCR activation with inward cation current and cellular activity. Using RNAi silencing of genes encoding signaling molecules in bursicon neurons, I found that Gαq proteins, phospholipase C (PLC) enzymes, and TRP channels in bursicon neurons play essential roles in setting proper timing of pupal ecdysis onset, whereas suppression of $IP_3$ signaling has no effect. Silencing of these signaling molecules likewise are required for proper timing of calcium mobilization in bursicon neurons. My study thus demonstrates the link between GPCR activation and TRP channel gating and suggests that signal transduction in endocrine and photoreceptor cells likely operates via a common intracellular signaling cascade.


### Introduction

During development, insects pass through sequential life stages, and each transition requires performance of a fixed action pattern (FAP) called ecdysis or shedding of old cuticle. The stereotypic ecdysis behavioral sequence is regulated by the circulating neuropeptide ecdysis

triggering hormone (ETH), synthesized and released from epitracheal gland Inka cells (Roller et al., 2010; Zitnan et al., 1996; Zitnan et al., 2003). Knockout of the ETH gene in the fruit fly *Drosophila melanogaster* causes early lethality through failure of larval ecdysis, a phenotype rescuable by injection of synthetic ETH (DmETH1), indicating the importance of ETH signaling in juvenile development (Park et al., 2002). ETHRs are present in distinct central neuronal ensembles, which are recruited according a precise schedule leading to performance of sequential behavioral steps (Diao et al., 2016; Kim et al., 2006a; Kim et al., 2006b).

*Drosophila* pupal ecdysis is performed in three behavioral subunits: pre-ecdysis, ecdysis, and post-ecdysis (Figure IV.1A). Pre-ecdysis is characterized by anteriolateral rolling contractions of the abdomen following a strong anterior contraction. The switch to ecdysis is recognized by anterior swinging of the abdomen. Head eversion (HE) occurs shortly after onset of ecdysis. Post-ecdysis is characterized by posterior swinging following stretch-compression of the abdomen (Kim et al., 2006b). Recent studies revealed that kinin-secreting neurons regulate pre-ecdysis scheduling, and that CAMB (CCAP, AstCC, MIP, and bursicon) neurons initiate ecdysis (Kim et al., 2015). In particular, ETHR expressing bursicon (burs and pburs) neurons trigger the switch from pre-ecdysis to ecdysis upon TRP-M8 activation. Suppression of ETHR expression by RNA knockdown in bursicon neurons delays onset of ecdysis, whereas ETHR overexpression in these neurons promotes acceleration of the ecdysis onset.

In a broad range of insect species, the ETHR gene encodes two functional subtypes of G protein-coupled receptors (ETHR-A and ETHR-B) (Park et al., 2003; Roller et al., 2010). Previous studies showed robust $Ca^{2+}$ dynamics in ETHR-expressing neurons elicited by ETH, suggesting that Gαq signaling is involved in neuronal activity (Kim et al., 2015; Kim et al., 2006b). However little is known about signal transduction mechanisms leading to ETH-induced activity of ETHR neurons.

In this study, we investigated roles of intracellular signal transduction molecules in regulation of pupal ecdysis onset in ETHR-expressing bursicon neurons of *Drosophila*. Utilizing genetic manipulation and functional $Ca^{2+}$ monitoring, we found that expression of Gαq proteins and downstream signal cascade molecules are essential for correct timing of ecdysis onset. Of great interest is the finding that $Ca^{2+}$ influx through TRP channels is essential for proper scheduling of the behavioral switch rather than release of $Ca^{2+}$ from intracellular stores, which has been considered as the main source for $Ca^{2+}$ elevation of these neurons. These results demonstrate the signal transduction pathway mediating TRP channel activation in endocrine bursicon neurons is similar to that originally described in fly photoreceptors.

## Materials and Methods

**Fly Stock.** *Drosophila melanogaster* stocks were maintained at room temperature on standard agar/cornmeal/yeast media under a 12-hour L/D regimen. Genetic background of wild-type flies was *w1118*. *Pburs-GAL4* (S3) was provided by Dr. Jae H. Park (Univ. Tennessee, Knoxville, TN). A combination line *Pburs-GAL4;UAS-GCaMP3* was obtained from Dr. Young-Joon Kim (GIST, South Korea). *UAS-mCD8-GFP*, *UAS-GCaMP5*, *norpA* RNAi line (31113), and *sl* RNAi lines (32385, 32906) were obtained from the Bloomington Drosophila Stock Center (BDSC, Indiana Univ., Bloomington, IN). The following RNAi lines were obtained from the Vienna Drosophila RNAi Center (VDRC): *CG17759* (50729, 105300), *CG17760* (52308, 107613), *CG30054* (4643, 102887), *PLC21C* (26557, 108395), *norpA* (105676), *$IP_3R$* (6484, 106982), *trp* (1365, 1366), *trpγ* (9337, 105280), and *trpl* (35571, 104450). All UAS-transgene bearing flies were crossed with wild-type flies (Canton-S) to create heterozygous controls.

**Behavioral Analysis of Pupal Ecdysis.** For behavioral analysis of each fly line, I collected late stage buoyant pharate pupae (approximately 2-hour prior to pupal ecdysis (Bainbridge and Bownes, 1981)) and placed 5-7 pharate pupae ventral-side up in a small recording chamber containing wet filter-paper strips. Ecdysis was recorded under a dissection microscope (Wild Heerbrugg) using a ExwaveHad digital video-camera (SONY) attached to a personal computer. To synchronize experimental conditions, the same number of UAS-transgene heterozygous control and test transgenic animals were placed on the plate, and filmed at 21℃ (EchothermTM Chilling/Heating plate, Torrey Pines Scientific Inc.) under dim transmitted light. Statistical analyses for ecdysis assays are described in the figure legend (Figure IV.2).

**Functional $Ca^{2+}$ Imaging and Immunohistochemistry.** For functional $Ca^{2+}$ imaging of bursicon neurons, the CNS of buoyant *Pburs-GAL4*/*UAS-GCaMP5* animals was dissected in ice-chilled fly saline, then placed on a petri dish ventral-side up in fly saline. $Ca^{2+}$-associated fluorescence responses of bursicon neurons were visualized with a CCD camera (TILL-Imago) mounted on an Olympus BX51W1 microscope and captured with Live Acquisition software (FEI). Excitation (488 nm; 50/1,000 msec excitation/duration) was provided by a Polychrome V monochromator. Following 4 min of pre-application sampling, 600 nM DmETH1 was applied to the saline bathing the CNS. Statistical analysis for multiple recordings is described in the figure legend.

For immunohistochemical staining of bursicon neurons, I prepared *Pburs-GAL4*/*UAS-mCD8-GFP* flies. The prepupal CNS was dissected in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS overnight at 4℃. After washing with PBST (0.5% Triton X-100 in PBS) and blocking with 5% NGS (normal goat serum) in PBST at room temperature, samples were incubated with rabbit anti-Pburs (1:1000) and mouse anti-GFP for two days at 4℃. Tissues were then washed with PBST and incubated with Alexa Fluor 633 goat anti-rabbit IgG antibody

and Alexa Fluor 488 goat anti-mouse IgG antibody (1:500 each). After washing with PBST and PBS, tissues were mounted in Aqua Poly/Mount (Polysciences Inc.). Confocal images were acquired using a Zeiss LSM 510 confocal microscope.

## Results

**Expression of Gαq proteins in bursicon neurons is required for proper scheduling of ecdysis onset.** Exposure of the isolated CNS to ETH *in vitro* results in robust intracellular $Ca^{2+}$ elevation in bursicon neurons, whose activity triggers the switch from preecdysis to ecdysis the pupal ecdysis behavioral sequence (Kim et al., 2015; Kim et al., 2006b). Timing of this behavioral switch is highly invariant, occurring about 10 minutes after onset of preecdysis behavior. I hypothesized that Gαq signaling may be important in activation of bursicon neurons.

I first tested whether RNA silencing of Gαq proteins in bursicon neurons affects timing of ecdysis onset by utilizing a bursicon-neuron specific driver, *Pburs-GAL4* (Figure IV.1B). A recent study demonstrated that *Drosophila* has three genes encoding functional Gαq subtypes: *CG17759*, *CG17760*, and *CG30054* (Yamanaka et al., 2015). Although *CG17759* gene had been considered as a highly conserved and conventional Gαq protein-encoding gene, the two related genes *CG17760* and *CG30054* encode highly homologous proteins to *CG17759* product (Figure IV.2A). Bursicon neuron-specific RNA knockdown of each Gαq type significantly delayed switch to ecdysis from the pre-ecdysis period (Figure IV.2B). Silencing *CG17759* in bursicon neurons delays ecdysis onset by approximately 2 min; silencing *CG17760* delays ecdysis onset by 1.8-2.9 min; silencing *CG30054* delays ecdysis onset by 2.3-2.9 min by comparison with the ecdysis onset of UAS controls. This suggests that 1) bursicon neurons express all Gαq subtypes, and 2) all three Gαq subtypes play a role in neuronal activity leading to ecdysis onset.

**PLC-β plays a role in bursicon neuronal function.** I next asked if suppressing signaling molecules in the Gαq cascade affect timing of pupal ecdysis onset. Gαq-coupled receptors signal via phospholipase C (PLC) isoforms that catalyze hydrolysis of phosphatidylinositol biphosphate ($PIP_2$), resulting in the production of inositol triphosphate ($IP_3$) and diacylglycerol (DAG). Liberation of $IP_3$ mobilizes intracellular $Ca^{2+}$ from the endoplasmic reticulum (ER). $Ca^{2+}$-dependent and PKC-dependent pathways participate in diverse signaling to evoke a variety of cellular events (Hubbard and Hepler, 2006).

Molecules directly downstream of activated Gαq proteins are PLC enzymes, and three genes encode PLC subtypes in *Drosophila* (Shortridge and McKay, 1995). Bursicon neuron-specific RNA knockdown of *PLC21C* and *norpA* (PLC-β-type enzymes) resulted in significant delay of ecdysis onset (Figure IV.3A): Silencing *PLC21C* in bursicon neurons delays the onset of pupal ecdysis 2.8-3.9 min; silencing *norpA* delays 2.8-3.3 min, compared with ecdysis onset timing of UAS controls. However, RNAi silencing of a PLC-γ class enzyme encoding gene *sl* (*small wing*) in same neurons did not significantly affect the ecdysis onset phenotype. These findings indicate that PLC-β type enzymes function as signaling molecules in the Gαq signaling cascade in bursicon neurons.

**Inhibiting $IP_3$ signaling in bursicon neurons does not affect the behavioral switch.** I hypothesized that intracellular $Ca^{2+}$ mobilization from ER by the Gαq-PLC-$IP_3$ cascade would be essential for activation of bursicon neurons and timing of the switch to ecdysis behavior. I thus tested whether suppression of $IP_3$ signaling in these neurons would affect pupal ecdysis onset by silencing the $IP_3$ receptor. Unexpectedly, RNAi silencing of $IP_3$ receptor gene encoding channel proteins, which is the major source for internal $Ca^{2+}$ release in bursicon neurons, did not alter timing of ecdysis onset (Figure IV.3B), indicating that $Ca^{2+}$ release through $IP_3$ receptor from ER is not involved in activation of bursicon neurons. These data thus suggest that an alternative second

messenger pathway downstream of PLC-β activation plays a role in the bursicon neuron-mediated behavioral switch.

**ETH-triggered $Ca^{2+}$ activation of bursicon neurons depends on extracellular $Ca^{2+}$.** Data presented in the previous section suggests that $IP_3$ receptor signaling is unnecessary for $Ca^{2+}$ elevation leading to bursicon neuron activation. What then, is the source of $Ca^{2+}$ underlying bursicon neurons responses to ETH? I hypothesized that ETH-driven GPCR activity may promote entry of extracellular $Ca^{2+}$. To test this hypothesis, I performed *in vitro* functional $Ca^{2+}$ imaging of bursicon neurons expressing the $Ca^{2+}$ indicator GCaMP5 in the prepupal CNS. In normal external fly saline containing 2.0 mM $Ca^{2+}$, bursicon neurons exposed to 600 nM *Drosophila* ETH1 (DmETH1) show robust $Ca^{2+}$-associated fluorescence spikes, which decreases gradually over an hour-long period. However, in low $Ca^{2+}$ saline (0.2 mM), $Ca^{2+}$-associated fluorescence responses are drastically reduced (Figure IV.4A). In particular, rapid-onset, high-amplitude $Ca^{2+}$-associated fluorescence responses are significantly suppressed. This external $Ca^{2+}$ level-dependent fluorescence reduction was observed in all bursicon neurons tested (AN2 to AN4) (Figure IV.4B). These data strongly indicate that influx of extracellular $Ca^{2+}$, rather than that released from intracellular stores, is responsible for bursicon neuron activation.

Next, I tested if pharmacological blocking of membrane-localized channels affects the $Ca^{2+}$ signal dynamics of bursicon neurons in response to ETH application. In a broad range of cell types, manganese ($Mn^{2+}$) has been used as an effective $Ca^{2+}$ influx blocker (Cao and Banks, 1990; Pumain et al., 1987; Shukla and Wakade, 1991; Streifel et al., 2013). I investigated the $Ca^{2+}$-associative fluorescence changes in prepupal bursicon AN2 cells (Figure IV.1C) in response to DmETH1 (600 nM) treatment with or without $Mn^{2+}$ (1 mM) in normal fly saline. Interestingly, in bursicon AN2 cells, $Mn^{2+}$ eliminated initial $Ca^{2+}$-associated fluorescence spikes evoked by ETH (Figure IV.5). However, I observed slow and gradual elevation of the GCaMP fluorescence signal following ETH

treatment in all AN2 cells exposed to $Mn^{2+}$. This suggests that ETHR-mediated signaling induces $Ca^{2+}$ mobilization via both external and internal sources. In particular, $Ca^{2+}$ influx from the extracellular space likely contributes to fast $Ca^{2+}$-associated fluorescence spikes, whereas release of $Ca^{2+}$ from intracellular stores is responsible for slow, low amplitude fluorescence changes (Figure IV.4).

**Bursicon neuron TRPC channels are essential for proper timing of the switch to ecdysis.** Transient receptor potential (TRP) cation channels play critical roles in sensory neurons involved in photosensation, olfaction, mechanosensation, thermosensation, etc. Based on amino acid sequence comparisons, TRP channels are classified into Group-1 (TRPC, TRPV, TRPM, TRPN, and TRPA) and Group-2 (TRPP and TRPML). However, the physiology of these channels between species is remarkably similar. Each TRP channel subtype is triggered by distinct external stimuli. In *Drosophila*, TRPA channels are activated by heat, while TRPV channels are sensitive to osmolarity (Montell, 2005). Interestingly, TRPC channels (three subtype proteins encoded by *trp*, *trpl*, and *trpγ*) critical for photoreceptor responses are activated by phospholipase C (Xu et al., 2000; Xu et al., 1997). Previous studies showed that a TRPC subtype channel in mammalian astrocytes is highly sensitive to block by $Mn^{2+}$ (Streifel et al., 2013). These findings suggest that that TRPC channels may function in bursicon neuron activation.

I thus tested if suppressing expression of TRPC channels in bursicon neurons alters timing of pupal ecdysis behavior. Indeed, RNAi silencing of TRPC channels significantly delays ecdysis onset (Figure IV.6): silencing TRP causes a 1.4 min delay, silencing of TRPL delays onset by 2.4 min, and silencing TRPγ causes a 1.7-3.0 min delay compared to UAS controls. These data argue that *Drosophila* TRPC type channels mediate activation of bursicon neurons and timing of the switch to ecdysis.

**RNAi knockdown of GPCR cascade molecules alters timing of $Ca^{2+}$ dynamics in bursicon neurons.** To test if disruption of Gαq-mediated GPCR signaling affects timing of $Ca^{2+}$ mobilization in bursicon neurons, I performed functional imaging of bursicon cells in the isolated CNS following ETH application. For consistency, I focused on $Ca^{2+}$ mobilization in AN2-α cells (Figure IV.7), which are homologs of peptidergic NS27 cell in the moth *Manduca sexta* (Kim et al., 2006a; Mena et al., 2016). I found that RNA silencing of Gαq proteins, two PLC-β types and TRPC-type channels causes significant delays in $Ca^{2+}$ mobilization of AN2-α cells following exposure of the CNS to ETH1 (600 nM; representative traces in Figure IV.7 and analysis in Figure III.8). Knockdown of Gαq subtypes in bursicon cells delays timing of $Ca^{2+}$ mobilization (Figure IV.8A) and reduces peak $Ca^{2+}$-associated fluorescence amplitudes (Figure IV.8B). Although the delay induced by RNA silencing of *CG30054* failed to reach significance, a significant trend in that direction was registered ($P = 0.0721$).

Interestingly, although silencing PLC21C delays onset of $Ca^{2+}$ mobilization and also reduces peak fluorescence, knockdown of norpA affects only the $Ca^{2+}$-associated amplitude. Likewise, silencing TRP and TRPγ significantly delays timing of $Ca^{2+}$ mobilization, whereas TRPL silencing results only reduced in $Ca^{2+}$ elevation. Knockdown of $IP_3$ receptor expression leads to no significant change in both latency and peak amplitude. Since knockdown of $IP_3$ receptor expression does not affect timing of ecdysis onset (Figure IV.3B), these results are consistent with the idea that $Ca^{2+}$ mobilization in bursicon neurons leads to their activation.

## Discussion

Insect ecdysis, or shedding of old cuticle at the end of each molt, occurs through execution of sequential motor patterns orchestrated by ETH via downstream central peptidergic ensembles.

Timing of the switch from preecdysis to ecdysis behavior, a highly invariant event in the behavioral sequence, is centrally-patterned and coincident with activation of ETHR-expressing bursicon neurons. Although considerable studies in *Drosophila* have identified ETHR-expressing neuronal architectures and chemically-induced neuronal activity by ETH (Diao et al., 2016; Kim et al., 2015; Kim et al., 2006b; Mena et al., 2016), intracellular signal transduction mechanisms underlying ETHR signaling are still largely based on logical inference. ETH promotes robust intracellular $Ca^{2+}$ mobilization in ETHR-target neurons, suggesting that Gαq signaling proteins are involved in neuronal activation. In our recent study, overexpression of a conventional Gαq protein (*CG17759*) in kinin neurons prolongs pre-ecdysis behavior, whereas overexpression in CAMB (CCAP, AstCC, MIP, and Bursicon) neurons accelerates ecdysis onset (Kim et al., 2015). Here, I have demonstrated that specific GPCR signaling cascade molecules are involved in ETH-triggered activity in bursicon neurons. Also, I provide evidence that ETH-mediated $Ca^{2+}$ mobilization in bursicon neurons depends mainly on influx of extracellular $Ca^{2+}$ rather than release from internal release. Finally, I found that TRPC group channels play a critical role in timing of both $Ca^{2+}$ mobilization in bursicon neurons and timing of ecdysis behavior onset. My findings are summarized (Figure IV.9) and discussed below.

**Second messenger signaling molecules as regulators of pupal ecdysis onset. The** Gαq-PLC-mediated signal transduction cascade is perhaps the most common means of $Ca^{2+}$ mobilization in GPCR target cells, although there are reports of considerable cross-talk between other signaling cascades triggered by GPCR activation (Prezeau et al., 2010; Selbie and Hill, 1998). As I hypothesized, knockdown of Gαq–encoding genes in bursicon neurons delays timing of ecdysis onset. Unexpectedly, all knockdown of multiple Gαq subtypes in these neurons leads to delays in pupal ecdysis onset. It is possible that off-target effects of RNAi lines could have caused some false-positive phenotypes. However most UAS-RNAi lines are specific for the target gene: one

exception is the RNAi line against *CG30054* (4643) which targets *CG17759* variants as well (Yamanaka et al., 2015). Therefore, I hypothesis that 1) individual Gαq protein types function with different physiology, or 2) Gαq protein types have different expression patterns among multiple bursicon neurons.

**PLC-β enzyme activation is crucial for ecdysis timing.** In this study, RNA silencing of PLC21C and norpA, but not PLC-γ type delayed ecdysis onset, indicating that PLC-β enzymes are important for signaling downstream of Gαq activation in bursicon neurons. Notably, conventional Gαq-PLC-$IP_3$ signaling cascade-mediated $Ca^{2+}$ release from the ER is not likely the main source for bursicon neuron activation, since knockdown of the $IP_3$ receptor gene had no effect on ecdysis timing. This suggests that $Ca^{2+}$ mobilization contributing to neuronal activity comes from an alternative source, namely the extracellular space.

**ETH signaling leads to $Ca^{2+}$ influx from extracellular space.** *In vitro* CNS $Ca^{2+}$ imaging experiments provide evidence that ETH-mediated cytoplasmic $Ca^{2+}$ mobilization in bursicon neurons originates mainly from external $Ca^{2+}$ sources, rather than intracellular organelles, and that this likely involves $Ca^{2+}$ influx across the plasma membrane (Figure IV.4 and 5). ETH signaling promotes two phases of $Ca^{2+}$ mobilization: fast and slow. Reduction of external $Ca^{2+}$ levels attenuated "fast" $Ca^{2+}$-mediated spikes (Figure IV.4), an effect mimicked by $Mn^{2+}$ (Figure IV.5). Rapid $Ca^{2+}$ entry into bursicon neurons may be essential for acute neurotransmitter release both within the CNS and at target neuromuscular junctions for the precise muscle control (Loveall and Deitcher, 2010). The gradual "slow" $Ca^{2+}$ elevation – likely from ER via Gαq-PLC-$IP_3$ signaling - may contribute to further signal amplification and additional $Ca^{2+}$-mediated long-term biophysiological processes such as modulation of gene expression (Dolmetsch et al., 1998; Li et al., 1998) and induction of enzyme activity (Kakiuchi and Yamazaki, 1970; Raraty et al., 2000).

**PLC-dependent TRPC type channels are required for bursicon neural function.** I found that expression of TRPC type channels in bursicon neurons is required for proper timing of pupal ecdysis onset. Since activity of these channels is induced by PLC (Montell, 2012), my data suggest a molecular link between G protein-mediated PLC enzyme activity and $Ca^{2+}$ entry via TRP channels. In *Drosophila* visual transduction, light-stimulated rhodopsin activates Gαq and the PLC subtype encoded by norpA. Considerable studies have revealed functions of *Drosophila* TRPC type channels in phototransduction cascades. There is clear evidence that TRP- and TRPL-mediated inward cation currents carried by $Na^+$ and $Ca^{2+}$ in photoreceptors are required for light sensing (Hardie and Minke, 1992; Montell et al., 1985; Montell and Rubin, 1989; Niemeyer et al., 1996) and TRPγ likely forms a heterodimer with TRPL (Xu et al., 2000).

Although the mechanisms linking PLC-β class enzymes with TRPC channel activity are not clear, previous findings suggest three prevailing models. First, activation of PLC promotes $PIP_2$ hydrolysis, followed by liberation of $IP_3$ and DAG. A DAG lipase encoded by *inaE* hydrolyzes DAG to produce polyunsaturated fatty acids (PUFAs). Electrophysiological studies utilizing whole-cell and single-channel recordings showed that TRPL gating is mediated by PUFAs (Chyb et al., 1999; Delgado and Bacigalupo, 2009; Leung et al., 2008). Second, TRP channels are directly activated by "inaction, no after-potential D" (INAD), which consists of multiple protein-interacting modules connecting PLC, PKC, and TRP channels. Previous studies showed that the interaction between PLC and INAD alters the scaffold of INAD to contact TRP channels (Chevesich et al., 1997; Li and Montell, 2000; Liu et al., 2011; Mishra et al., 2007; Shieh and Zhu, 1996; Tsunoda et al., 1997; Venkatachalam et al., 2010; Wes et al., 1999; Xu et al., 1998). Third, a study showed that hydrolyzed "inhibitory" $PIP_2$ promotes TRP- and TRPL- channel activities (Huang et al., 2010). This would be similar with actions of PLC-mediated $PIP_2$ hydrolysis that promotes gating of thermo-sensing TRPV1 (Brauchi et al., 2007; Cao et al., 2013; Gao et al., 2016; Kim et al., 2008;

Rohacs et al., 2008), and opposite mechanism with PLC-mediated $PIP_2$ function on M-channel activity (Suh and Hille, 2002; Winks et al., 2005; Zhang et al., 2003). Similar with my finding for pupal ecdysis, $IP_3$ signal-mediated $Ca^{2+}$ mobilization is not likely functional for signal amplification during phototransduction (Acharya et al., 1997; Hardie and Raghu, 1998; Raghu et al., 2000).

Knockdown of PLC enzymes and TRP channels led to two changes in $Ca^{2+}$ responses of bursicon cells to ETH exposure (Figure IV.7 and IV.8): in one group (PLC21C, TRP, and TRPγ knockdown) latency was prolonged and amplitude was reduced, but in another group (norpA and TRPL silencing) amplitude was reduced, but no significant change in latency $Ca^{2+}$ elevation was observed. Since knockdown of $IP_3$ receptor expression did not alter $Ca^{2+}$ mobilization in target bursicon cells in response to ETH, we suggest that ETH-driven $Ca^{2+}$ mobilization, mediated by G protein-PLC-TRP channel signaling, triggers onset of ecdysis behavior. I hypothesize that the cumulative $Ca^{2+}$ levels in bursicon cells within optimal time range after the pre-ecdysis period may be critical for precise motor control, and the G protein - PLC enzymes - TRPC-type channel signaling cascade is responsible for this.

In summary, my work provides evidence that timing of *Drosophila* pupal ecdysis onset is regulated by the Gαq-PLC-TRP signaling cascade in bursicon neurons. Therefore, I suggest that this model is not specific to photosensory transduction, but also plays an important role in endocrine regulation of motor control. Given the discovery that distinct $Ca^{2+}$ mobilization patterns in bursicon cells induced by ETH depend on distinct ion sources, additional dissection of molecular dynamics responsible for the kinetics of $Ca^{2+}$ mobilization will be important for understanding $Ca^{2+}$-dependent neurophysiology.

## References


Acharya, J.K., Jalink, K., Hardy, R.W., Hartenstein, V., and Zuker, C.S. (1997). InsP3 receptor is essential for growth and differentiation but not for vision in Drosophila. Neuron 18, 881-887.

Brauchi, S., Orta, G., Mascayano, C., Salazar, M., Raddatz, N., Urbina, H., Rosenmann, E., Gonzalez-Nilo, F., and Latorre, R. (2007). Dissection of the components for PIP2 activation and thermosensation in TRP channels. Proc Natl Acad Sci U S A 104, 10246-10251.

Cao, E., Cordero-Morales, J.F., Liu, B., Qin, F., and Julius, D. (2013). TRPV1 channels are intrinsically heat sensitive and negatively regulated by phosphoinositide lipids. Neuron 77, 667-679.

Cao, L.Q., and Banks, R.O. (1990). Cardiovascular and renal actions of endothelin: effects of calcium-channel blockers. Am J Physiol 258, F254-258.

Chevesich, J., Kreuz, A.J., and Montell, C. (1997). Requirement for the PDZ domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex. Neuron 18, 95-105.

Chyb, S., Raghu, P., and Hardie, R.C. (1999). Polyunsaturated fatty acids activate the Drosophila light-sensitive channels TRP and TRPL. Nature 397, 255-259.

Delgado, R., and Bacigalupo, J. (2009). Unitary recordings of TRP and TRPL channels from isolated Drosophila retinal photoreceptor rhabdomeres: activation by light and lipids. J Neurophysiol 101, 2372-2379.

Diao, F., Mena, W., Shi, J., Park, D., Diao, F., Taghert, P., Ewer, J., and White, B.H. (2016). The splice isoforms of the Drosophila ecdysis triggering hormone receptor have developmentally distinct roles. Genetics 202, 175-189.

Dolmetsch, R.E., Xu, K., and Lewis, R.S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392, 933-936.

Gao, Y., Cao, E., Julius, D., and Cheng, Y. (2016). TRPV1 structures in nanodiscs reveal mechanisms of ligand and lipid action. Nature 534, 347-351.

Hardie, R.C., and Minke, B. (1992). The trp gene is essential for a light-activated Ca2+ channel in Drosophila photoreceptors. Neuron 8, 643-651.

Hardie, R.C., and Raghu, P. (1998). Activation of heterologously expressed Drosophila TRPL channels: Ca2+ is not required and InsP3 is not sufficient. Cell Calcium 24, 153-163.

Huang, J., Liu, C.H., Hughes, S.A., Postma, M., Schwiening, C.J., and Hardie, R.C. (2010). Activation of TRP channels by protons and phosphoinositide depletion in Drosophila photoreceptors. Curr Biol 20, 189-197.

Hubbard, K.B., and Hepler, J.R. (2006). Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. Cell Signal 18, 135-150.

Kakiuchi, S., and Yamazaki, R. (1970). Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain studies on cyclic 3',5'-nucleotide phosphodiesterase (3). Biochem Biophys Res Commun 41, 1104-1110.

Kim, A.Y., Tang, Z., Liu, Q., Patel, K.N., Maag, D., Geng, Y., and Dong, X. (2008). Pirt, a phosphoinositide-binding protein, functions as a regulatory subunit of TRPV1. Cell 133, 475-485.

Kim, D.H., Han, M.R., Lee, G., Lee, S.S., Kim, Y.J., and Adams, M.E. (2015). Rescheduling behavioral subunits of a fixed action pattern by genetic manipulation of peptidergic signaling. PLoS Genet 11, e1005513.

Kim, Y.J., Zitnan, D., Cho, K.H., Schooley, D.A., Mizoguchi, A., and Adams, M.E. (2006a). Central peptidergic ensembles associated with organization of an innate behavior. Proc Natl Acad Sci U S A 103, 14211-14216.

Kim, Y.J., Zitnan, D., Galizia, C.G., Cho, K.H., and Adams, M.E. (2006b). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr Biol 16, 1395-1407.

Leung, H.T., Tseng-Crank, J., Kim, E., Mahapatra, C., Shino, S., Zhou, Y., An, L., Doerge, R.W., and Pak, W.L. (2008). DAG lipase activity is necessary for TRP channel regulation in Drosophila photoreceptors. Neuron 58, 884-896.

Li, H.S., and Montell, C. (2000). TRP and the PDZ protein, INAD, form the core complex required for retention of the signalplex in Drosophila photoreceptor cells. J Cell Biol 150, 1411-1422.

Li, W., Llopis, J., Whitney, M., Zlokarnik, G., and Tsien, R.Y. (1998). Cell-permeant caged InsP3 ester shows that Ca2+ spike frequency can optimize gene expression. Nature 392, 936-941.

Liu, W., Wen, W., Wei, Z., Yu, J., Ye, F., Liu, C.H., Hardie, R.C., and Zhang, M. (2011). The INAD scaffold is a dynamic, redox-regulated modulator of signaling in the Drosophila eye. Cell 145, 1088-1101.

Loveall, B.J., and Deitcher, D.L. (2010). The essential role of bursicon during Drosophila development. BMC Dev Biol 10, 92.

Mena, W., Diegelmann, S., Wegener, C., and Ewer, J. (2016). Stereotyped responses of Drosophila peptidergic neuronal ensemble depend on downstream neuromodulators. Elife 5.

Mishra, P., Socolich, M., Wall, M.A., Graves, J., Wang, Z., and Ranganathan, R. (2007). Dynamic scaffolding in a G protein-coupled signaling system. Cell 131, 80-92.

Montell, C. (2005). Drosophila TRP channels. Pflugers Arch 451, 19-28.

Montell, C. (2012). Drosophila visual transduction. Trends Neurosci 35, 356-363.

Montell, C., Jones, K., Hafen, E., and Rubin, G. (1985). Rescue of the Drosophila phototransduction mutation trp by germline transformation. Science 230, 1040-1043.

Montell, C., and Rubin, G.M. (1989). Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. Neuron 2, 1313-1323.

Niemeyer, B.A., Suzuki, E., Scott, K., Jalink, K., and Zuker, C.S. (1996). The Drosophila light-activated conductance is composed of the two channels TRP and TRPL. Cell 85, 651-659.

Park, Y., Filippov, V., Gill, S.S., and Adams, M.E. (2002). Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development 129, 493-503.

Park, Y., Kim, Y.J., Dupriez, V., and Adams, M.E. (2003). Two subtypes of ecdysis-triggering hormone receptor in Drosophila melanogaster. J Biol Chem 278, 17710-17715.

Prezeau, L., Rives, M.L., Comps-Agrar, L., Maurel, D., Kniazeff, J., and Pin, J.P. (2010). Functional crosstalk between GPCRs: with or without oligomerization. Curr Opin Pharmacol 10, 6-13.

Pumain, R., Kurcewicz, I., and Louvel, J. (1987). Ionic changes induced by excitatory amino acids in the rat cerebral cortex. Can J Physiol Pharmacol 65, 1067-1077.

Raghu, P., Colley, N.J., Webel, R., James, T., Hasan, G., Danin, M., Selinger, Z., and Hardie, R.C. (2000). Normal phototransduction in Drosophila photoreceptors lacking an InsP(3) receptor gene. Mol Cell Neurosci 15, 429-445.

Raraty, M., Ward, J., Erdemli, G., Vaillant, C., Neoptolemos, J.P., Sutton, R., and Petersen, O.H. (2000). Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. Proc Natl Acad Sci U S A 97, 13126-13131.

Rohacs, T., Thyagarajan, B., and Lukacs, V. (2008). Phospholipase C mediated modulation of TRPV1 channels. Mol Neurobiol 37, 153-163.

Roller, L., Zitnanova, I., Dai, L., Simo, L., Park, Y., Satake, H., Tanaka, Y., Adams, M.E., and Zitnan, D. (2010). Ecdysis triggering hormone signaling in arthropods. Peptides 31, 429-441.

Selbie, L.A., and Hill, S.J. (1998). G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. Trends Pharmacol Sci 19, 87-93.

Shieh, B.H., and Zhu, M.Y. (1996). Regulation of the TRP Ca2+ channel by INAD in Drosophila photoreceptors. Neuron 16, 991-998.

Shortridge, R.D., and McKay, R.R. (1995). Invertebrate phosphatidylinositol-specific phospholipases C and their role in cell signaling. Invert Neurosci 1, 199-206.

Shukla, R., and Wakade, A.R. (1991). Functional aspects of calcium channels of splanchnic neurons and chromaffin cells of the rat adrenal medulla. J Neurochem 56, 753-758.

Streifel, K.M., Miller, J., Mouneimne, R., and Tjalkens, R.B. (2013). Manganese inhibits ATP-induced calcium entry through the transient receptor potential channel TRPC3 in astrocytes. Neurotoxicology 34, 160-166.

Suh, B.C., and Hille, B. (2002). Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. Neuron 35, 507-520.

Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M., and Zuker, C.S. (1997). A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature 388, 243-249.

Venkatachalam, K., Wasserman, D., Wang, X., Li, R., Mills, E., Elsaesser, R., Li, H.S., and Montell, C. (2010). Dependence on a retinophilin/myosin complex for stability of PKC and INAD and termination of phototransduction. J Neurosci 30, 11337-11345.

Wes, P.D., Xu, X.Z., Li, H.S., Chien, F., Doberstein, S.K., and Montell, C. (1999). Termination of phototransduction requires binding of the NINAC myosin III and the PDZ protein INAD. Nat Neurosci 2, 447-453.

Winks, J.S., Hughes, S., Filippov, A.K., Tatulian, L., Abogadie, F.C., Brown, D.A., and Marsh, S.J. (2005). Relationship between membrane phosphatidylinositol-4,5-bisphosphate and receptor-mediated inhibition of native neuronal M channels. J Neurosci 25, 3400-3413.

Xu, X.Z., Chien, F., Butler, A., Salkoff, L., and Montell, C. (2000). TRPgamma, a drosophila TRP-related subunit, forms a regulated cation channel with TRPL. Neuron 26, 647-657.

Xu, X.Z., Choudhury, A., Li, X., and Montell, C. (1998). Coordination of an array of signaling proteins through homo- and heteromeric interactions between PDZ domains and target proteins. J Cell Biol 142, 545-555.

Xu, X.Z., Li, H.S., Guggino, W.B., and Montell, C. (1997). Coassembly of TRP and TRPL produces a distinct store-operated conductance. Cell 89, 1155-1164.

Yamanaka, N., Marques, G., and O'Connor, M.B. (2015). Vesicle-mediated steroid hormone secretion in Drosophila melanogaster. Cell 163, 907-919.

Zhang, H., Craciun, L.C., Mirshahi, T., Rohacs, T., Lopes, C.M., Jin, T., and Logothetis, D.E. (2003). PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. Neuron 37, 963-975.

Zitnan, D., Kingan, T.G., Hermesman, J.L., and Adams, M.E. (1996). Identification of ecdysis-triggering hormone from an epitracheal endocrine system. Science 271, 88-91.

Zitnan, D., Zitnanova, I., Spalovska, I., Takac, P., Park, Y., and Adams, M.E. (2003). Conservation of ecdysis-triggering hormone signalling in insects. J Exp Biol 206, 1275-1289.

**Table IV.1. RNAi testing of genes involved in GPCR signal transduction in bursicon neurons.**

| Classification | Gene Name | CG Number | RNAi Lines |
|---|---|---|---|
| Gαq | *Gαq (49B)* | CG17759 | $50729^{V}$, $105300^{V}$ |
| | *CG17760* | CG17760 | $52308^{V}$, $107613^{V}$ |
| | *CG30054* | CG30054 | $4643^{V}$, $102887^{V}$ |
| Phospholipase C (PLC) | *PLC21C* | CG4574 | $26557^{V}$, $108395^{V}$ |
| | *norpA* | CG3620 | $31113^{B}$, $105676^{V}$ |
| | *PLC-γ (sl)* | CG4200 | $32385^{B}$, $32906^{B}$ |
| ER $Ca^{2+}$ channel | *$IP_3R$* | CG1063 | $6484^{V}$, $106982^{V}$ |
| TRPC channels | *TRP* | CG7875 | $1365^{V}$, $1366^{V}$ |
| | *TRP- γ* | CG5996 | $9337^{V}$, $105280^{V}$ |
| | *TRPL* | CG18345 | $35571^{V}$, $104450^{V}$ |

UAS-RNAi lines from Vienna Drosophila RNAi Center $(VDRC)^{V}$ and Bloomington Drosophila Stock Center $(BDSC)^{B}$ were crossed to *Pburs-GAL4* line to induce tissue-specific RNA knockdown of each component in bursicon neurons. Two RNAi lines were tested for each gene to minimize off-target effects of double-stranded RNA silencing.

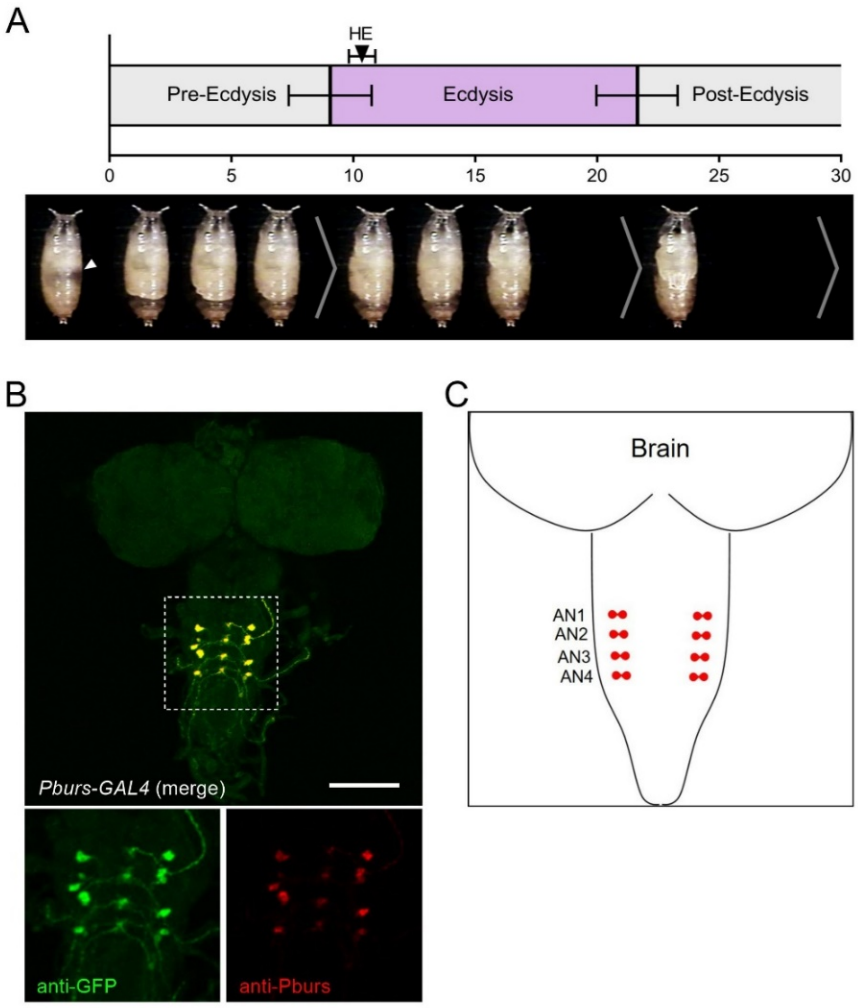
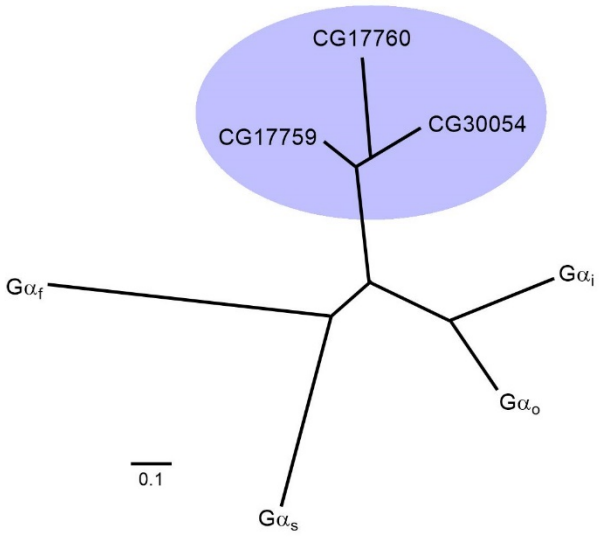



**Figure IV.1. Mapping bursicon neurons in prepupal stage of *Drosophila*.** (A) The *Drosophila* pupal ecdysis sequence. Behaviors were recorded from wild-type (*w1118*), buoyant stage pre-pupae (bottom left, an arrowhead indicates an internal air burble). All time points are relative to the onset of pre-ecdysis. Pre-ecdysis is characterized by anteriolateral rolling contractions of the abdomen following a strong anterior contraction. HE, head eversion. (B) Immunohistochemical staining of *Pburs-GAL4*/*UAS-mCD8-GFP* prepupal CNS to verify specificity of GAL4 expression in bursicon neurons. Scale bar, 50 μm. (C) Schematic diagram showing position of bursicon neurons (AN1-4). AN, abdominal neuromeres.

A


CG17760
CG17759
CG30054
Gαf
Gαi
Gαo
Gαs
0.1

B

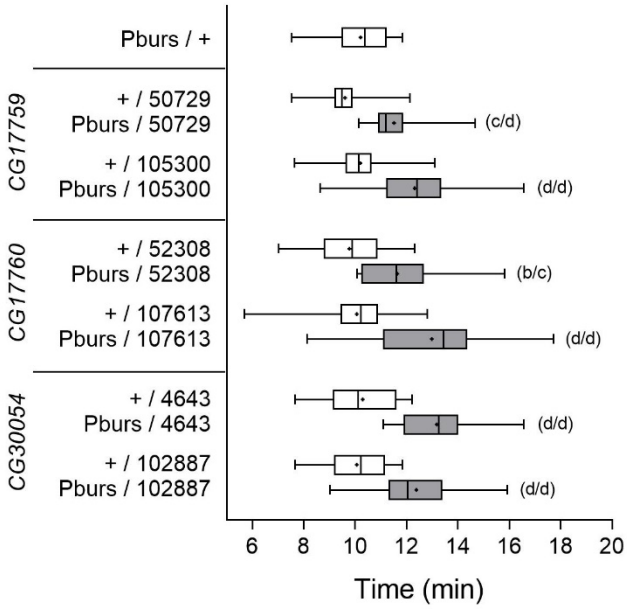

Pburs / +
CG17759
+ / 50729
Pburs / 50729
(c/d)
+ / 105300
Pburs / 105300
(d/d)
CG17760
+ / 52308
Pburs / 52308
(b/c)
+ / 107613
Pburs / 107613
(d/d)
CG30054
+ / 4643
Pburs / 4643
(d/d)
+ / 102887
Pburs / 102887
(d/d)
6
8
10
12
14
16
18
20
Time (min)

**Figure IV.2. Suppressing expression of Gαq proteins alters timing of pupal ecdysis onset.** (A) Phylogenetic tree of Gα proteins in *Drosophila* melanogaster. Two genes (*CG17760* and *CG30054*) encode Gα proteins homologous to Gαq (*CG17759*, also known as *49B*). The scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position. The tree is adapted from a previous study (Yamanaka et al., 2015). (B) Gαq subtypes play a role in bursicon neuronal function. RNA knockdown of Gαq subtypes in bursicon neurons using a *Pburs-GAL4* driver and independent UAS-RNAi lines against *CG17759*, *CG17760*, and *CG30054*. In each genotype, a dot represents the mean, the midline represents the median, the box represents SEM, and the error bars indicate 1-99% percentile or data. Data was analyzed using one-way ANOVA, Tukey's multiple comparison for data of each genotype to GAL4 or UAS controls (Test to GAL4/Test to UAS) (n = 17-25, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$).

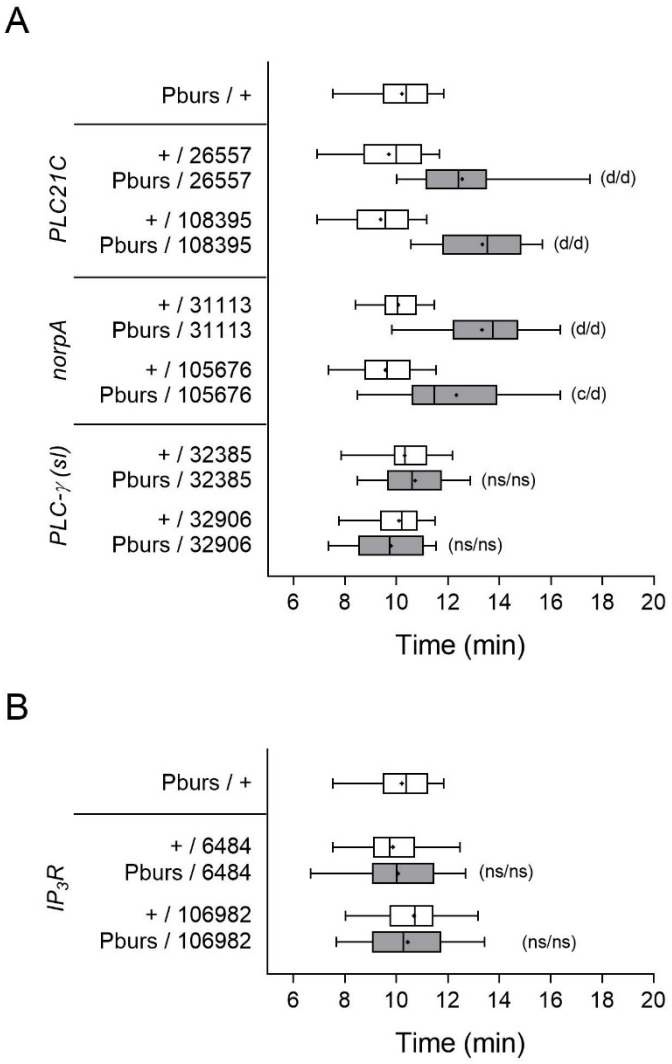



**Figure 3. Suppressing expression of PLC-β enzymes, but not $IP_3$ receptor delays onset of pupal ecdysis.** (A) Knockdown of all PLC-β enzyme isoforms tested (PLC21C, norpA, and PLC-γ (sl)) delays onset of pupal ecdysis behavior (n = 18-22). (B) $IP_3$-mediated signaling does not required for the bursicon neuronal function to trigger ecdysis onset. *Drosophila* $IP_3$ receptor ($IP_3R$) expression was suppressed in bursicon neurons. (n = 19-25).

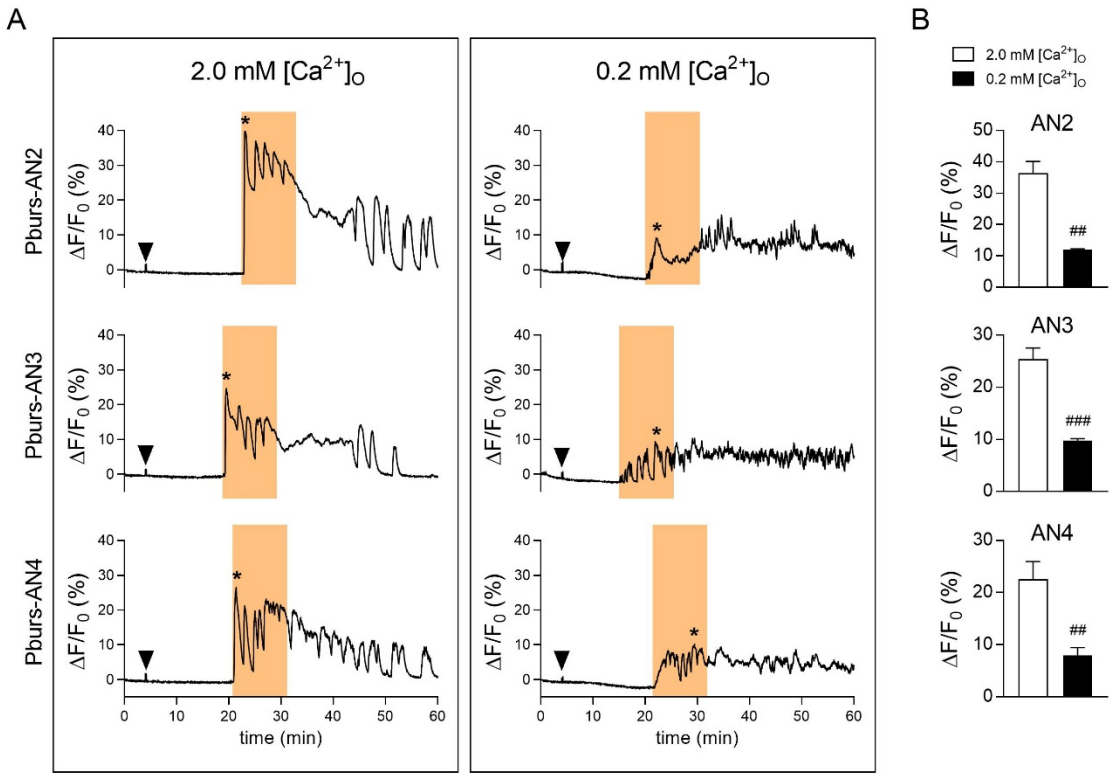



**Figure IV.4. ETH signaling-driven $Ca^{2+}$ dynamics in bursicon neurons are dependent upon extracellular $Ca^{2+}$ level.** (A) Representative intracellular $Ca^{2+}$-associated fluorescence signals of bursicon neurons (AN2-4 of *Pburs-GAL4*/*UAS-GCaMP5*) recorded from the isolated CNS of prepupae. ETH1 (600 nM) was applied to the isolated pupal CNS in 2.0 mM $Ca^{2+}$-containing normal saline (left box) or 0.2 mM $Ca^{2+}$ media. Arrowheads indicate timing of ETH treatment. (B) Peak fluorescence amplitudes in bursicon AN2-4 cells within 10 min (orange box in each representative graph of A) from the first point of oscillation (asterisks in A) following ETH treatment (Student's *t*-Test, n = 3-4, $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$).

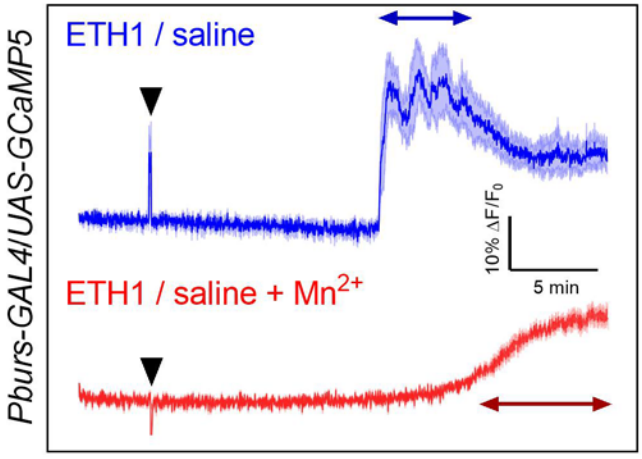



**Figure IV.5. $Mn^{2+}$ treatment blocks "fast" $Ca^{2+}$ mobilization but maintains "slow" $Ca^{2+}$ elevation in bursicon cells in response to ETH.** $Ca^{2+}$-associated fluorescence signals of bursicon AN2 neurons following ETH1 (600 nM) treatment were recorded from the pupal CNS of *Pburs-GAL4*/*UAS-GCaMP5* animals in 2.0 mM $Ca^{2+}$-containing normal fly saline without (above, blue) or with (below, red) 1.0 mM $Mn^{2+}$. Values plotted indicate average ± SEM (n = 3).

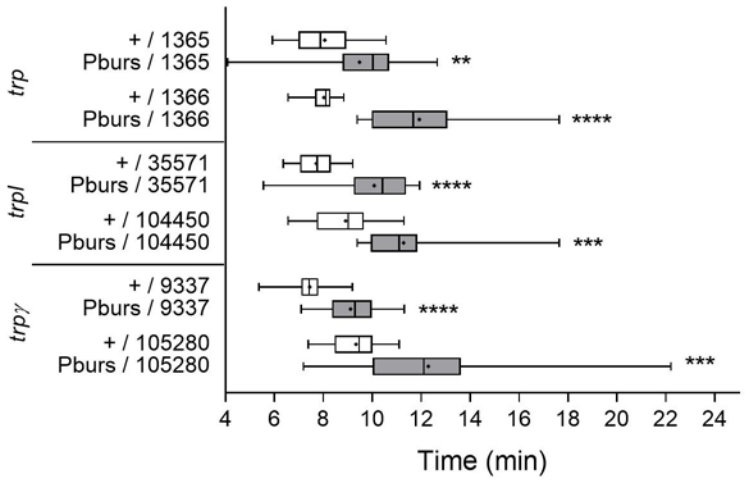



**Figure IV.6. *Drosophila* TRPC channels play a role in timing of bursicon-mediated onset of ecdysis behavior.** RNA Knockdown of TRPC subtypes in bursicon neurons using *Pburs-GAL4* driver and independent UAS-RNAi lines against specific sequences of trp, trpl, and trpγ. Data was analyzed using unpaired Student *t*-Test of each genotype to UAS. (n = 15-22, ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

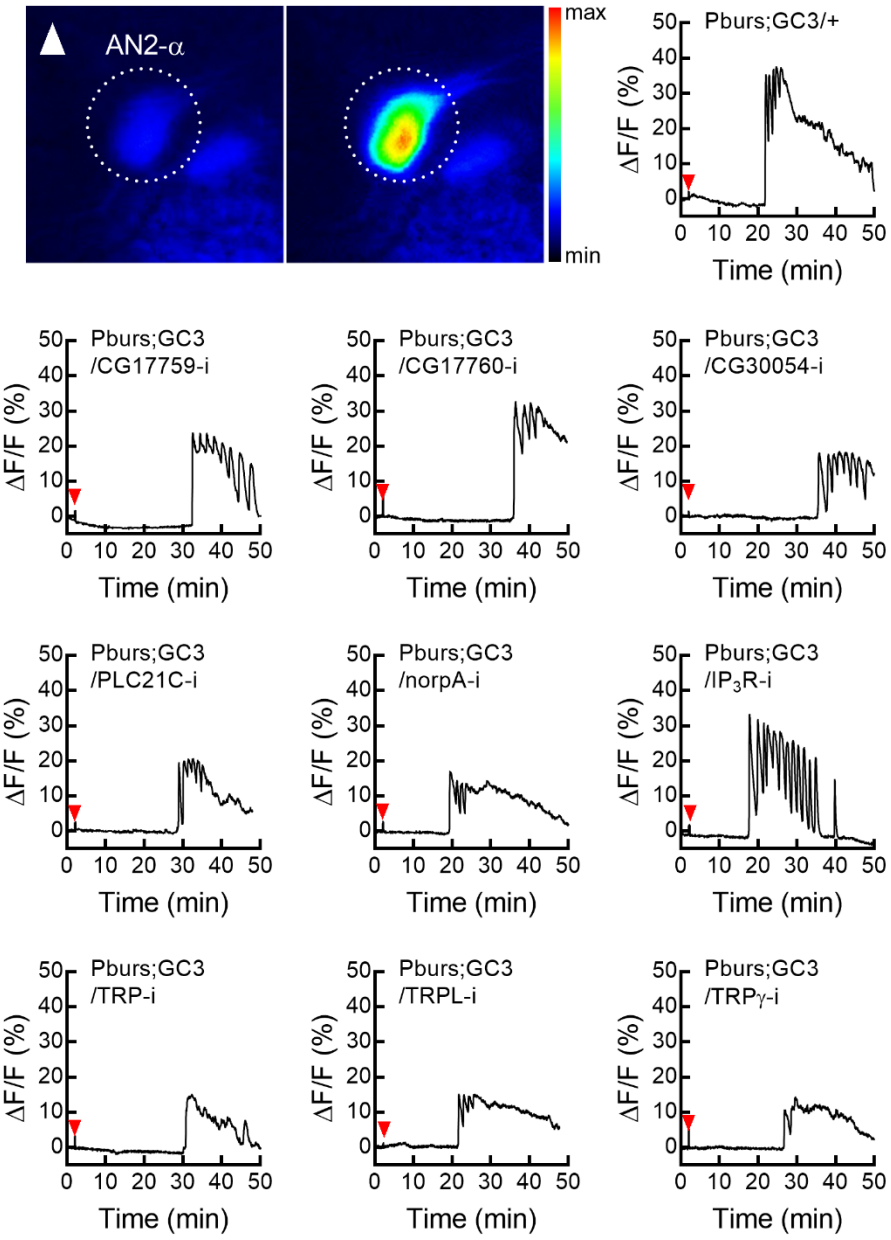

AN2-α
max
min
Pburs;GC3/+
Pburs;GC3 /CG17759-i
Pburs;GC3 /CG17760-i
Pburs;GC3 /CG30054-i
Pburs;GC3 /PLC21C-i
Pburs;GC3 /norpA-i
Pburs;GC3 /IP3R-i
Pburs;GC3 /TRP-i
Pburs;GC3 /TRPL-i
Pburs;GC3 /TRPγ-i
ΔF/F (%)
Time (min)

**Figure IV.7. Representative $Ca^{2+}$ responses of bursicon AN2-α cells after 600 nM ETH1 treatment.** Upper pictures show an AN2-α cell of *Pburs-GAL4;UAS-GCaMP3* prepupae before (left) and after (right) ETH application. A white arrowhead directs anterior side. The right panel shows representative $Ca^{2+}$-associated fluorescence in response to ETH, and below plots are presented as representative traces of $Ca^{2+}$ responses of AN2-α cells of signaling molecule-silenced prepupae. Red arrowheads in each plot represent the ETH treatment.

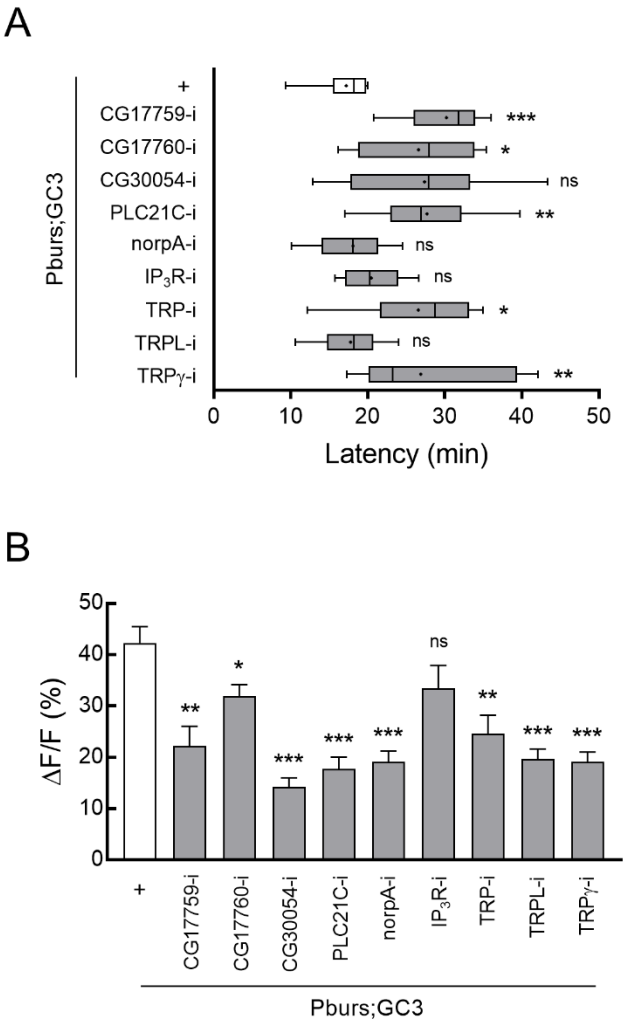



**Figure IV.8. Analysis of $Ca^{2+}$ dynamics in AN2-α cells responding to ETH application.** (A) Latency to first increase of fluorescence following ETH application. In each genotype, a dot represents the mean, the midline represents the median, the box represents SEM, and the error bars indicate min-max of data (Mann-Whitney *U* Test, n = 5-8, **P* < 0.05; ***P* < 0.01; ****P* < 0.001 ns: not significant). (B) Mean ± SEM maximum fluorescence amplitudes of AN2-α cells exposed to 600 nM ETH1 (Student *t*-Test, n = 5-8, **P* < 0.05; ***P* < 0.01; ****P* < 0.001 ns: not significant).

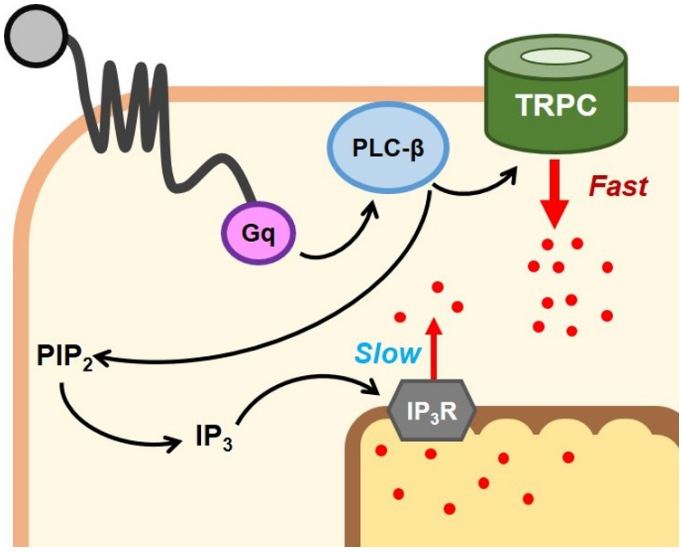



**Figure IV.9. Schematic illustration of G protein-mediated $Ca^{2+}$ mobilization in bursicon neurons.** 1) Gαq proteins activate PLC-β enzymes following activation of ETHR by ETH. 2) PLC-β mediates the signaling cascade directly or indirectly activating TRPC type channels, thereby promoting $Ca^{2+}$ influx, which plays a critical role in the activation of bursicon neurons. 3) PLC-mediated $PIP_2$ hydrolysis causes slow, gradual $Ca^{2+}$ mobilization from ER to cytoplasm through $IP_3$ signaling; this type of $Ca^{2+}$ mobilization may not play a critical role in neuronal activation.

# CHAPTER V

## Inhibition of the CCAP Neuronal Function Impairs *Drosophila* Reproductive Behaviors


### Abstract

Although basic physiological mechanisms underlying reproduction of animals have been described extensively, neural pathways modulating effectiveness of reproductive behaviors are less well-known. Here, I show that neuropeptide crustacean cardioactive peptide (CCAP)-expressing neurons are important in modulation of reproductive behaviors of both male and female fruit fly, *Drosophila melanogaster*. Electrical silencing of CCAP neurons in males leads to elevated male-male courtship, as does block of synaptic release from these neurons. In particular, synaptic block leads to selective increase in copulation attempts, which is not observed following neuronal silencing. In recently mated females, inhibition of the CCAP neuronal function leads to loss of ethanol preference while ovipositing and disinhibition of egg laying. Based on these observations, I propose a sexually-conserved role for CCAP neurons in decision-making, but also sexual dimorphic tuning of these neurons for increased effectiveness of reproduction based on distinct interests of *Drosophila* sexes.

.


### Introduction

Animals are born with set of innate behaviors that require finely modulated neural mechanisms. It is not only limited to the body plan or fixed action pattern (FAP) during development, but also essential for adult behaviors. In *Drosophila*, among adult innate behaviors, male courtship and female oviposition have been considerably studied (Joseph et al., 2009; Pan and Baker, 2014; Wu

et al., 2015; Yamamoto and Koganezawa, 2013; Yang et al., 2008). However, how neural pathways modulate these behaviors is still not fully understood.

*Drosophila* male courtship is composed of multiple behavioral subroutines: orienting, tapping, singing, licking, and attempting to copulate (Greenspan and Ferveur, 2000). Several genetic and neural architectures are involved in decision-making to modulate each subtype, thereby avoiding futile reproductive effort and to achieve reproductive success. When a male encounters another male, he shows little attraction: a wild-type male generally spends less than 10% time for courting other males, during 10 minutes-long pairings. Instead, he is usually not interested in other males or even displays aggression, particularly when he is starved, to defend a food source. In this decision making, multiple sensory stimulus and modulatory circuits are at operational (Hu et al., 2015; Moon et al., 2009; Thistle et al., 2012).

In females, selection of appropriate oviposition sites is critical for progeny fitness and survival. In particular, ethanol preference of a mated female has several benefits for progeny. Although high ethanol consumption by fly larvae entails risk, lower concentrations of ethanol increase body weight, which may result in metabolic fitness (Devineni and Heberlein, 2013; Geer et al., 1993; McClure et al., 2011; Ranganathan et al., 1987). Furthermore, a recent study revealed that low concentrations of ethanol in hemolymph of larvae protects them against the infection by natural parasites (Milan et al., 2012). This decision-making of females is also promoted by several signal inputs and neural modulations (Azanchi et al., 2013; Kacsoh et al., 2013).

As the name indicates, the neuropeptide crustacean cardioactive peptide (CCAP) has cardioacceleratory propterties in insects (Estevez-Lao et al., 2013; Nichols et al., 1999). Under specific conditions, CCAP induces cardiac reversal (Dulcis and Levine, 2003; Dulcis et al., 2005). CCAP serves other functions as a neurotransmitter and neurohormone (Lange and Patel, 2005; Mikani et al., 2015; Sakai et al., 2006; Suggs et al., 2016). Strong circumstantial evidence

implicates CCAP in ecdysis motor control of juvenile insects (Ewer, 2005; Gammie and Truman, 1997; Kim et al., 2006; Lee et al., 2013; Zitnan and Adams, 2000). Previous findings showed that CCAP, but not CCAP neuronal innervation, acts on muscular contractions of oviduct and spermatheca in female locusts, indicating additional functions for CCAP during the adult stage of insects (da Silva and Lange, 2006; Donini et al., 2001; Donini and Lange, 2002).

In this study, I used genetic and ethological approaches to identify roles of CCAP neurons in reproductive behaviors of both male and female *Drosophila*. Both silencing and blocking CCAP neurons increases male sexual avidity toward other males, but only blocking synaptic transmission elevates a specific subroutine of courtship behavior, namely copulation attempts. In mated females, silencing or blocking CCAP neurons negatively affects ethanol preference in oviposition and also increases egg production.

## Materials and Methods

**Fly Strains.** Fly lines were raised on standard cornmeal-agar media at 25°C in a 12:12 L:D cycle and aged 5-7 days after eclosion. *Canton-S*, *wCS*, and *w1118* were used as wild-types. Since GAL4, and UAS lines have *w1118* genetic background, all heterogygous genetic controls were prepared by crossing with the w1118 line. All animals were collected individually during the pupal period to prevent any pretest social interaction. *CCAP-GAL4;Pburs-GAL80* line was obtained from Dr. Young-Joon Kim (GIST, South Korea). *CCAP-GAL4*, *UAS-mCD8-GFP*, *UAS-Kir2.1*, *UAS-TeTx* lines were obtained from Bloomington Stock Center.

**Courtship Assays.** Single-pair courtship assays were described previously (Demir and Dickson, 2005). Briefly, a test male and a partner were paired in a gating-based courtship chamber of 10 mm diameter and 4 mm height for 10 min. For the male mating assay, a day-4 male was paired with a

mature (day 4-5) *Canton-S* virgin female and recorded with a high-frame digital camcorder for 10 min. Copulation was analyzed every 2 min. For courtship activity assays of males, a male was placed with an immobilized (decapitated) Canton-S virgin female ($F_{dv}$) and courtship behaviors were recorded 10 min. For male-male courtship assays, we used day 4 *wCS* males as partners to distinguish a test male's behavior, since test males have red eyes. All behaviors were scored blindly. Courtship index (CI) includes all courting activities: CI (%) = {[courtships]/600 s} X 100. Copulation attempts were also counted at the same time.

**Female Two-choice Oviposition Assay.** Preparation of the oviposition arena was modified from a prior study (Azanchi et al., 2013). A virgin female was paired with a *Canton-S* male in a courtship arena for 1 hour. Only mated females during the pairing were individually transferred to the 35 mm two-choice food plate (Figure V.4A). Two-choice food plates were prepared by combining normal food and 5% ethanol-containing food. Flies were left in the dark at 25℃ to lay eggs for 18 hours. After removing a female, the number of eggs on the plate were counted (food + $H_2O$, midline, and food + 5% ethanol) under a stereomicroscope.

**Immunohistochemistry.** The CNS of *CCAP-GAL4*/*UAS-mCD8-GFP* flies was dissected in phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde in PBS overnight at 4℃. After washing with PBST (0.5% Triton X-100 in PBS) and blocking with 5% NGS (normal goat serum) in PBST at room temperature, samples were incubated with rabbit anti-CCAP (1:1000) and mouse anti-GFP (1: 500) for two days at 4℃. Tissues were then washed with PBST and incubated with Alexa Fluor 633 goat anti-rabbit IgG antibody and Alexa Fluor 488 goat anti-mouse IgG antibody (1:500 each). After washing with PBST and PBS, tissues were prepared on slides in mounting medium (Aqua Poly/Mount, Polysciences Inc.). For Figure V.1A, rabbit anti-GFP (1:500) and mouse anti-nc82 (1:50) were used as primary antibodies, and Alexa Fluor 488 goat anti-rabbit (1:500) IgG and Alexa Fluor 568 goat anti-mouse (1:500) IgG were used for secondary antibodies.

Confocal images were acquired using a Zeiss LSM 510 confocal microscope. For higher resolusion of neuronal structure in Figure V.1A, color was inverted by utilizing a raster graphics software (Adobe Photoshop CC).

## Results

**Inhibition of CCAP neuronal activity increases male-male courtship.** Although CCAP neurons of *Drosophila* are broadly present through the juvenile CNS (Kim et al., 2006), a mature adult has two sets of CCAP neurons in the CNS. A GFP-expressing *CCAP-GAL4* line shows a labeling pattern consisting of a pair of brain neurons projecting posteriorly and 13-14 neurons located in abdominal neuromeres of the fused thoracic ganglion (Figure V.1A). All neurons exhibit strong CCAP-like immunoactivity (Figure V.1B).

To investigate the role of CCAP neurons in normal courtship, I first tested whether inhibition of CCAP neuronal function changes male courtship avidity toward females. I ectopically expressed the inward-rectifier potassium channel Kir2.1 using the *CCAP-GAL4* driver. Since a few bursicon neurons that also express CCAP have a critical role during ecdysis, Kir2.1 expression was suppressed specifically in these neurons by combining *CCAP-GAL4* and *Pburs-GAL80*. I found that CCAP-, but not bursicon neuron-silenced mature males show courtship activity similar to genetic control groups when paired with virgin wild-type females (Table V.1).

To investigate the role of CCAP neurons during male-male interactions, I paired *CCAP-GAL4;Pburs-GAL80*/*UAS-Kir2.1* males with wild-type males, *wCS10*, which can be distinguished by having white eyes. Electrical silencing of non-bursicon CCAP neurons significantly increased rates of male-male courtship (Figure V.2). Likewise, blocking synaptic output from CCAP neurons through ectopic expression of tetanus toxin light chain (TeTx) (Sweeney et al., 1995) significantly

elevated male-male courtship rates, indicating that CCAP neurons are involved in courtship decision-making of males encountering other males.

**Synaptic transmission from CCAP neurons plays a role in regulation of a specific courtship behavior subroutine: copulation attempts.** Male courtship behavior is composed of a series of sequential behavioral subroutines, such as orienting, tapping, singing, leaking, and attempting to copulate (Greenspan and Ferveur, 2000). In particular, abdominal bending associated with attempted copulation is easily observed. Since copulation attempt is the final courtship step following multiple decision-making, normal males barely show this behavior toward a male target (Figure V.3). Electrical silencing of CCAP neurons in males (*CCAP-GAL4;Pburs-GAL80*/*UAS-Kir2.1*) did not induce significant changes in frequency of this behavior, compared to wild-type and genetic control groups. However, blocking synaptic transmission from non-bursicon CCAP neurons (*CCAP-GAL4;Pburs-GAL80*/*UAS-TeTx*) resulted in an increased number male-male courtship attempts. This suggests that a specific behavioral subroutine, copulation attempt, is regulated by chemical signal transmission from CCAP neurons, possibly in the absesnce of electrical activity.

**Inhibition of the CCAP neuronal function causes abnormal oviposition of mated females.** Female oviposition assay was performed using a two-choice food arena: one half of the plate is filled with normal fly food and the other half is filled with food containing 5% ethanol (Figure V.4A). I tested whether either electrically silencing (*UAS-Kir2.1*) or blocking synaptic transmission (*UAS-TeTx*) affects female oviposition behavior using the *CCAP-GAL4;Pburs-GAL80* line (Figure V.4B). Newly mated females were individually placed in the arena and each female was allowed to lay eggs for 18 hours under dark conditions.

I found that either electrical silencing (*UAS-Kir2.1*) or blocking vesicle release (*UAS-TeTx*) from CCAP neurons increased egg production significantly (Figure V.4C). *CCAP-GAL4;Pburs-*

*GAL80*/*UAS-Kir2.1* females produced 33-48% more eggs and *CCAP-GAL4;Pburs-GAL80*/*UAS-TeTx* females produced 48-61% more eggs than their genetic control groups.

In the case of ethanol preference for oviposition, similar to the previous report (Azanchi et al., 2013), wild-type and genetic control groups laid approximately 60% more eggs on the 5% ethanol-containing food side. However, when CCAP neurons were subjected to electrical silencing or block of vesicle release, ethanol preference while laying eggs was abolished (Figure V.4D). Thus, my data show that female CCAP neurons play a significant role in both control of egg production choice of oviposition site.

## Discussion

Inhibiting functions of CCAP neurons resulted in reproductive behavior abnormalities in both male and female *Drosophila*, suggesting that these neurons have multiple roles in behavioral control. The overall findings in this study is summarized in Figure V.5A.

**Roles of CCAP neurons during male courtship behavior in male-male pairing.** Electrical silencing of CCAP neurons increased male-male courtship frequency (Figure V.2), whereas no changes in male-female courtship behavior was observed. Block of exocytotic release from CCAP neurons also increased courting activity of males, but only with regard to a specific behavioral subroutine: attempted copulation with a male partner (Figure V.3). This behavior was extremely rare in normal (wild-type) males when paired with another males, since this behavior is the last step following prior courtship behaviors that normally permit recognition of proper mating partners, such as orienting, tapping, singing, and leaking. During prior courtship sequences, multiple sensory inputs are integrated for optimal decision-making (Krstic et al., 2009). Males with electrically silenced CCAP neurons showed normal frequency of copulation attempts. This indicates specific

and separate roles for electrical firing and chemical transmission of CCAP neurons in regulation of a specific behavior.

A pair of brain CCAP neurons may play a role in decision-making of males paired with other males, and electrically-induced neuronal activity likely promotes chemical communication of these neurons with other modulatory or output circuit neurons localized in deep brain areas such as mushroom body and central complex (Figure V.1). In contrast, CCAP neurons present in abdominal ganglia appear to play roles in inhibition of muscular contractions involved in regulation of copulation attempts. Electrically-driven neuronal activity may not be necessary for suppressing muscular contraction, whereas exocytotic release from these neurons likely plays a role. This suggests that presynaptic inputs may regulate CCAP release exclusive of electrical activity in CCAP neurons. In *Aplysia* motor circuits (Frost and Kandel, 1995), serotonergic interneurons innervating axon termini modulate synaptic transmission, thereby regulating downstream motor circuits. In this aspect, a "second player" may play a compensatory role for motor control even after the suppression of electrical firing of CCAP neurons through overexpression of potassium channels. One possible mechanism underlying this hypothesis could involve ligand-gated $Ca^{2+}$ channels localized on axon terminals. I thus hypothesize that synaptic communication between abdominal CCAP neurons and their cellular targets in motor circuits could be regulated by facilitatory neuronal inputs innervating to presynaptic terminals, thereby activating a putative inhibitory muscle contraction circuit (Figure V.5B).

**Roles of female CCAP neurons for egg-laying.** Suppressing CCAP neuron electrochemical functions also affects female behaviors. Both silencing electrical activity and blocking exocytotic release from of CCAP neurons abolishes female preference for ethanol during oviposition. This is likely a consequence of failure of recognition or decision-making for an appropriate egg-laying site. A recent study identified neural pathways important for female ethanol preference (Azanchi et al.,

2013). In particular, a dopaminergic PPM3 cluster regulates 5% ethanol preference through innervation of the ellipsoid body, a component of the central complex. Immunohistochemical staining of CCAP using a dopaminergic driver *TH-GAL4* shows that brain CCAP neurons are not dopaminergic (figure not shown), suggesting that the CCAP neural circuit in the female brain may connect to the PPM3 circuit or share the target area, or both. Inhibition of CCAP neurons also increased egg production. This may also be attributed to the role of inhibition, similar to that invoked for motor output neurons in male abdominal ganglia. The *Drosophila* female reproductive apparatus involves three types of muscles. Each ovary is surrounded by a contractile peritoneal sheath and individual ovarioles are covered by a contractile epithelial sheath, while the oviduct also contains epithelial muscle layers. Previous studies revealed that the monoamine octopamine controls contractions of the peritoneal sheath and relaxation of the oviduct muscle (Lee et al., 2009; Middleton et al., 2006), and this cooperation permits ovulation. I hypothesize that the electrochemical action of ganglionic CCAP neurons may involve oviduct contraction or relaxation. In particular, CCAP may induce contractions of muscle layers localized in the common oviduct, playing an opposite role to that of octopamine (Figure V.5C). Precise control of sequential muscle contraction is important for effectiveness of the behavior, thereby minimizing any waste of the animal's energy resources.

In conclusion, I investigated roles of CCAP neurons in adult *Drosophila*, and found that loss-of-function in electrical and secretory activities of CCAP neurons results in abnormalities in reproductive behaviors of both sexes. Phenotypes affected are summarized in Figure V.6. Since it has been previously reported that peripheral CCAP neurons have a function in adults (Dulcis and Levine, 2003), I still cannot rule out the effect of peripheral input in these phenotypes. Also, there may be additional neural/molecular components interacting with CCAP-driven pathways.

Although several questions are left for future studies, my results provide possible neural components crucial to regulation of sexually dimorphic behavioral plasticity.

## References


Azanchi, R., Kaun, K.R., and Heberlein, U. (2013). Competing dopamine neurons drive oviposition choice for ethanol in Drosophila. Proc Natl Acad Sci U S A 110, 21153-21158.

da Silva, R., and Lange, A.B. (2006). The association of crustacean cardioactive peptide with the spermatheca of the African migratory locust, Locusta migratoria. J Insect Physiol 52, 399-409.

Demir, E., and Dickson, B.J. (2005). fruitless splicing specifies male courtship behavior in Drosophila. Cell 121, 785-794.

Devineni, A.V., and Heberlein, U. (2013). The evolution of Drosophila melanogaster as a model for alcohol research. Annu Rev Neurosci 36, 121-138.

Donini, A., Agricola, H., and Lange, A.B. (2001). Crustacean cardioactive peptide is a modulator of oviduct contractions in Locusta migratoria. J Insect Physiol 47, 277-285.

Donini, A., and Lange, A.B. (2002). The effects of crustacean cardioactive peptide on locust oviducts are calcium-dependent. Peptides 23, 683-691.

Dulcis, D., and Levine, R.B. (2003). Innervation of the heart of the adult fruit fly, Drosophila melanogaster. J Comp Neurol 465, 560-578.

Dulcis, D., Levine, R.B., and Ewer, J. (2005). Role of the neuropeptide CCAP in Drosophila cardiac function. J Neurobiol 64, 259-274.

Estevez-Lao, T.Y., Boyce, D.S., Honegger, H.W., and Hillyer, J.F. (2013). Cardioacceleratory function of the neurohormone CCAP in the mosquito Anopheles gambiae. J Exp Biol 216, 601-613.

Ewer, J. (2005). Behavioral actions of neuropeptides in invertebrates: insights from Drosophila. Horm Behav 48, 418-429.

Frost, W.N., and Kandel, E.R. (1995). Structure of the network mediating siphon-elicited siphon withdrawal in Aplysia. J Neurophysiol 73, 2413-2427.

Gammie, S.C., and Truman, J.W. (1997). Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, Manduca sexta. J Neurosci 17, 4389-4397.

Geer, B.W., Heinstra, P.W., and McKechnie, S.W. (1993). The biological basis of ethanol tolerance in Drosophila. Comp Biochem Physiol B 105, 203-229.

Greenspan, R.J., and Ferveur, J.F. (2000). Courtship in Drosophila. Annu Rev Genet 34, 205-232.

Hu, Y., Han, Y., Shao, Y., Wang, X., Ma, Y., Ling, E., and Xue, L. (2015). Gr33a modulates Drosophila male courtship preference. Sci Rep 5, 7777.

Joseph, R.M., Devineni, A.V., King, I.F., and Heberlein, U. (2009). Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in Drosophila. Proc Natl Acad Sci U S A 106, 11352-11357.

Kacsoh, B.Z., Lynch, Z.R., Mortimer, N.T., and Schlenke, T.A. (2013). Fruit flies medicate offspring after seeing parasites. Science 339, 947-950.

Kim, Y.J., Zitnan, D., Galizia, C.G., Cho, K.H., and Adams, M.E. (2006). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr Biol 16, 1395-1407.

Krstic, D., Boll, W., and Noll, M. (2009). Sensory integration regulating male courtship behavior in Drosophila. PLoS ONE 4, e4457.

Lange, A.B., and Patel, K. (2005). The presence and distribution of crustacean cardioactive peptide in the central and peripheral nervous system of the stick insect, Baculum extradentatum. Regul Pept 129, 191-201.

Lee, D., Orchard, I., and Lange, A.B. (2013). Evidence for a conserved CCAP-signaling pathway controlling ecdysis in a hemimetabolous insect, Rhodnius prolixus. Front Neurosci 7, 207.

Lee, H.G., Rohila, S., and Han, K.A. (2009). The octopamine receptor OAMB mediates ovulation via $Ca^{2+}$/calmodulin-dependent protein kinase II in the Drosophila oviduct epithelim. PLoS One 4, e4716.

McClure, K.D., French, R.L., and Heberlein, U. (2011). A Drosophila model for fetal alcohol syndrome disorders: role for the insulin pathway. Dis Model Mech 4, 335-346.

Middleton, C.A., Nongthomba, U., Parry, K., Sweeney, S.T., Sparrow, J.C., and Elliott, C.J.H. (2006). Neuromuscular organization and aminergic modulation of contractions in the Drosophila ovary. BMC Biol 4, 17.

Mikani, A., Watari, Y., and Takeda, M. (2015). Brain-midgut cross-talk and autocrine metabolastat via the sNPF/CCAP negative feed-back loop in the American cockroach, Periplaneta americana. Cell Tissue Res 362, 481-496.

Milan, N.F., Kacsoh, B.Z., and Schlenke, T.A. (2012). Alcohol consumption as self-medication against blood-borne parasites in the fruit fly. Curr Biol 22, 488-493.

Moon, S.J., Lee, Y., Jiao, Y., and Montell, C. (2009). A Drosophila gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. Curr Biol 19, 1623-1627.

Nichols, R., McCormick, J., Cohen, M., Howe, E., Jean, C., Paisley, K., and Rosario, C. (1999). Differential processing of neuropeptides influences Drosophila heart rate. J Neurogenet 13, 89-104.

Pan, Y., and Baker, B.S. (2014). Genetic identification and separation of innate and experience-dependent courtship behaviors in Drosophila. Cell 156, 236-248.

Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., and Ewer, J. (2003). Targeted ablation of CCAP neuropeptide-containing neurons of Drosophila causes specific defects in execution and circadian timing of ecdysis behavior. Development 130, 2645-2656.

Ranganathan, S., Davis, D.G., and Hood, R.D. (1987). Developmental toxicity of ethanol in Drosophila melanogaster. Teratology 36, 45-49.

Sakai, T., Satake, H., and Takeda, M. (2006). Nutrient-induced alpha-amylase and protease activity is regulated by crustacean cardioactive peptide (CCAP) in the cockroach midgut. Peptides 27, 2157-2164.

Suggs, J.M., Jones, T.H., Murphree, S.C., and Hillyer, J.F. (2016). CCAP and FMRFamide-like peptides accelerate the contraction rate of the antennal accessory pulsatile organs (auxiliary hearts) of mosquitoes. J Exp Biol 219, 2388-2395.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C.J. (1995). Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14, 341-351.

Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., and Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during Drosophila courtship. Cell 149, 1140-1151.

Wu, C.L., Fu, T.F., Chou, Y.Y., and Yeh, S.R. (2015). A single pair of neurons modulates egg-laying decisions in Drosophila. PLoS One 10, e0121335.

Yamamoto, D., and Koganezawa, M. (2013). Genes and circuits of courtship behaviour in Drosophila males. Nat Rev Neurosci 14, 681-692.

Yang, C.H., Belawat, P., Hafen, E., Jan, L.Y., and Jan, Y.N. (2008). Drosophila egg-laying site selection as a system to study simple decision-making processes. Science 319, 1679-1683.

Zitnan, D., and Adams, M.E. (2000). Excitatory and inhibitory roles of central ganglia in initiation of the insect ecdysis behavioural sequence. J Exp Biol 203, 1329-1340.

**Table V.1. Male-female pairing assay: copulation rates and courtship activity**

| Genotype | [a]Copulation in 10 min, % | [b]CI (%) |
|---|---|---|
| *CCAP-GAL4;Pburs-GAL80/+* | 95 | 82.3 ± 3.4 (20) |
| *+/UAS-Kir2.1* | 90 | 76.9 ± 4.3 (20) |
| *CCAP-GAL4;Pburs-GAL80/UAS-Kir2.1* | 87.5 | 85.5 ± 7.3 (20) |

Animals of the indicated genotypes were single-raised day 4 males (see Material and Methods).

a. The total copulation rates were tested by counting the number of males copulated with virgin females in 10 minutes.

b. The courtship indices were analyzed from the time of courting activities of males toward an unreceptive immobilized virgin female in 10 minutes.

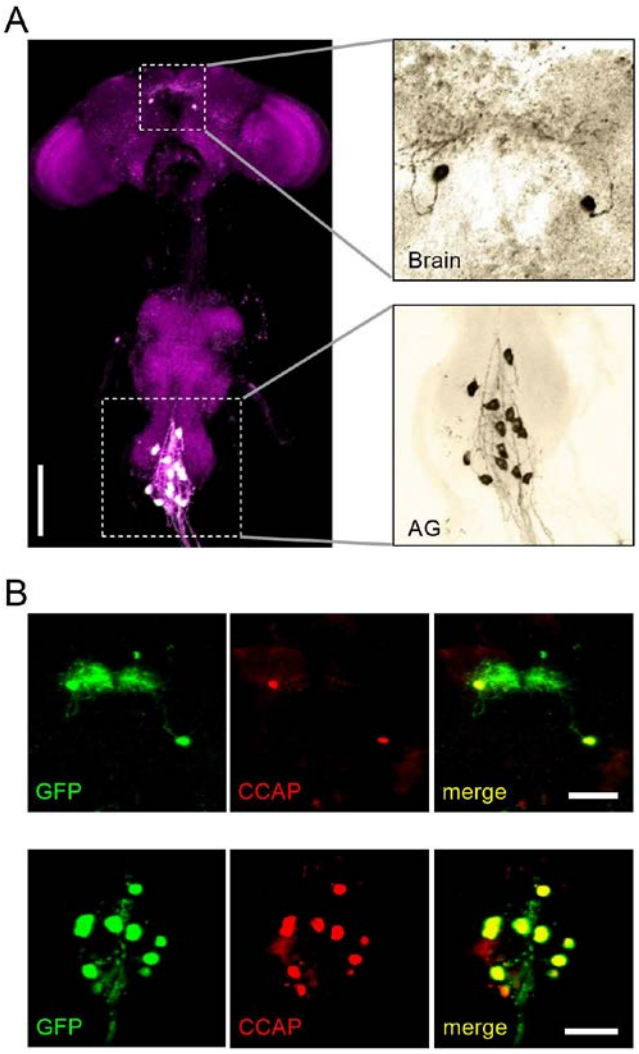



**Figure V.1. Mapping CCAP neurons in the adult male CNS.** (A) Representative images for post-eclosion day 4 *CCAP-GAL4*/*UAS-mCD8-GFP* CNS along with magnified images of the highlighted regions (posterior view, brain/abdominal ganglion (AG)). Scale bar represents 100 μm. (B) Adult CCAP neurons are co-localized with CCAP-immunoactive cells. In the adult CNS, intense CCAP-immunoactivity was detected in cell bodies in the brain (above) and AG (below). Scale bars indicate 50 μm.

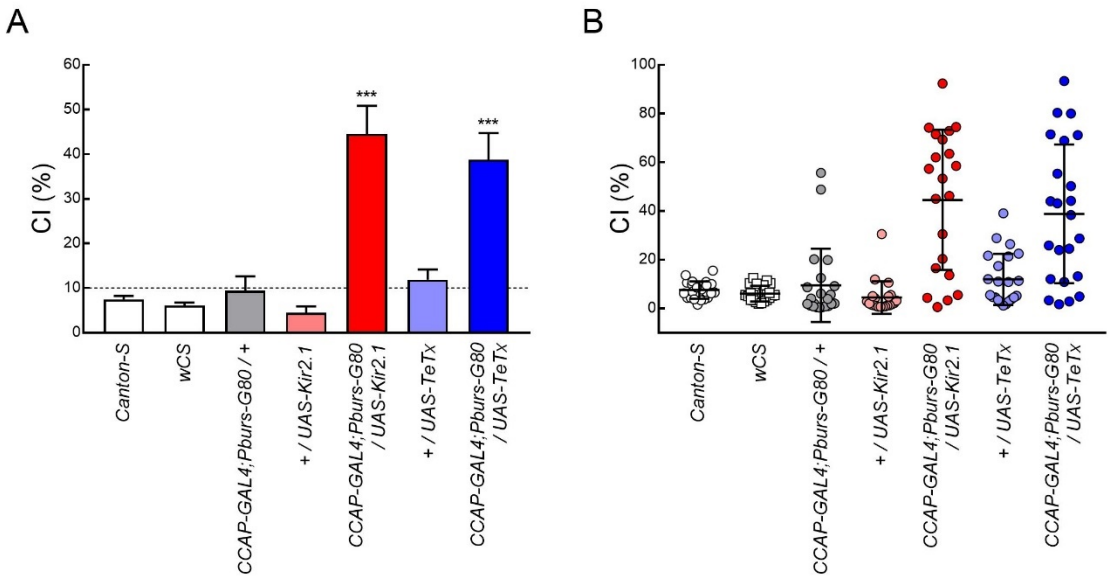



**Figure V.2. Electrical silencing of male CCAP neurons elevates male-male courtship.** The role of non-bursicon CCAP neurons in male-male courtship was tested by either electrically silencing neurons (*CCAP-GAL4;Pburs-GAL80*/*UAS-Kir2.1*) or blocking synaptic transmission (*CCAP-GAL4;Pburs-GAL80*/*UAS-TeTx*). (A) Mean CI of individual test males paired with single *wCS* males. (B) Same data as in (A) plotted according to individual CI distributions. (n = 21-23, ***$P < 0.001$, Kruskal-Wallis ANOVA test). Error bars in (A) indicate SEM, while those in (B) represent SD; middle bars in (B) indicate mean scores.

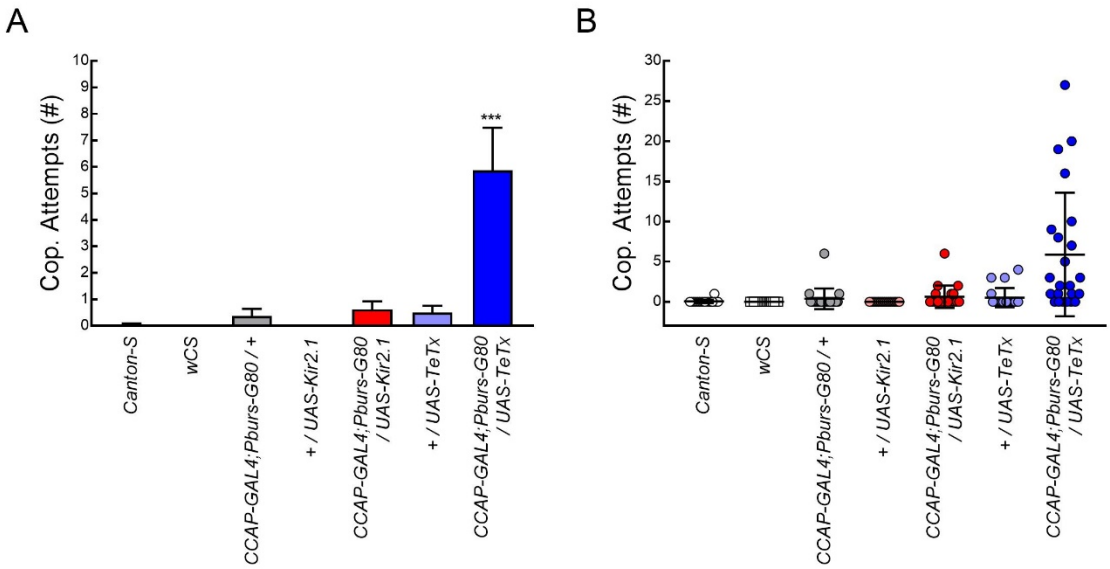



**Figure V.3. Blocking synaptic release from CCAP neurons induces abnormal attempted copulation.** The role of non-bursicon CCAP neurons in male-male courtship was tested by either electrically silencing neurons (*CCAP-GAL4;Pburs-GAL80*/*UAS-Kir2.1*) or blocking synaptic transmission (*CCAP-GAL4;Pburs-GAL80*/*UAS-TeTx*). (A) Mean CI of individual test males paired with single *wCS* males. (B) Same data as depicted in (A) plotted as individual CI distributions. (n = 21-23, ***$P < 0.001$, Kruskal-Wallis ANOVA test). Error bars in (A) depict SEM, while those in (B) show SD; middle bars in (B) indicate mean scores.

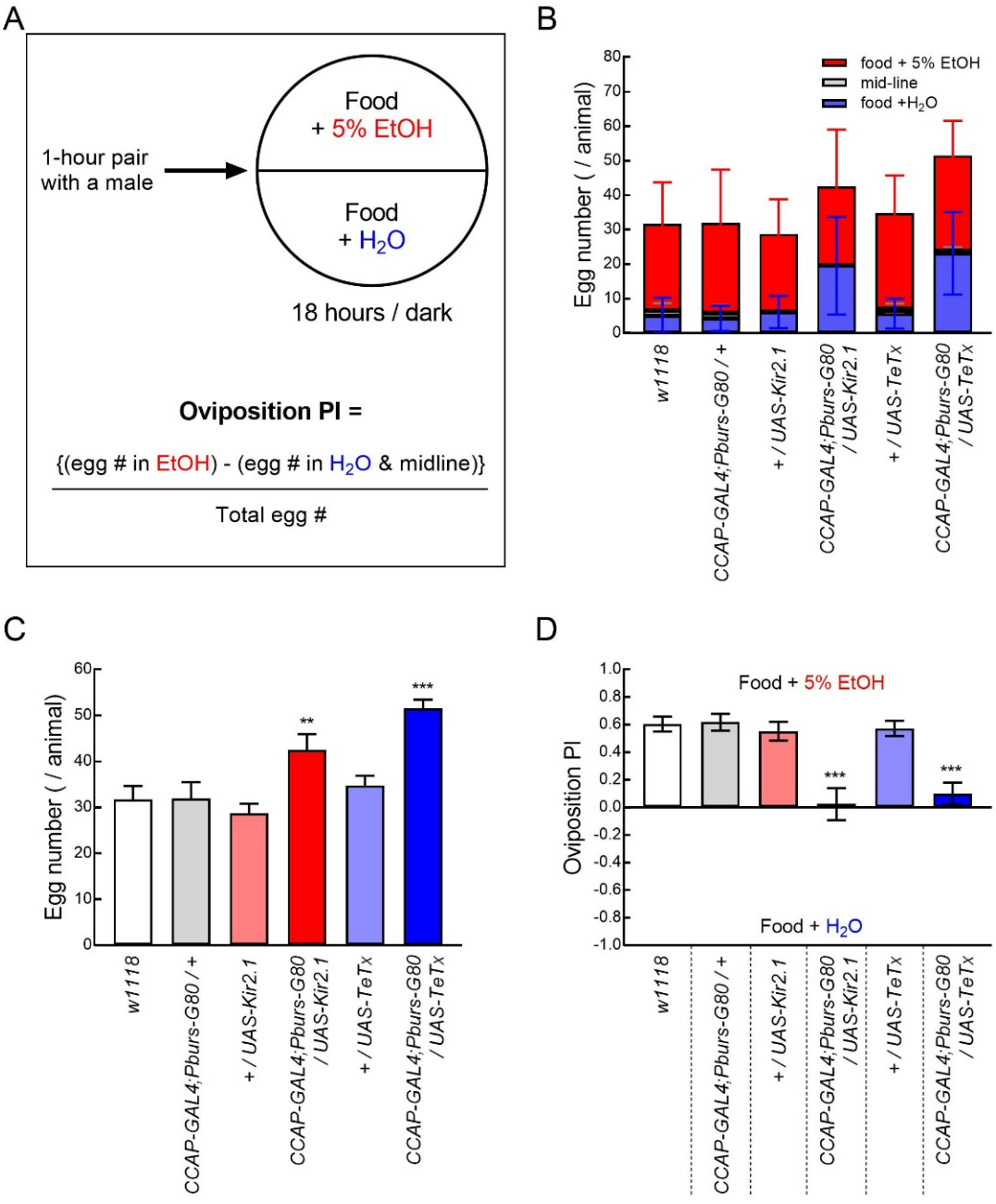

A
Food
+ 5% EtOH
1-hour pair
with a male
Food
+ H2O
18 hours / dark
Oviposition PI =
{(egg # in EtOH) - (egg # in H2O & midline)}
Total egg #
B
food + 5% EtOH
mid-line
food +H2O
Egg number ( / animal)
w1118
CCAP-GAL4;Pburs-G80 / +
+ / UAS-Kir2.1
CCAP-GAL4;Pburs-G80 / UAS-Kir2.1
+ / UAS-TeTx
CCAP-GAL4;Pburs-G80 / UAS-TeTx
C
Egg number ( / animal)
**
***
D
Food + 5% EtOH
Food + H2O
Oviposition PI
***
***

**Figure V.4. Electrical silencing of CCAP neurons influences female oviposition behavior.** (A) Diagram illustrating oviposition assay. Mated female preference for 5% ethanol was tested using a two-choice assay. Oviposition assays were performed by placing recently mated females in an oviposition arena for 18 hours. (B) Egg-laying phenotypes were obtained by electrically silencing CCAP neurons (*CCAP-GAL4;Pburs-GAL80*/*UAS-Kir2.1*) or blocking presynaptic release from CCAP neurons (*CCAP-GAL4;Pburs-GAL80*/*UAS-TeTx*). Error bars indicate SD (n = 21-26). (C) Total egg number in scored in the oviposition arena. (D) Oviposition preference for 5% ethanol. Error bars indicate SEM (One-way ANOVA, $^{**}P < 0.01$; $^{***}P < 0.001$).

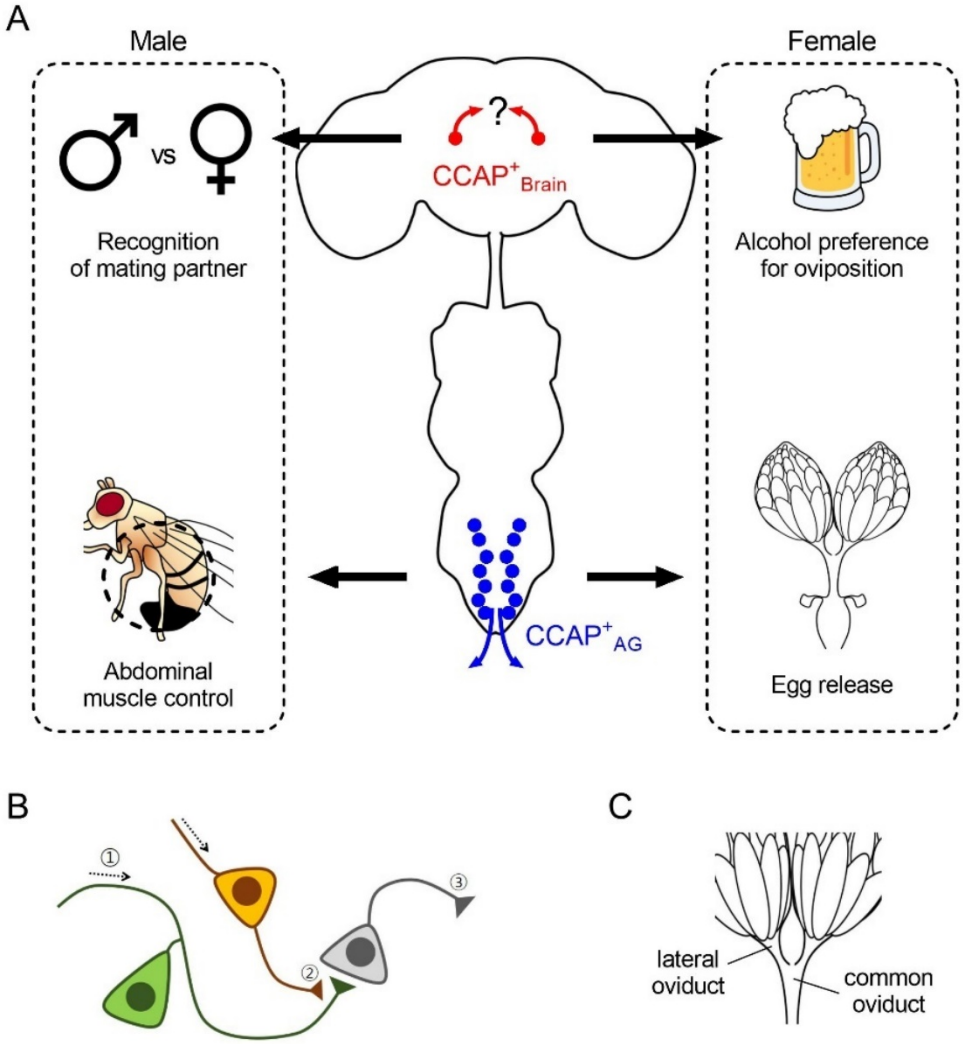

A
Male
Female
vs
?
CCAP+ Brain
Recognition
of mating partner
Alcohol preference
for oviposition
Abdominal
muscle control
CCAP+ AG
Egg release
B
①
②
③
C
lateral
oviduct
common
oviduct

**Figure V.5. Summary of functions for CCAP neurons in reproductive behaviors.** (A) CCAP neurons play diverse roles in both male and female *Drosophila*. In males, CCAP neurons contribute to (i) recognition of mating partners, which may be related to decision-making circuits in brain, and (ii) muscular control during the copulation attempts through exocytotic release from CCAP neurons. In females, these neurons are involved in finding appropriate oviposition sites possibly having functional conservation with dopaminergic circuits in brain. Also, electrochemical action of AG neurons likely suppresses egg release while laying eggs. (B) A hypothetical model for CCAP action on male abdominal muscle contractions. ① An aversive signal input stimulates the CCAP neuron, but neurotransmitter release fails to occur without additional input. ② An "second player" neuron stimulates presynaptic terminals, promoting synaptic release from CCAP neuron to a target neuron. ③ The target neuron of the chemical transmission inhibits muscle contractions. (C) Possible target regions of CCAP neurons for ovulation control. CCAP neurons may play a role opposite to octopamine in control of contraction/relaxation of lateral or common oviducts.

# CHAPTER VI

## General Conclusions

Animal behaviors are precisely controlled through cooperation of anatomical and molecular components in the nervous system. Although circulating hormones have been considered as promoters and modulators of neuronal activity, mechanisms underlying the endocrine-neural network are still illusive. A series of ecdysis behaviors, centrally patterned fixed action patterns in juvenile insects, are initiated upon release of the peptide hormone ETH from epitracheal Inka cells (Zitnan et al., 1996; Zitnan et al., 1999). The periphery-originated ETH promotes sequential activities of central peptidergic neurons through activation of G protein-mediated calcium dynamics and the precise motor controls (Kim et al., 2015; Kim et al., 2006), indicating that this circulating peptide acts on the nervous system as a command chemical by orchestrating multiple neural functions. Although molecular components of ETH signaling are still present in the adult stage (Graveley et al., 2011; Meiselman et al., 2017; Park et al., 2002) have critical functions in adult reproduction (Areiza et al., 2014; Meiselman et al., 2017), post-developmental functions of this peptide are largely unknown. Thus, identification of ETH signaling functions in adult behavior will help us to elucidate important neural elements modulating behaviors. In my studies, the genetically modifiable model organism, *Drosophila melanogaster* was utilized to investigate hormonal regulation of adult behavioral plasticity.

In this dissertation, I focused mainly on the allatotropic role (promoting JH synthesis) of ETH during adulthood, which is essential for *Drosophila* memory processes. For this purpose, I utilized the courtship conditioning memory paradigm (McBride et al., 1999; Siegel and Hall, 1979). In particular, Chapter II described the role of the ETH-JH hormonal cascade in short-term memory retention. In adult males, ETH binds to ETH receptors (ETHRs) present in corpora allata (CA) cells

and induces calcium mobilization. RNA knockdown of ETHRs in the CA attenuated not only calcium mobilization, but also increased latency to the response to ETH exposure. As a consequence, ETHR silencing in JH levels by more than 70%. JH-deficient males exhibited short-term memory deficits induced by 1-hour training with a mated female, and memory impairment was rescuable by JH analog treatment. Although a recent study showed JH deficiency likely causes impairment of pheromone sensing in males (Lin et al., 2016) , I showed that JH action in short-term courtship memory is mainly related to behavioral cues (i.e., rejection by a mated female) while the training. Precise genetic manipulations and pharmacological treatments revealed that influences of the ETH-JH signaling cascade occur during a critical period: the first 3 days after the eclosion. Lastly, I showed that JH targets dopaminergic neurons to maintain short-term memory.

In Chapter III, I described the role of ETH signaling in formation of long-term memory induced by a 5-hour aversive sexual experience. Suppression of ETH signaling and the ETH-JH cascade impaired memory formation. Thermogenetically reinforced Inka cell activity during the training upregulated memory performance of males and reduced the training period required for 24-hour memory maintenance. I also showed that enhanced memory resulting from Inka cell reinforcement lasts significantly longer than that of genetic control groups. This Inka cell activity-dependent memory formation is mediated by new protein synthesis. To identify targets of ETH for memory formation, I employed RNA knockdown of ETHRs in the nervous system and found that ETH directly targets mushroom body γ neurons and a pair of memory consolidation neurons (DAL neurons, (Chen et al., 2012)). Functional calcium imaging showed gradual calcium mobilization by ETH in these neurons, suggesting the requirement of ETH-dependent calcium signaling in the memory process. Similar to my findings in short-term memory studies, the ETH-JH cascade also is essential for long-term memory. However, dopaminergic neurons labeled by the TH-Gal4 driver, which are essential for short-term memory, are not the main target of JH to regulate long-term

memory formation. Instead, expression of the JH receptor Met in mushroom body γ neurons is required for memory formation, providing a hormonal convergence model for regulation of memory formation, which was suggested previously in the mammalian limbic system (Ferry et al., 1999; McGaugh, 2004; Roozendaal et al., 1999).

Together, Chapter II and Chapter III described the essential role of hormonal states in regulation of *Drosophila* learning and memory. I summarize these findings with a new model of parallel endocrine pathways. As I described above, the ETH-JH-DA cascade is essential for STM maintenance (>10 min memory induced by 1-hour exposure to the sexual rejection by a mated female). Since ETHR-mediated JH synthesis has an early adult-specific (first three days) critical period, which is correlated with age-dependent JH fluctuation in males, neural maturation of dopaminergic memory circuits appears to depend on critical endocrine influences. In contrast, ETH-mediated JH synthesis has a role in regulating long-term memory (24-hour memory following 5-hour training) in mature males, indicating that this hormonal cascade acts independent of the STM memory process. In addition to the ETH-JH cascade, ETH signaling also directly affects the function of LTM memory circuits. ETH-driven G protein signaling is involved in calcium-dependent mechanisms in the CA, and memory circuits and it will be required for both JH production and protein synthesis. Since it has been previously reported that levels of the steroid hormone ecdysone are elevated in males upon long-term experience of courtship failure (Ishimoto et al., 2009), and that ETH levels are regulated by ecdysone (Cho et al., 2014), I provide a model of ecdysone-ETH-JH endocrine pathway for long-term memory formation. Furthermore, my observation of hormonal convergence in the mushroom body of *Drosophila* reveals a memory mechanism similar to that described for convergence of norepinephrine and glucocorticoids in the mammalian amygdala.

In Chapter IV, I described the molecular pathway underlying ETH signaling by utilizing pupal ecdysis as a model system. Our previous studies provided evidence that ETH promotes calcium mobilization and activation of peptidergic (e.g., CCAP, bursicon, kinin, etc) neurons (Kim et al., 2015; Kim et al., 2006). In particular, ETHR knockdown in bursicon-producing neurons delays the behavioral switch from pre-ecdysis to ecdysis (Kim et al., 2015). However, the signal transduction mechanism in target cells induced by ETH signaling has not been studied in detail. I found that G proteins and PLC enzymes in bursicon neurons are crucial for timing of the switch to ecdysis. However, $IP_3$ receptor expression, which has been considered as the main calcium source regulated by G proteins, was not necessary for ecdysis timing, suggesting that another source of calcium is responsible the the ecdysis switch. Functional calcium imaging data suggests that ETH-driven rapid calcium influx from the extracellular space is important for this process. In normal fly saline solution, a set of bursicon neurons showed robust calcium dynamics in response to ETH exposure. They first showed a strong calcium-associated fluorescence peak following ETH treatment, which gradually subsided over a period of ~1 hour. In contrast, lowering the external calcium concentration markedly reduced the magnitude of this first fluorescence peak. Signficantly, inclusion of manganese in the extracellular bathing medium resulted in a similara reduction, suggesting that ETH signaling likely mediates influx of extracellular calcium. Lastly, RNA silencing of TRPC-type channels in bursicon neurons also delays timing of pupal ecdysis onset, suggesting the interaction between G protein signaling and membrane channels, which has been revealed in the fly phototransduction system (Montell, 2012). Results from functional imaging experiments provide a strong correlation between $Ca^{2+}$ dynamics in bursicon cells and timing of the ecdysis switch following ETH exposure. My study provides new information regarding signal transduction in endocrine cells, which will be helpful in understanding mechanisms underlying G protein-mediated regulation of neuronal activity.

In Chapter V, I switched my topic to the function of CCAP neurons. As the name indicates, CCAP controls cardiac function. Although CCAP serves other functions as a neuromodulator in juvenile and adult stages, its function in adult behaviors has not been reported. Using the GAL4/UAS system and immunostaining, I confirmed presence of CCAP-immunoreactive neurons in the mature adult CNS of both male and female *Drosophila*. I also found that CCAP neurons play distinct functional roles in males and females. Genetically inhibiting CCAP neuronal activity resulted in abnormally increased male courting behavior toward other males. The dramatic increase in attempted copulation behavior induced by blocking synaptic transmission, but not by electrical silencing, suggests that independent neural mechanisms underly these male courtship behaviors. Electrochemical activity of female CCAP neurons also plays important roles in oviposition. Both electrical silencing and inhibition of exocytosis in female CCAP neurons impairs their ethanol preference during oviposition and increases the number of eggs produced. Although detailed mechanisms underlying these phenotypes are not clear, these findings contribute to our understanding of neuropeptide-based sexual dimorphism for reproductive behaviors.

In summary, I investigated functional roles played by the endocrine system for control of simple and complex *Drosophila* behaviors by utilizing diverse technques such as transgenesis, precise behavioral analysis, quantitative molecular analysis, immunohistochemistry, pharmacology, and functional imaging. Combining these experimental approaches provided new insights into neurohormonal interactions underlying behavior changes and their mechanistic underpinnings. Functional imaging demonstrated calcium mobilization induced by ETH signaling in target cells. These findings suggest that G protein-mediated calcium signaling mechanisms powerfully influence cellular activity. In particular, JH levels in male flies of different ages is strongly correlated to the critical period governing JH actions in memory performance. Combining targeted

RNAi silencing with calcium imaging of neural elements reveals influences of hormone-neural interacting pathways and underlying signal mechanisms on changes of behavior.

As I described in Chapter I, behavioral plasticity of animals is processed through various neural components, and the endocrine system plays significant roles in provoking or modulating neural circuits to change behavior. My studies reported in this dissertation provide not only evidence for functions of hormones in simple and complex behaviors, but also the cellular changes that underly them. I hope that my findings inspire other researchers in neuroscience and endocrinology to unveil further the contributions of endocrine-neural networks to animal and human behaviors. I want to finish my dissertation with an inspirational paragraph from a writing of Charles Darwin (Darwin, 1871): *"Nevertheless the difference in mind between man and higher animals, great as it is, certainly is one of degree and not of kind. We have seen that the senses and intuitions, the various emotions and faculties, as love, memory, attention, curiosity, imitation, reason, etc., of which man boasts, may be found in an incipient, or even sometimes in a well-developed condition in lower animals"*.

## References


Areiza, M., Nouzova, M., Rivera-Perez, C., and Noriega, F.G. (2014). Ecdysis triggering hormone ensures proper timing of juvenile hormone biosynthesis in pharate adult mosquitoes. Insect Biochem Mol Biol 54, 98-105.

Chen, C.C., Wu, J.K., Lin, H.W., Pai, T.P., Fu, T.F., Wu, C.L., Tully, T., and Chiang, A.S. (2012). Visualizing long-term memory formation in two neurons of the Drosophila brain. Science 335, 678-685.

Cho, K.H., Daubnerova, I., Park, Y., Zitnan, D., and Adams, M.E. (2014). Secretory competence in a gateway endocrine cell conferred by the nuclear receptor betaFTZ-F1 enables stage-specific ecdysone responses throughout development in Drosophila. Dev Biol 385, 253-262.

Darwin, C. (1871). The descent of man, and selection in relation to sex. London: John Murray. 1st ed., p.105.

Ferry, B., Roozendaal, B., and McGaugh, J.L. (1999). Role of norepinephrine in mediating stress hormone regulation of long-term memory storage: a critical involvement of the amygdala. Biol Psychiatry 46, 1140-1152.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., et al. (2011). The developmental transcriptome of Drosophila melanogaster. Nature 471, 473-479.

Ishimoto, H., Sakai, T., and Kitamoto, T. (2009). Ecdysone signaling regulates the formation of long-term courtship memory in adult Drosophila melanogaster. Proc Natl Acad Sci U S A 106, 6381-6386.

Kim, D.H., Han, M.R., Lee, G., Lee, S.S., Kim, Y.J., and Adams, M.E. (2015). Rescheduling behavioral subunits of a fixed action pattern by genetic manipulation of peptidergic signaling. PLoS Genet 11, e1005513.

Kim, Y.J., Zitnan, D., Galizia, C.G., Cho, K.H., and Adams, M.E. (2006). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr Biol 16, 1395-1407.

Lin, H.H., Cao, D.S., Sethi, S., Zeng, Z., Chin, J.S., Chakraborty, T.S., Shepherd, A.K., Nguyen, C.A., Yew, J.Y., Su, C.Y., et al. (2016). Hormonal modulation of pheromone detection enhances male courtship success. Neuron 90, 1272-1285.

McBride, S.M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G., and Siwicki, K.K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron 24, 967-977.

McGaugh, J.L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci 27, 1-28.

Meiselman, M., Lee, S.S., Tran, R.T., Dai, H., Ding, Y., Rivera-Perez, C., Wijesekera, T.P., Dauwalder, B., Noriega, F.G., and Adams, M.E. (2017). Endocrine network essential for reproductive success in Drosophila melanogaster. Proc Natl Acad Sci U S A 114, E3849-E3858.

Montell, C. (2012). Drosophila visual transduction. Trends Neurosci 35, 356-363.

Park, Y., Filippov, V., Gill, S.S., and Adams, M.E. (2002). Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development 129, 493-503.

Roozendaal, B., Williams, C.L., and McGaugh, J.L. (1999). Glucocorticoid receptor activation in the rat nucleus of the solitary tract facilitates memory consolidation: involvement of the basolateral amygdala. Eur J Neurosci 11, 1317-1323.

Siegel, R.W., and Hall, J.C. (1979). Conditioned responses in courtship behavior of normal and mutant Drosophila. Proc Natl Acad Sci U S A 76, 3430-3434.

Zitnan, D., Kingan, T.G., Hermesman, J.L., and Adams, M.E. (1996). Identification of ecdysis-triggering hormone from an epitracheal endocrine system. Science 271, 88-91.

Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., and Adams, M.E. (1999). Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. Neuron 23, 523-535.

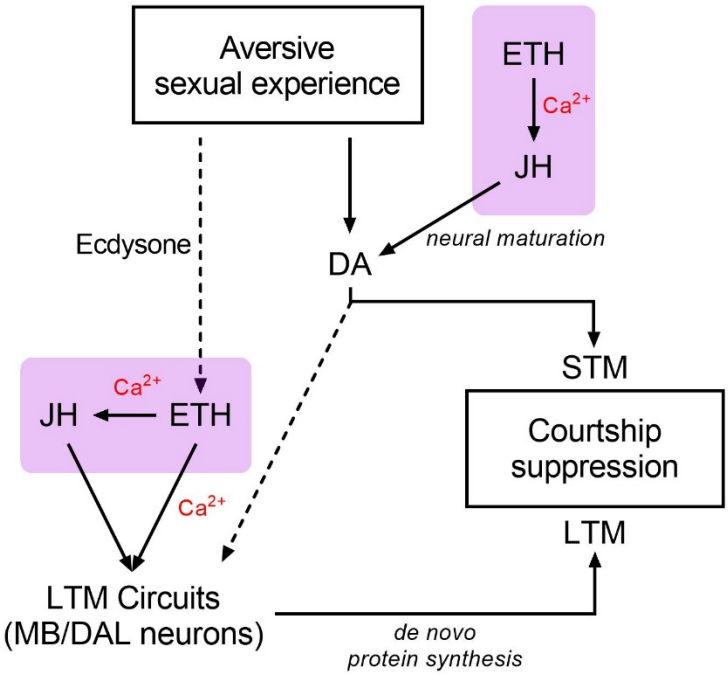



**Figure VI.1. A summary of endocrine-neural interactions contributing to *Drosophila* memory processes induced by courtship conditioning.** Dashed lines represent hypothetical functional connections based on prior studies.