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Courtship and Reproduction Through an Endocrine Lens: The Role and Regulation of Ecdysis-Triggering Hormone in Behavior of *Drosophila melanogaster* Adults

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ABSTRACT OF THE DISSERTATION

Courtship and Reproduction Through an Endocrine Lens: The Role and Regulation of Ecdysis-Triggering Hormone in Behavior of *Drosophila melanogaster* Adults

by

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Endocrine state is an emergent phenomenon; hormonal coregulation in multicellular organisms generally establishes a malleable endocrine "backbone" which allows individuals to coordinate a systemic response to environmental and physiological stimuli. While endocrine networks regulating reproduction in vertebrate systems are very well-defined, the post developmental networks in arthropoda lack clarity. Using the model insect *Drosophila melanogaster*, with its exceptional genetic tractability, this dissertation elucidates a novel hormone network regulating reproduction and courtship, and demonstrates how this network affords the organism versatile control of reproductive behaviors. Developmental hormones 20-hydroxyecdysone (20E) and juvenile hormone (JH) have well-defined roles as gonadotropins among insects, but ecdysis triggering-hormone (ETH) has been largely ignored, despite its prevalence and critical role in development. This work here provides evidence that ETH provides a link between these

two hormones, and relays hierarchical information regarding endocrine state to a variety of targets which regulate male and female reproduction. Taken together, data from this dissertation support the hypothesis that Drosophila reproduction is tightly regulated by 20E, ETH and JH, which work in tandem to ensure coordination of physiology and environment.

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Introduction

An early milestone in modern endocrinology is attributed to the experiments of Kopec and Wigglesworth, who, through ligation experiments were able to show neurosecretory cells in the brain were able to synthesize and release a blood-borne chemical signal with competence to stimulate molting. At the time, hormones were assumed to be the product solely of the gonads, and the idea of a brain-derived hormone was, at that point, not under consideration. Since then, the insects, and molting in particular, have proven to be a convenient model to study how hormone systems can work together to coordinate physiological response and activity.

The arthropod phylum is the largest and most successful in the animal kingdom, representing three quarters of all known species, extinct or extant. This is thanks in large part to their integumentary system and ability to protect themselves against the environment and xenobiotics. Their rigid, chitinous exoskeleton does an excellent job of this, but it must be remade for each life stage as the insects grow. This process is called molting, and the motor program that activates the terminal behavioral sequence is known as ecdysis.

After the last ecdysis at eclosion, a variety of works have provided strong evidence that rather than disappear or face obsolescence, 20E and JH are repurposed as regulators of the adult-specific behavior of reproduction (Jindra, Palli, & Riddiford, 2013; Uryu, Ameku, & Niwa, 2015). Juvenile hormone (JH), produced in the adult corpus allatum, has been shown to regulate learning and memory, diapause, innate immunity and yolk protein uptake into vitellogenic oocytes (Denlinger, 1985; Flatt et al., 2008; Raikhel,

Brown, & Belles, 2005). Ecdysone (hereafter 20E) has an equally critical role, regulating longevity, male courtship, neuronal activity, stress resistance, circadian rhythm and oogenesis (Uryu et al., 2015). However, there has been no proposed function for ETH, despite its documented persistence into the adult stage(Park, Filippov, Gill, & Adams, 2002).

Unlike the aforementioned molting hormones, Ecdysis Triggering Hormone (ETH) is an amidated peptide hormone. The Adams lab discovered this hormone 20 years ago for its role, as the nomenclature suggests, in triggering the ecdysis behavior. ETH is processed from a preprohormone to ETH1, ETH2 and ETHAP. ETH1 is a 10-fold more potent activator than ETH2 to their receptors, ETH receptor A and ETH receptor B, while the third product of the preprohormone, ETH-AP, does not activate the receptors(Park, Kim, Dupriez, & Adams, 2003). These two alternatively spliced versions of the ETHR gene share 4 of the 5 ETHR exons, and are both high affinity Gαq coupling GPCRs. They differ both in binding affinity (ETHR-B is about 200-fold more sensitive than ETHR-A) and their expression pattern ETHR-A and B have been shown to express in mutually exclusive neurons during the developmental stages (Diao et al., 2016). Both ETH and ETHR genes are tightly regulated by 20E.

During my graduate research I was able to use the wealth of research conducted on endocrine co-regulation during the developmental stages as an "endocrine scaffold." I used this scaffold to build a network of dependency around ETH that allowed for the establishment of new hormonal axes, and a deeper understanding of innate reproductive behaviors in *Drosophila melanogaster* adults.

Endocrine Network Essential for Reproductive Success in *Drosophila melanogaster*

As organisms grow and develop, they encounter temporally isolated challenges that demand dynamic biological response. As larvae, holometabolous insects need only be concerned with feeding and survival. As adults, the same insects have to be able to reproduce, an entirely new behavior, using the same genetic tools. In large part, this change is facilitated by hormones. In humans, gonadotropic hormones are mostly dormant during childhood. In females, when the body is prepared for adulthood with adequate fat stores, leptin activates the hypothalamus to release gonadotropin releasing hormone, which causes the release of gonadotropins follicle stimulating hormone and luteinizing hormone, activating the growth of the first follicle and steroidogenesis(Knobil & Neill, 2006). When this process, menarche, occurs, it sets in motion a complex endocrine network, feeds back to itself, integrates external cues, and dominates the female reproductive system throughout the individuals entire reproductive life(Knobil & Neill, 2006).

At the time of my arrival, very little was clear about the endocrine circuitry of Drosophila melanogaster; though most of the key players (insulin, JH, 20E) had been identified, scant attention had been paid to how they interacted with one another, and that attention had yielded no sequential hierarchy as we find in the mammalian system(Badisco, Van Wielendaele, & Broeck, 2013).

Prior to my arrival in the Adams lab it was determined that ETH stimulated a calcium response in the corpora allata (CA), and illumina sequencing suggested that the

ETH receptor was present in the corpora allata (Meiselman et al., 2017)(Adams Lab, Unpublished). The first three years of my work focused heavily on establishing ETH as an allatotropin. I was able to show that silencing ETHR in the CA, or elimination of the Inka cells and consequent elimination of the ETH peptide was sufficient to significantly reduce whole body JH levels. I was also able to show that this resulted in stunted vitellogenesis and reduction in progeny produced in females. In addition these depleted levels of JH resulted in less fecund males, a consequence of reduced accessory gland protein synthesis. As mentioned above, it was known that ETH and ETHR expression were dependent on 20E levels during the developmental stages, so I sought to examine whether ETH and ETHR remained under the influence of 20E as adults. Finally, I showed that elimination of the ecdysone receptor in the Inka cells caused deficiencies in reproduction, analogous to ETH deficiency.

In so doing, I was able to establish a cyclic hormone core on which reproduction and reproductive timing depends during the adult stage.

Altered Endocrine State Induced by Environmental Stressors Mediates Reproductive Arrest through Suppressed Oogenesis and Ovulation

With a role for ETH and the endocrine network in which it fits firmly established, I next asked the succeeding question: What is the role for this system? Generally, the purpose of a hormone is to translate a signal to a discreet subset of cells, allowing for a coordinated response to a stimulus (Mac E Hadley, 1996). Without an understanding of why hormone levels fluctuate, little can be gleaned from even groups of hormones and an

understanding of their targets. To better understand our network, we needed more information on both inputs and outputs. I accomplished this by delving more deeply into the physiology of ETH-deficient females.

By killing the Inka cells after eclosion, we were able to see a multitude of reproductive phenotypes, most of which could be rescued by topical application of methoprene. Several phenotypes, however, could not be explained by JH deficiency. In ETH deficient flies, I observed a drastic increase in mature eggs retained in the ovaries, even more drastic considering the ETH deficient flies' reduced capacity to produce mature eggs (Meiselman et al., 2017). As octopamine plays a well-established role in oviduct relaxation and ovary contraction (Lee, Seong, Kim, Davis, & Han, 2003; Middleton et al., 2006), I tested the hypothesis that ETH targets the octopaminergic neurons innervating the ovaries and oviduct. Indeed, ETH was necessary and sufficient for octopamine release in the vicinity of the female reproductive tract, and for ovulation.

It has been shown that anovulation is a common phenomenon in high 20E flies, and also a number of studies suggested elevation of circulating ecdysone was a translational element in reproductive arrest during stress (Gruntenko, Bownes, Terashima, Sukhanova, & Raushenbach, 2003; Hirashima, Rauschenbach IYu, & Sukhanova MJh, 2000; Ishimoto, Sakai, & Kitamoto, 2009; Schwedes & Carney, 2012; Sedore Willard, Koss, & Cronmiller, 2006; Terashima, Takaki, Sakurai, & Bownes, 2005). ETH was a clear candidate link, as high 20E blocks Inka cell secretory competence in the developmental stages (Cho, Daubnerová, Park, Zitnan, & Adams, 2014), and high 20E during stress could result in decreases in ETH, and ovulation as a

result. Using the heat-stress paradigm, I found that oogenesis and ovulation, two processes I have shown depend upon ETH, are interrupted after stress exposure. I have also shown that elevation of ETH levels, either by activation of the Inka cells or ETH injection, have capacity to rescue depressed fecundity, but only during stress. The Inka cells have more ETH immunoreactivity during stress, arguing for the canonical 20E suppression of ETH release. Using genetics, I also showed that interrupting the molecular components of secretory competence repression, EcR and Bftz-F1, rescue heat stress-induced attenuation of oogenesis, ovulation and fecundity. Finally, elevated 20E and repression of ETH levels were detected and confirmed using enzyme immunoassay.

As systemic clarity had been ascertained, I examined a novel stressor; presence of conspecific carcasses. I found that females exposed to carcasses for a mere 24 hours showed an increase in mature eggs retained in their ovaries and also elevated 20E levels. Moreover, I was able to identify the modality by which carcasses affected healthy females, olfaction.

Here, I showed that the hormonal network I established affords systemic plasticity. In situations unfavorable to reproduction, the dependency of elements of the reproductive system on hormones allows for rapid attenuation. This network allows critical energetic resources to be shunted toward survival rather than be wasted on offspring unlikely to thrive in the stressful environment.

Courtship Discrimination Depends Upon Ecdysis Triggering Hormone Signaling

Reproduction depends not only on functional reproductive organs, but upon successful identification of potential mates. Sexual attraction is contingent upon ability to distinguish conspecifics and their sex. Of course, the cost of reproduction is magnitudes higher in females, but males must also be able to integrate external and internal cues with the decision to reproduce. With the establishment of the E-ETH-JH axis, we turn our attention to the male.

The role of JH and 20E are less clear in male reproductive physiology than in the female, but their influence on male courtship has received a little more attention. It has been shown that 20E is a critical component in male courtship discrimination: male to male courtship increases 5-fold in EcR mutants (Ganter et al., 2007). It has also been demonstrated that fruitless olfactory sensory neurons, which are critical for pheromone detection, depend upon 20E signaling(Dalton, Lebo, Sanders, Sun, & Arbeitman, 2009). JH is also a critical regulator of adult male courtship, and has been studied extensively(Lin, Cao, Sethi, Zeng, Chin, Chakraborty, Shepherd, Nguyen, Yew, Su, & Wang, 2016b; Wijesekera, Saurabh, & Dauwalder, 2016; Wilson, DeMoor, & Lei, 2003). While 20E's role in courtship has been relatively simplistic, JH excess or absence can stimulate or attenuate courtship depending on age and context (Argue, Yun, & Neckameyer, 2013; Lin, Cao, Sethi, Zeng, Chin, Chakraborty, Shepherd, Nguyen, Yew, Su, & Wang, 2016a).

We find here that their linker, ETH, acts as a courtship inhibitor in the adult stage, stimulating several neurons on which courtship inhibition depends. Using the Trojan

ETHR-Gal4, we identified OR67D-expressing olfactory neurons, GR32A-expressing taste neurons, and antennal lobe interneurons known to tune pheromone sensation. Silencing ETHR in any of these subsets significantly disrupts male courtship behavior. We also find that silencing ETHR in OR67D neurons or elimination of ETH from the hemolymph eliminates the post-copulation refractory period in male flies. In sum, male courtship behavior is critically dependent upon ETH signaling.

Ancillary Consequences of ETH deficiency

Ecdysis triggering hormone is a critical regulator of adult reproductive behaviors. With a depth of understanding of its targets and the effects of excess and deficient ETH signaling, we can begin to unravel the purpose of the hormone, as well as conserved principles in endocrinology. When I eliminated the Inka cells specifically in the adult stage, I saw several phenotypes beyond deficient oogenesis, ovulation, and male courtship behavior. In addition to the previously described deficiencies, hyperactivity, a drop in hatch rate or egg viability in females, and an increase in virgin female egg laying were all observed in ETH deficient flies, and each warranted investigation.

Hyperactivity

I found that ETH and JH deficiency results in hyperactivity. While the effect of ETH and JH deficiency on hyperactivity have been suggested (Argue et al., 2013; Liu, Li, Prasifka,

Jurenka, & Bonning, 2008; Yu et al., 2016), it remained unclear whether this effect was developmental or physiological. ETH injection has capacity to induce hypoactivity within 1 hour, suggestin an acute, physiological regulation of motor speed. I go on to suggest that because ETH deficiency results in broad catecholamine deficiency, it presents an excellent model for ADHD in flies, going beyond mere dopamine signaling impairment current models possess (Lebestky et al., 2009; van Swinderen & Brembs, 2010). As ETH levels likely decline during starvation, I suggest a new model for starvation-induced food seeking behavior.

Decrease in Female Hatch Rate

Elimination of the Inka cells caused fecundity to drop significantly. We found that oogenesis and ovulation were significantly perturbed, but upon rescue with topical application of methoprene, number of eggs laid returned to normal, but 40% of the eggs laid did not hatch. I found that low hatch rate associated with Inka cell elimination was due to poor fertilization, evidenced by diffuse DAPI staining and by lack of paternal gene expression. Silencing the ETH receptor ubiquitously or in the oviduct epithelium seemed to decrease hatch rate, but variability in results was concerning. Further, I suggest that an unknown pathway in late oogenesis may be contributing as rescuing ETH deficient females with methoprene results in "small eggs," a result of kep1 impairment which also impairs fertilization (Di Fruscio et al., 2003). While a target remains unclear, an Inka cell product, which is likely ETH, is necessary for proper fertilization.

ppk^+ Neuron

While JH deficiency reduces egg production, ETH-deificient females showed no change in virgin female egg laying. When I silenced ETHR panneuronally, and ultimately with the ppk-Gal4 driver, virgin female egg laying increased significantly. The dependency of the ppk^+ neurons on ETH is particularly interesting because in addition to virgin female egg laying, the activity of ppk^+ neurons inhibits ecdysone release into the hemolymph. This suggests an interesting model for endocrine state change after mating, wherein sex peptide inhibits ppk^+ neurons after mating, leading to ecdysteroidogenesis and an inhibition of ETH release, elevating 20E levels long term in mated females.

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Chapter 2

An Endocrine Network Essential for Reproductive Success In *Drosophila melanogaster*

Abstract

Ecdysis triggering hormone (ETH) was originally discovered and characterized as a molt termination signal in insects through its regulation of the ecdysis sequence. Here we report that ETH persists in adult *Drosophila melanogaster*, where it functions as an obligatory allatotropin to promote juvenile hormone (JH) production and reproduction. ETH signaling deficits lead to sharply reduced JH levels and consequent reductions of ovary size, egg production, and yolk deposition in mature oocytes. Males deficient in ETH signaling suffer significant reduction in reproductive potential. Expression of ETH and ETH receptor genes is in turn dependent on ecdysone (20E), which promotes ETH gene expression, especially in females. Furthermore, 20E receptor (EcR) knockdown specifically in Inka cells reduces fecundity and male reproductive potential. Our findings indicate that the canonical developmental roles of 20E, ETH and JH during juvenile stages are repurposed to function as a hormonal triad essential for reproductive success of both males and females during adulthood.

Introduction

The life history of insects is characterized by radical morphogenetic transformations, whereby tissues are reorganized and hormones are repurposed for roles associated with

stage-specific functions. During development, larvae complete each molt by shedding the cuticle under control of ecdysis triggering hormones (ETHs) targeting central peptidergic ensembles to orchestrate an innate behavioral sequence (Y.-J. Kim, Zitnan, Galizia, Cho, & Adams, 2006; White & Ewer, 2014; Žitňan & Adams, 2012). Previous observations that Inka cells, the sole source of ETHs, persist into the adult stage (Park, Filippov, Gill, & Adams, 2002), suggests possible reproductive functions for these peptides.

We hypothesized that ETHs regulate juvenile hormone (JH) levels, based on the report of ETH receptor (ETHR) expression in *corpora allata* (CA) of the silkworm, *Bombyx mori* (Yamanaka et al., 2008). Evidence that ETH functions as an allatotropin in the yellow fever mosquito *Aedes aegypti* came from a recent study showing its activation of JH acid methyltransferase (Areiza, Nouzova, Rivera-Perez, & Noriega, 2014).

JH is a sesquiterpenoid hormone with well-known morphogenetic and gonadotropic functions. In *Drosophila*, adult phenotypes resulting from reduction of JH levels have been characterized through induction of cell death in the CA or through enhancement of its degradation (Gruntenko et al., 2010; Liu, Li, Prasifka, Jurenka, & Bonning, 2008). Based on evidence from studies on *Bombyx* and *Aedes*, we investigated whether ETH functions as an allatotropin in adult *Drosophila* and the extent to which it may be necessary for reproductive functions.

Previous studies showed that 20E regulates synthesis and release of ETH and expression of ETHR during larval stages of moths and mosquitoes (Dai & Adams, 2009; Kingan & Adams, 2000; Žitňan, Kim, Zitnanova, Roller, & Adams, 2007; Žitňan et al., 1999). More recently, STARR-Seq data confirms that 20E induces EcR enhancer activity

in promoters of both ETH and ETHR genes (Shlyueva et al., 2008). Since circulating 20E levels are of major physiological and reproductive relevance (Schwedes & Carney, 2012), we also asked whether 20E influences ETH gene expression during the adult stage.

Here we describe functional roles for 20E, ETH, and JH as a hormonal triad essential for reproductive success in *Drosophila*. In particular, we confirm persistence of ETH signaling throughout adulthood and demonstrate its obligatory functional roles in regulating reproductive physiologies of both male and female flies through maintenance of normal JH levels.

Results

Inka cells and ETH signaling persist throughout adulthood in Drosophila

Previous evidence indicates that Inka cells persist into the adult stage of *Drosophila melanogaster* (Park et al., 2002), but little is known about their number, distribution, or sexual dimorphism. We therefore examined their spatiotemporal distribution in both males and females. To visualize Inka cells, we drove expression of the nucleus-targeted RedStinger protein using an Inka cell-specific Gal4 driver and performed immunostaining for ETH in the adult stage (Figure 1). Inka cells are perched on branch points of the tracheal system, whose fragility made mapping their positions difficult through traditional dissection. Whole flies therefore were washed in 30% hydrogen peroxide for 4 hours to visualize of Inka cells through the cuticle *in vivo* (Figure 2). We

observed two pairs of Inka cells in the ventral thorax, and seven pairs in the dorsal abdomen, five of which are clustered in the caudal region. As in larval stages (Park et al., 2002), adult Inka cells are bilaterally paired and positioned just inside the body wall.

We next measured relative levels of ETH and ETH receptor transcripts in male and female adults using reverse transcriptase PCR (RT-PCR) (Figure 1B, C). Expression of ETH, ETHR-A and ETHR-B is robust both pre- and post-eclosion and remains strong in both males and females through day 20. The temporal pattern of expression is sexually dimorphic (Figure 1B, C). In virgin females, all three transcripts fluctuate in phase during the first 3 weeks of adulthood, increasing in intensity through day 5, dropping on days 8 and 10, and increasing again to day 20. In contrast, the male expression pattern shows a steady increase during the first 2 weeks of adulthood and remains strong through day 20. In general, expression of ETH and ETHR is in phase, suggesting a common upstream regulator.

Inka cells of *Drosophila* larvae are the sole source of ETH (Park et al., 2002). We asked whether the same is true during adulthood by conditionally ablating Inka cells in post-eclosion flies using an Inka cell-specific Gal4 driver to express the apoptosis gene *reaper* (*ETH-Gal4>tubulin-Gal80^{ts}/UAS-rpr*). Flies were raised at 18^{o}C, at which temperature Gal80 inhibits Gal4 expression. If flies were moved to 29^{o}C ~10 hours prior to eclosion to inactivate Gal80, over 95% lethal eclosion deficiency was observed. Escapers were deficient in tanning, likely related to insufficient release of bursicon, known to be regulated by ETH (D.-H. Kim et al., 2015; Lahr, Dean, & Ewer, 2012). When the temperature shift was postponed until after eclosion, all flies survived, but were

completely devoid of ETH transcript (Figure 3). These data confirm that Inka cells are the sole source of ETH in adult *Drosophila*.

ETH induces calcium mobilization in corpora allata

ETHR transcripts have been detected in CA of *Bombyx* and *Aedes*, suggesting these glands are targets of ETH (Areiza et al., 2014; Yamanaka et al., 2008). CA of both males and females both appeared to both be labeled by ETHR-Gal4 and by neurons innervating the tissue from the oesophagus (Figure 4A-B). When we drove UAS-Redstinger with the ETHR-Gal4 driver, we found the 20 large CA cells did not label, but cells on the surface of the gland were clearly visible (Figure 4C). We also notice the neurons innervating the CA also had processes innervating the crop (Figure 4D).

We found that exposure of CA excised from day 3 adults to ETH1 produced robust calcium mobilization (Figure 5). Using two different genotypes (*Aug21-Gal4>UAS-GCaMP3*; Figure 5B, C or *JHAMT-Gal4>UAS-GCaMP5*; Figure 5A, D, E) calcium mobilization was observed in both males and females for more than 45 minutes.

Latency to ETH-induced calcium mobilization, defined as the time elapsed between ETH treatment and the first peak with amplitude greater than background activity (established during the 4 minutes of recording prior to treatment), was concentration-dependent and sexually dimorphic. CA responses occurred within 1000 seconds in 58/60 experiments; the two non- responders were females at the lowest concentrations of ETH tested (200 nM). When latencies for each sex and dose were compared using a 2X3 Factorial ANOVA, both factors

were found to be significant effectors of latency at $p < 0.001$. Latency to calcium mobilization was significantly shorter for male CA compared to those of females and latency was inversely proportional to concentration (Figure 5E).

Calcium mobilization in the CA following ETH exposure depends on level of ETHR expression

Upon RNAi silencing of ETHR using the genotype *JHAMT-Gal4>UAS-GCaMP5/UAS-ETHR-Sym*, the percentage of female responders exposed to 5 µM ETH decreased from 100% in controls to 66% and from 100% to 83% in males. Among the responders, latency and variance were significantly increased in both sexes after RNA silencing ($p < 0.05$) (Figure 5E). Thus, calcium mobilization in the CA in response to ETH exposure depends upon relative abundance of ETHR transcripts.

To verify presence of ETHR transcript in the CA, we performed qPCR on isolated glands of male and female 4-day old adult flies of genotype *JHAMT-Gal4>UAS-CD4-tdGFP/UAS-ETHR-Sym*. We observed GFP labeling solely in the CA, which were carefully extirpated under fluorescence optics. Analysis by qPCR revealed presence of ETHR transcript in both male and female CA; relative transcript abundance was significantly higher in males ($p<0.05$). Expression of *UAS-ETHR-Sym* resulted in significant knockdown of ETHR in CA of both sexes ($p < 0.05$) (Figure 5F).

ETH is an obligatory allatotropin in both males and females

We tested whether ETH is required for maintenance of JH levels using two genetic approaches to interrupt signaling: 1) RNAi silencing of ETHR in the CA using the *JHAMT-Gal4* driver, and 2) conditional ablation of Inka cells using the apoptosis gene

reaper (*rpr*). RNA knockdown of ETHR expression in the CA (genotype *JHAMT-Gal4>UAS-ETHR-Sym*)
led to a ~70% reduction of JH levels in males and ~85% reduction in females (Figure 6A). We observed no defects in body size, head eversion, or time to eclosion in ETHR-silenced flies.

Conditional ablation of Inka cells was accomplished using *ETH-Gal4>tubulin-Gal80^{ts}/UAS-rpr*. Since ablation of Inka cells during larval or pupal stages leads to lethal ecdysis defects, flies were moved to Gal80-inactivating warmer temperatures only after eclosion to the adult stage (within 8 hr post-eclosion). Both males and females subjected to Inka cell ablation exhibited markedly depressed levels of JH, 94% in males and over 99% in females (Figure 6B).

Disruption of ETH signaling reduces fecundity and impairs vitellogenesis

We observed clear reproductive phenotypes associated with impaired ETH signaling. ETHR silencing using the *JHAMT-Gal4* driver and two double stranded RNA constructs directed toward mutually exclusive sequences in the ETHR gene (*UAS-ETHR-Sym* and *UAS-ETHR-IR2*; see Methods for details) resulted in a 35% decrease in egg production in mated female flies (Figure 6C). Fecundity was restored to normal levels following topical application of 3.4 pg of the JH analog methoprene, a known agonist of the *Drosophila* JH receptors Met and Gce, on the day of eclosion (Jindra, Uhlirova, Charles, Smykal, & Hill, 2015). Likewise, ablation of Inka cells, the source of ETH, led to a ~30% drop in egg production and methoprene treatment again rescued egg production to normal levels (Figure 6D). ETHR silencing in the CA (*JHAMT-Gal4>UAS-ETHR-Sym*) or

conditional Inka cell ablation (*ETH-Gal4>tubulin-Gal80^{ts}/UAS-rpr*) reduced ovary size in day 5 virgin females. Interestingly, despite their smaller size, ovaries from Inka cell-ablated flies retained more mature (Stage 14) eggs than controls (Figure 7). Reduction in ovary size resulting from either treatment was rescued by topical treatment with methoprene, whereas mature egg number was unaffected (Figure 8A, B, Figure 7).

In order to investigate this seemingly conflicting dichotomy, we examined oocytes and ovarioles of affected flies. First, we scored numbers of eggs at successive stages of oogenesis (staging according to (Spradling, n.d.)) and found that, while development through stage 7 was normal, a significantly greater number of oocytes from ETH deficient flies degenerated during stages 8-9, suggesting that the balance between 20E and JH was perturbed (Soller, Bownes, & Kubli, 1999)(Figure 8C, D; Figure 9). Furthermore, progressing oocytes in stages 9-13 were present in much lower numbers following Inka cell ablation (Figure 8D; Figure 9). A balance between JH and 20E determines whether oogenesis will progress beyond the mid-oogenesis checkpoint stage (7-8). JH deficiency results in activation of caspases and apoptosis, marked by DNA fragmentation, obvious with DAPI and TUNEL staining (Pritchett, Tanner, & McCall, 2009; Soller et al., 1999). After staining, we observed more degeneration of oocytes in ETH deficient flies than in controls (Figure 8C, D).

We also found that stage 14 eggs were thinner, relatively translucent, and often did not activate (incomplete inflation) in PBS (Sartain & Wolfner, 2013). Eggs were depleted or devoid of yolk (Figure 8E). Protein (Bradford) assays showed marked reduction of soluble protein in eggs from ETHR-deficient or Inka cell-ablated flies (Figure 8F).

JH levels are directly correlated to yolk protein gene transcription (R. Yamamoto, Bai, Dolezal, Amdam, & Tatar, 2013). In order to determine whether yolk protein transcription was diminished in ETH-CA interrupted flies we performed QPCR for yolk protein genes in 4-day-old virgin females (Figure 8G). Yp1 and Yp2 were significantly reduced in both Inka cell ablated and CA ETHR RNAi flies.

Impaired ETH signaling reduces male reproductive potential

Previous studies demonstrated that JH is necessary for normal male accessory gland functions in a variety of insects(P. Chen, 1984; Gold & Davey, 1989; Parthasarathy et al., 2009), and that JH has the ability to induce accessory gland protein synthesis in *Drosophila melanogaster* (Herndon et al., 1997; K. Yamamoto, Chadarevian, & Pellegrini, 1988). However, reproductive impairment associated with JH deficiency in male *Drosophila* has not been described. We disrupted ETH signaling in post-eclosion males via ETHR knockdown in the CA or Inka cell ablation. Day 4 males were paired with wild type females of the same age, placed in a 1 cm diameter courtship chamber,and observed for 30 minutes to confirm copulation. Immediately after mating, inseminated females were isolated and allowed to lay eggs for three days. After 72 hours, we removed the female counted larvae and unhatched eggs over the next 24 hours to assess both egg laying and mate quality. We observed significant reduction (~40%) in egg number in females mated with males subjected to either ETHR-silencing in the CA or Inka cell ablation (Figure 10 A, B). There was no change in egg viability, suggesting sperm quality was unaffected (Figure 10E). We also found Inka cell-ablated males and CA ETHR-silenced males have accessory glands that appear less full than controls (Figure

10F,G). Reductions were rescued by topical treatment of males with methoprene. While previous studies, as well as this study, have confirmed wild-type, mated female fecundity is unaffected by methoprene treatment at this dose, males were treated at eclosion (4 days prior to mating) on the dorsal thorax which should not contact the female during mating, with 1.7pg of methoprene as a precaution to avoid appreciable transference. Females mated to day 10 males showed no difference in total eggs laid compared to controls (Fig. 10D).

20E Regulates ETH signaling during the adult stage

During juvenile stages, expression of genes encoding ETH and ETHR is induced by ecdysteroids (Dai & Adams, 2009; Shlyueva et al., 2008; Žitňan et al., 2007). We examined whether ETH and ETHR transcript levels are influenced by 20E in adult flies. Injection of 20E (150 pg) into male and female flies led to significant and sustained ~2-fold elevation of the ETH precursor transcript in both males and females (Figure 11). With regard to ETHR expression, 20E-injection elicited much stronger elevation of ETHR transcript in females compared to males. At 1 hour post-injection, we observed a 6-fold increase in females, but only 1.5-fold increase in males. However at 4 hrs post-injection, ETHR transcripts increased nearly 100-fold, whereas male transcript levels returned to baseline, if not slightly below control levels.

We then asked whether steroid signaling in Inka cells affects fecundity. We tested this by suppressing 20E receptor (EcR) expression in Inka cells specifically, either through RNAi silencing or expression of an EcR dominant negative allele. Indeed, both of these treatments led to significant reductions in both female fecundity and male reproductive

potential (Figure 11C, Figure 10C). Both of these phenotypes were rescued by topical application of methoprene, suggesting that reduced fecundity resulting from elimination of EcR in Inka cells is the result of JH deficiency, and that 20E acts through ETH from Inka cells, which in turn targets the CA to maintain normal JH levels.

Discussion

We have shown that Inka cells and expression of genes encoding ETH signaling molecules persist well into the adult stage of male and female *Drosophila*. Our findings indicate a vital functional role for ETH as an obligatory allatotropin for maintenance of JH levels required for normal vitellogenesis and fecundity in females and reproductive potential in males. A critical upstream signal for regulation of ETH gene expression is 20E. Thus 20E, ETH, and JH comprise a hormonal triad essential for normal reproductive physiology in both male and female flies.

ETH and ETHR transcripts and ETH-like immunoreactivity in Inka cells persist for at least 3 weeks post-eclosion in both males and females. ETH and ETHR transcripts appear to be in phase with one another and also following an infradian rhythmicity, with band intensity levels similar to reported peaks of fecundity (Wayne, Soundararajan, & Harshman, 2006). A total of 9 Inka cell pairs are present, 2 of which are located in the thorax and 7 in the abdomen. The pattern of Inka cell distribution in adults is particularly interesting. Unlike larval Inka cells, which are evenly distributed throughout the animal (Park et al., 2002), adult cells are more strategically located. In the thorax, an anterior pair is situated in close proximity to the corpora allata, consistent with the allatotropic

action of ETH described here. Abdominal Inka cells are more concentrated posteriorly, particularly in the female, where 4 of the 7 pairs are clustered in terminal segments closely associated with reproductive organs, thought to be the most prominent source of 20E (Uryu, Ameku, & Niwa, 2015).

I found that ETHR-Trojan-Gal4, a high-fidelity Gal4 system (Diao et al., 2015), expressed in cells innervating the CA, and possibly in the CA itself. Given that JHAMT-Gal4 expresses specifically in the CA and not in cells innervating it, it is likely the surface red stinger-labeled cells are JHAMT-expressing. This could suggest heterogeneity of the CA, but it is also possible the Gal4 labeling is low enough that we cannot detect it.

We present evidence that ETHR is expressed in the CA of *Drosophila*, in agreement with previous reports documenting ETHR expression in CA of the silkworm, *Bombyx mori* and yellow fever mosquito, *Aedes aegypti* (Areiza et al., 2014; Yamanaka et al., 2008). ETH has been previously shown to stimulate activity of the rate-limiting enzyme in JH biosynthesis, Juvenile Hormone Acid Methyl-Transferase via calcium release. RNAi knockdown of ETHR using the CA driver *JHAMT-Gal4* causes marked reduction of JH levels and reproductive loss-of-function phenotypes, including reduced fecundity (~35-40% reduction in egg production), decreased ovary size, and lower male reproductive potential, all of which are restored to normal levels by methoprene rescue. Indeed, the magnitude of reduced egg production in response to disrupted ETH signaling is comparable to that resulting from total ablation of the CA (Figure 12.) (Abdou et al., 2011; R. Yamamoto et al., 2013).

It has been proposed that oogenesis in *Drosophila* depends upon balanced levels of JH and 20E (Soller et al., 1999). Under normal conditions, JH stimulates yolk protein mRNA synthesis and acts on the follicle cell layer and enhances patency or endocytosis of yolk proteins, in primed follicles, into developing oocytes (Seidelmann, Helbing, Göbeler, & Weinert, 2016; R. Yamamoto et al., 2013). The combinatorial effect of both of these processes leads to adequate yolk deposition in mature oocytes and progression of oogenesis to the latter stages. However, during situations of stress, ecdysteroid levels rise, causing nurse cell apoptosis and follicle degeneration. We show marked follicle degeneration and a decrease in late stage oocytes following Inka cell ablation (Figure 8C, D, Figure 9). A previous study on the role of EcR in oogenesis using a temperature sensitive EcR mutant reported disruption in progression to late stage oogenesis as well as an increase in the number of stage 14 oocytes (Carney & Bender, 2000). Our present findings indicate that these defects could arise from reduction of EcR and ETH production in Inka cells, rather than reduced EcR in ovaries, as was previously concluded. Furthermore, these findings suggest that ETH is necessary for balancing 20E and JH levels. Unlike steroids and JH, hemolymph ETH concentration can change very rapidly, as it does over a matter of minutes during ecdysis (Park et al., 2002). Thus ETH may contribute increased plasticity to the stress response system, which is known to work over a span of hours or even days; further experiments are necessary to test such a prediction.

Ramifications of low JH in male flies have been described as “enigmatic” (R. Yamamoto et al., 2013). This is, in part, because although a variety of insects show

reduced accessory gland production and a reproductive cost stemming from reduced JH, previous studies have not translated this to reproductive potential of adult males (Wilson, DeMoor, & Lei, 2003). Following disruption of ETH signaling, we subjected males deficient in JH to a single mating to same-aged wild-type females on day 4 and observed a significant reduction in reproductive potential. This effect was not seen when males were raised in groups with females, nor was the effect obvious when males were mated on day 10. Although the mechanism of this impairment is currently unclear, investigations in other species, the clear link between JH and AG synthesis, and our experimental evidence suggest partner fecundity impairment could be due to the rate of accessory gland (AG) protein synthesis being reduced in low JH males. Accessory gland proteins have an indispensible role in stimulating female fecundity, sex peptide transfer can enhance reproductive output in females by stimulating JH synthesis, intestinal remodeling and germline stem cell proliferation in females, and ovulin stimulates growth of octopaminergic neurons that regulate ovulation (Ameku & Niwa, 2016; Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011; Moshitzky et al., 1996; Reiff et al., 2015; Rubinstein & Wolfner, 2013). By day 10 the JH deficient flies may have caught up to controls and "filled" accessory glands with fecundity-stimulating proteins, so that ejaculate contained an optimal dose. In groups, multiple matings can occur, and more matings may compensate for a reduced AG protein dose on the initial mating. Low JH males show no reproductive potential deficit (Gruntenko et al., 2010), which could be explained by lower sex peptide transfer from the male, resulting in a shorter time to re-

mating (Yapici, Kim, Ribeiro, & Dickson, 2008), whereby a fresh dose of oogenesis-stimulating accessory gland protein is released into the female.

Based on the work presented here, it appears that normal levels of JH in the hemolymph of *Drosophila* adults depend upon ETH signaling. Furthermore, our findings indicate that 20E regulates ETH in Inka cells via EcR activation, thus regulating JH levels indirectly. This interpretation is supported by the fact that reduced fecundity and male reproductive potential following RNAi knockdown of EcR in Inka cells was rescued by the JH analog, methoprene.

On the other hand, injection of 20E resulted in a sustained, approximately two-fold increase in ETH in both sexes, whereas ETHR transcript increased dramatically in females, while males exhibited only a small, transient increase. Nonetheless, while ETH transcript number in males may increase only slightly in response to 20E, its dual regulation of ETH and ETHR transcripts could have multiplicative effects on ETH target tissues.

ETH, previously known only for its regulation of ecdysis, now acquires a critical role in the adult stage as a reproductive stimulant. Three hormones interrelated by their canonical roles in morphogenesis are shown here to maintain their relationship despite dramatic reorganization of the body plan following metamorphosis. The diversity of allato-regulators in *Drosophila* including insulin-like peptides and biogenic amines dopamine, and octopamine (Gruntenko, Karpova, & Alekseev, 2007; Gruntenko et al., 2005; Tatar et al., 2001; Thompson, Yagi, Chen, & Tobe, 1990) also influence JH production and each may influence this network in a context-specific manner to

coordinate and optimize reproductive behaviors. The ability of the CA to integrate a variety of inputs including nutrition and steroid levels into a pro-reproductive signal bears a striking similarity to the mammalian GnRH neuron, which integrates complex hormonal information regarding stress, nutrition, and circadian rhythm into its activity; the emergent hormonal state determining whether reproduction is appropriate (Knobil & Neill, 2006).

Developmental signaling roles for ecdysteroids, ETH, and JH have been characterized in a number of holometabolous insect species (Jindra, Palli, & Riddiford, 2013; Roller et al., 2010; Yamanaka, Rewitz, & O'Connor, 2013). In particular, periodic molting and ecdysis are brought about by bouts of steroid (20E) surge and ebb. The 20E surge promotes synthesis of ETH in Inka cells via the transcription factors cryptocephal and EcR-B2 (Gauthier, VanHaaften, Cherbas, Cherbas, & Hewes, 2012). Meanwhile, 20E represses transcription of βFtz-F1, an orphan nuclear receptor necessary for secretory competence (Cho, Daubnerová, Park, Zitnan, & Adams, 2014). Subsequent decline in steroid levels de-represses βFTZ-F1, leading to acquisition of secretory competence and release of ETH.

While ETH is known to be under the control of 20E during the developmental stages (Žitňan et al., 1999; 2007), we show here that this relationship persists into the adult stage. It will be interesting to ascertain whether fluctuation of 20E levels during adulthood functions in a similar manner to trigger synthesis and release of ETH for reproductive functions. A reasonable prediction might be that ETH functions as a link between 20E and JH signaling to promote successive, non-overlapping surges of these

hormones, similar to cyclic hormonal fluctuations in mammals ("Knobil and Neill's Physiology of Reproduction," 2006).

We propose a model depicting chemical signaling among members of the 20E-ETH-JH triad (Figure 13). A 20E surge stimulates production of ETH in Inka cells and ETHR in target tissues, such as the CA. If the CA is primed by insulin and other queues, when ETH release occurs upon steroid ebb, active JHAMT stimulates JH biosynthesis and release from the CA. Normal JH levels elevate, promoting normal rates of egg production in females and reproductive potential in males. According to previous reports, circulating JH levels can inhibit 20E production during the adult stage (Schwartz, Kelly, Imberski, & Rubenstein, 1985; Terashima & Bownes, 2006; Terashima, Takaki, Sakurai, & Bownes, 2005). Indeed, mutually exclusive fluctuations of E75A and E75B are observed throughout the lifespan of *Drosophila*, and it has been proposed that these are indicators of hemolymph concentrations of JH and 20E, respectively (Dubrovsky, Dubrovskaya, & Berger, 2004). Coordinated fluctuations of 20E and JH could facilitate oogenesis through sequential steps of development in the ovariole (Dubrovsky, Dubrovskaya, & Berger, 2002; Uryu et al., 2015). Real time hormone measurements will be required to validate this model.

Materials and Methods

Reagents

Fly Strains

Flies used for immunohistochemistry, calcium imaging and CA ETHR silencing were raised at 23˚C on standard cornmeal-agar media under a 12:12 hr light:dark regimen. Inka cell ablated flies were raised at the Gal80^{ts} permissive temperature (18°C). Following eclosion, they were moved to the nonpermissive temperature (29°C) for 24 hours, then moved to 23°C until day 4. Corpora allata-ablated flies were reared as described previously (R. Yamamoto et al., 2013) at 29°C, isolated prior to eclosion, and transferred to isolated chambers held at 23°C prior to mating and fecundity analysis. The JHAMT-Gal4 fly line has been described recently (Wijesekera, Saurabh, & Dauwalder, 2016). Use of double-stranded RNA constructs for silencing of ETHR (UAS-ETHR-Sym; UAS-ETHR-IR2 line (VDRC transformant ID dna697) were described recently (D.-H. Kim et al., 2015). Aug21-Gal4 flies were obtained from Dr. Korge (Freie Universität Berlin). All other fly lines were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN): UAS-Red Stinger (BS no. 8574), UAS-mCD8-GFP (BS no. 5137), UAS-CD4-tdGFP (BS no. 35836), UAS-rpr (BS no. 8524), UAS-NiPp1.HA (BS no. 23711; referred to as UAS-NIPP1 henceforth), UAS-GCaMP3 (BS no. 32235), UAS-GCaMP5 (BS no. 42037), TubP-Gal80^{ts} (BS no. 7017), ETH-Gal4 (BS no. 51982), UAS-EcR-RNAi (BS no. 37059), UAS-EcR.B1 (BS no. 6869). All flies used for behavior experiments were backcrossed for at least five generations into the Canton-S background.

Visualization of Inka cells

We crossed *ETH-Gal4* transgenic flies with *UAS-Red Stinger* flies to produce progeny expressing RFP in endocrine Inka cells for double immunohistochemical staining. Day 4 adults were dissected in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS overnight at 4˚C. After washing with PBS-0.5% Triton X-100 (PBST) five times and blocking in 3% normal goat serum in PBST for 30 minutes at room temperature, samples were incubated with anti-ETH antiserum (1:1000 dilution in PBST; previously described in (D.-H. Kim et al., 2015)) for 2 days at 4˚C. Tissues were washed with PBST three times, incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) overnight, and washed 4 times for 10 minutes each in PBST before imaging. Immunofluorescence was recorded using a confocal microscope (Leica model SP2) with FITC filter in the Institute of Integrative Genome Biology core facility at UC Riverside.

Reverse transcriptase PCR (RT-PCR)

Fifteen *Canton-s* wild type flies were collected on the days relative to eclosion: -2, -1, 0, 3, 5, 8, 10, 13, 16 and 20 days after eclosion. Following homogenization of whole flies, cDNA was prepared using a Superscript III kit (Invitrogen). cDNA was normalized and incubated for 20 cycles with actin, ETH, ETHR-A, or ETHR-B primers and Invitrogen Taq polymerase. Inka cell ablation was confirmed by processing 15 day 4 females of Inka cell-ablated and control flies for immunostaining according to procedures described above. Band intensities were quantified using Adobe Photoshop and plotted.

Primer sequences were as follows:

Actin: Forward – TTCAACACACCCGCCATGTA,

Reverse – AGCCTCCATTCCCAAGAACG

ETH: Forward - AGCTGCTTGACAACAACGCTA

Reverse - CGAATACTCCACATCTCACAGG

ETHR-A: Forward - TCGACCAAGTTTCGAAGGGG

Reverse – GTCCGGCGTACGGAAACTAT,

ETHR-B: Forward – CCTACAGCGTGGAACCCTAC,

Reverse - TCGGTTTATTGACTTCTTCTGAGG

Quantitative RT-PCR (qPCR)

For qPCR of ETHR expression in the CA, 3 replicates were obtained from 30 corpora allata dissected from day 4 adult males and females *JHAMT-Gal4>UAS-ETHR-Sym;UAS-CD4-tdGFP* or *JHAMT-Gal4>UAS-CD4-tdGFP* (control). Care was taken to extirpate selectively fluorescent CA labelled with GFP. cDNA was prepared using the Superscript III kit (Invitrogen). Due to low tissue volume, cDNA for this experiment was preamplified using the SsoAdvanced Preamp Supermix (Biorad) kit using the manufacturers protocol.

For injections of 20E, day 5 male and female flies were injected with 47 nl of 1 µg/µl 20E in fly saline (final concentration estimated to be ~100 nM) or fly saline alone. After one or four hours, whole bodies were homogenized and mRNA samples were extracted using TRIzol (Life Technologies), following the manufacturer's protocol. cDNA was

prepared using the Superscript III kit (Invitrogen). In both experiments, cDNA was used as a template for expression analysis with SYBR green (cat # 170-8882 Bio-Rad) using the following PCR conditions: Step 1=95°C for 3 min. Step 2=95°C for 15 s, 61°C for 20 s and 72°C for 25 s; this step was repeated 45 times. Step 3=95°C for 1 min. This was followed by melt curve analysis. Quantitative real time PCR (qPCR) was done on an iCycler iQ (Bio-Rad). Primers for expression analysis are found below. The specificity of each primer set was validated by the construction of a melting curve. Actin mRNA expression was determined as housekeeping gene. The relative expression of target mRNA was normalized to the amount of actin by using the standard curve method, and compared using Mann-Whitney rank sum analysis.

Actin: Forward – GCGTCGGTCAATTCAATCTT

Reverse - AAGCTGCAACCTCTTCGTCA

ETH: Forward – TTCGCTCTTGGTGGGTCTTG

Reverse – CAAAGTTCTCGCCTCGCTTG

ETHR: Forward – TCCATCGTATATCCGCACAA

Reverse - GTTGCGCATATCCTTCGTCT

Calcium Imaging

Corpora allata and esophagus of 4-day males and females (n~24) were extirpated and placed in a petri dish. We used an imaging setup consisting of a Polychrome V monochromator (TILL Photonics/FEI) as light sources and a TILL Imago CCD camera. The microscope (Olympus Model BX51WI) was equipped with a 40x W NA 0.8 objective. Binning on the chip (8 X 8) was set to give a spatial sampling rate of 1

μm/pixel (image size 172 X 130 pixels, corresponding to 172 μm and 130 μm). Images were taken at a rate of 1 Hz. The excitation wavelength was 488 nm, and exposure time was 25 msec. Fluorescent light passing an excitation filter (370-510 nm) was directed onto a 500 nm DCLP mirror followed by a 515 LP emission filter for EGFP. One hr long continuous images were acquired from each CNS preparation and ETH was applied into a bathing media ~5 min after imaging onset. The volume of applied ETH was 3.6 μl. We used a cocktail of ETH1 and ETH2 for all experiments; 300 nM ETH (300 nM ETH1 plus 300 nM ETH2) and 600 nM ETH (600 nM ETH1 plus 600 nM ETH2) was added to a stagnant bathing bath with a micropipette. Fluorescence intensity was calculated as ΔF/F; mean fluorescence over the entire 100 frames was taken, for each pixel, as an estimate for F.

Latency Experiments

For dose response curves, CA from *JHAMT-Gal4>UAS-GCaMP5* were dissected as above using similar imaging settings, using ETH1 only. As considerable constitutive activity was observed using the more-sensitive GCaMP5, latency was defined as time to first atypical ΔF/F peak, as recorded by the software. After 240 seconds of recording, establishing baseline activity, 20 μl of 10 times the noted concentrations was added to a bath with 180μl fly saline and CA were recorded for 1000 seconds. Each data point contains latencies from 8-12 CA. ETHR-silenced imaging was performed with *JHAMT-Gal4>UAS-GCaMP5;UAS-ETHR-Sym* and *JHAMT-Gal4>UAS-GCaMP5* flies.

Methoprene Treatment

Within 24 hours of eclosion, adult males or females were cold anesthetized and treated topically on the dorsal side of the abdomen with 72 (females) or 36 (males) nl of either acetone, or 0.01% methoprene dissolved in acetone (~300 nM). The entire procedure took under 20 minutes, after which flies were returned to their housing.

Egg Production

Newly eclosed males and females of noted genotype were kept in incubators at 50% humidity in isolation vials until day 4, at which point they were paired with a wild type *Canton-S* mate of the opposite sex in courtship chambers. Following mating, females were isolated in 10x35mm dishes filled with 4 ml of apple juice diet supplemented with 0.5 g of yeast and allowed to lay eggs for three days at 23°C. Flies were then discarded and progeny were tallied. Larvae were counted immediately after removal of the female, and remaining eggs were given 24 hours to hatch, after which all eggs, hatched and unhatched, were counted. For TrpA1 experiments, females of given genotypes were kept in 23°C until day 4, at which point they were mated to *Canton-S* males and moved to incubators maintained at indicated temperatures.

Ovary size measurement

Four days after eclosion, ovaries were dissected from females of the genotypes *JHAMT-Gal4/+, UAS-ETHR-Sym/+, JHAMT-Gal4>UAS-ETHR-Sym, ETH-Gal4;tubulin-Gal80^{ts}/+, UAS-rpr /+, ETH-Gal4;tubulin-Gal80^{ts}>UAS-rpr*. Ovarioles were then scored

in a single blind manner with an ocular micrometer. In cases where ovaries were not symmetrical, ovaries were not used for size determination.

Egg Staging and protein content

Ovaries dissected from day 4 virgin females were immediately fixed for 1 hour in 4% paraformaldehyde, washed 5x in 0.5% PBST and incubated overnight in 0.5mg/ml DAPI and 2% NGS in PBST. Samples were washed 5x and TUNEL stained using the ROCHE *In Situ* Cell Death Detection Kit, TMR Red according to the manufacturer's protocol. Egg staging was performed as described by (Jia, Xu, Xie, Mio, & Deng, 2016). Ovaries of virgin day 5 females of indicated genotype were raised individually in isolation vials, dissected in PBS, and 10 mature, stage 14 oocytes were collected from 10 sample flies. Eggs were removed and placed in tubes containing MilliQ water, homogenized, and centrifuged at 15,000g for 5 min. The supernatant containing soluble protein was recovered and subjected to Bradford assay according to the protocol described by Thermo Scientific (https://www.thermofisher.com/order/catalog/product/23236) using a Nanodrop 2000 at the UCR Genomics Core Facility.

JH determination

JH III was extracted from flies, labelled with a fluorescent tag and analyzed by reversed phase high performance liquid chromatography coupled to a fluorescent detector (HPLC-FD) as previously described (Rivera-Perez, Nouzova, & Noriega, 2012), with 100 flies for each sex/genotype divided into two groups for statistical comparison (One way ANOVA).

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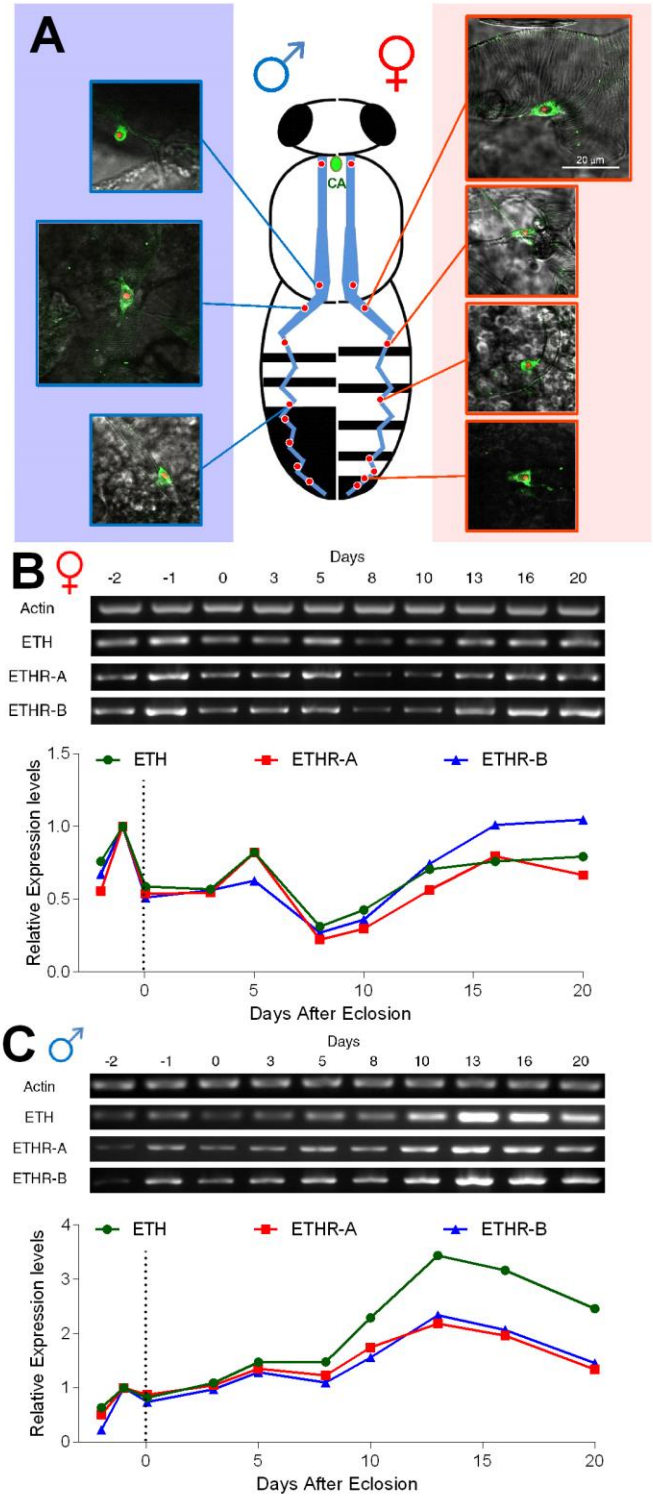
A
CA
20 μm
B
Days
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ETH
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ETH
ETHR-A
ETHR-B
Relative Expression levels
1.5
1.0
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Days After Eclosion
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Days
-2 -1 0 3 5 8 10 13 16 20
Actin
ETH
ETHR-A
ETHR-B
ETH
ETHR-A
ETHR-B
Relative Expression levels
4
3
2
1
0
0 5 10 15 20
Days After Eclosion

Figure 2.1. ETH signaling persists into the adult stage, evidenced by presence of Inka cells

(A) ETH immunoreactivity in *ETH-Gal4>UAS-RedStinger* males and females and schematic diagram showing relative locations of Inka cells; Scale bars=20 µM. (B-C) Patterns of ETH, ETHR-A and ETHR-B expression detected by RT-PCR of females (B) and males (C) on days -2, -1, 1 hour after eclosion (0), 1, 3, 5, 8, 10, 13, 16 and 20. Band intensity quantified and graphed below respective bands.

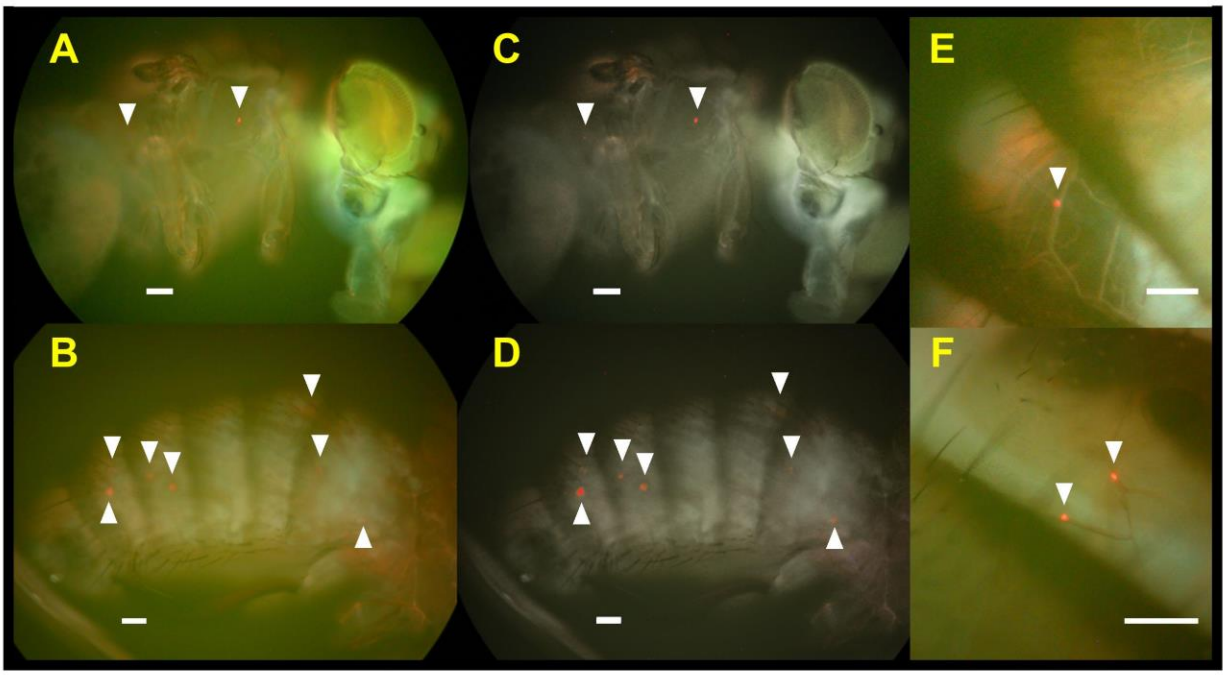


Figure 2.2. In vivo Inka cells Images of Inka cells *in vivo* in *ETH-Gal4>UAS-RedStinger* adults, Inka cell nuclei (red, indicated by white arrowheads) visible through the cuticle after 4 hours of H_2O_2 treatment in an adult female, locations of thoracic (A, C) and abdominal (B, D) cells in color (A-B) and black & white (C-D). Higher magnification images of Inka cells on trachea (E, F).

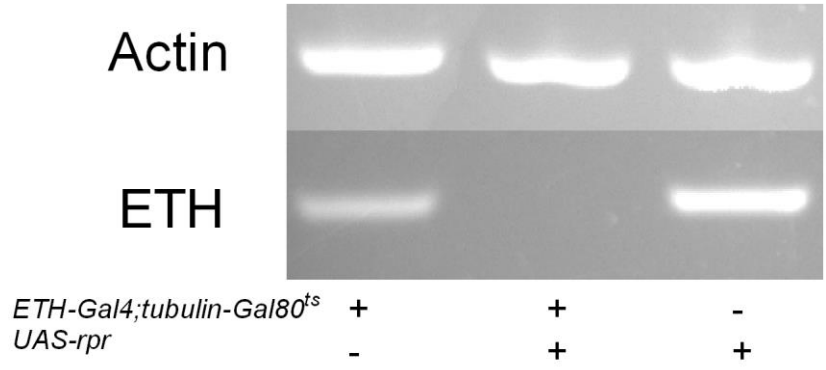


Figure 2.3. ETH transcript is absent in Inka cell ablated females RT-PCR of ETH transcript deficiency in *ETH-Gal4>UAS-rpr* flies. Inka cell-ablated flies showed no detectable levels of ETH mRNA on Day 4 post-eclosion.

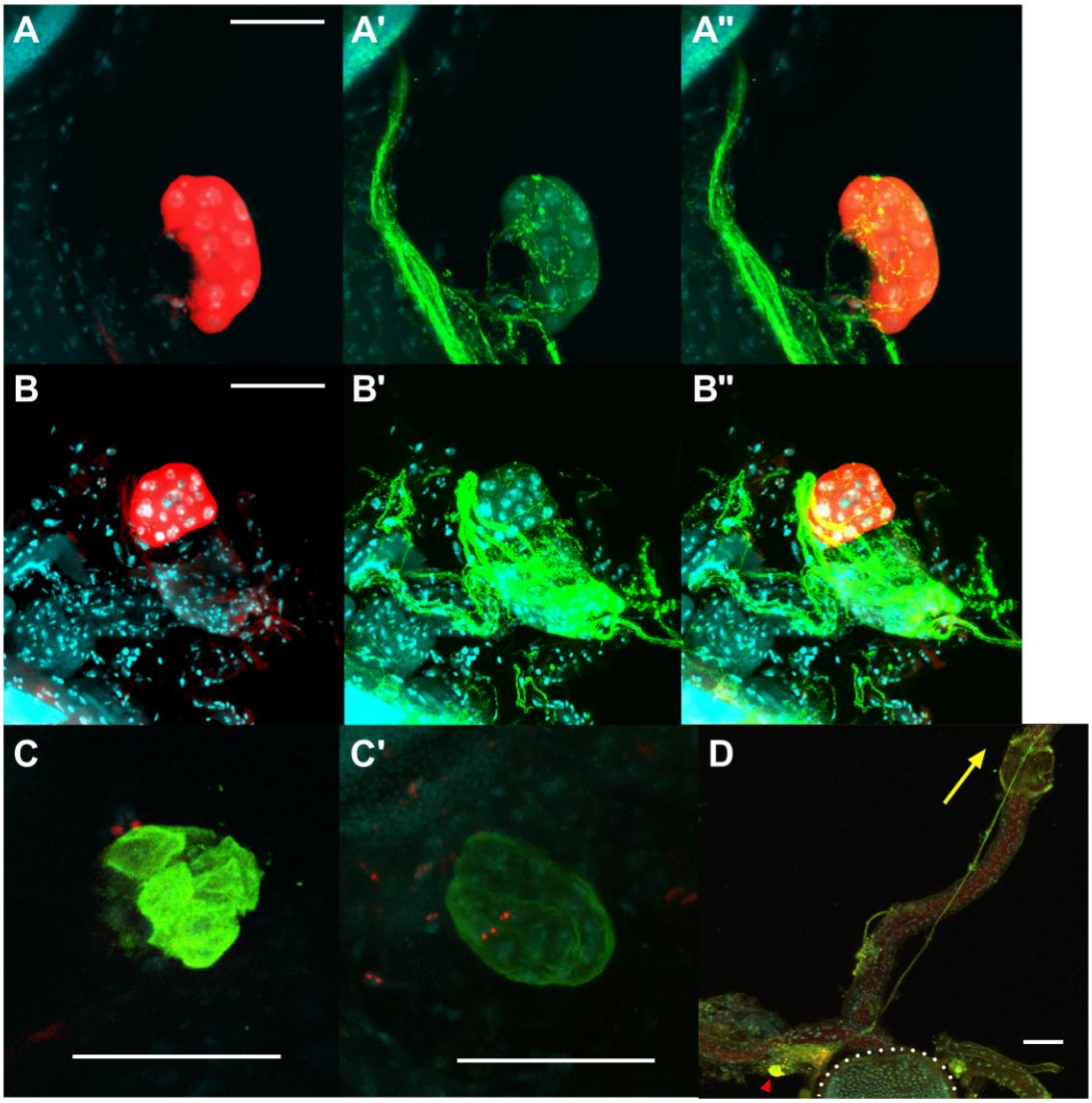
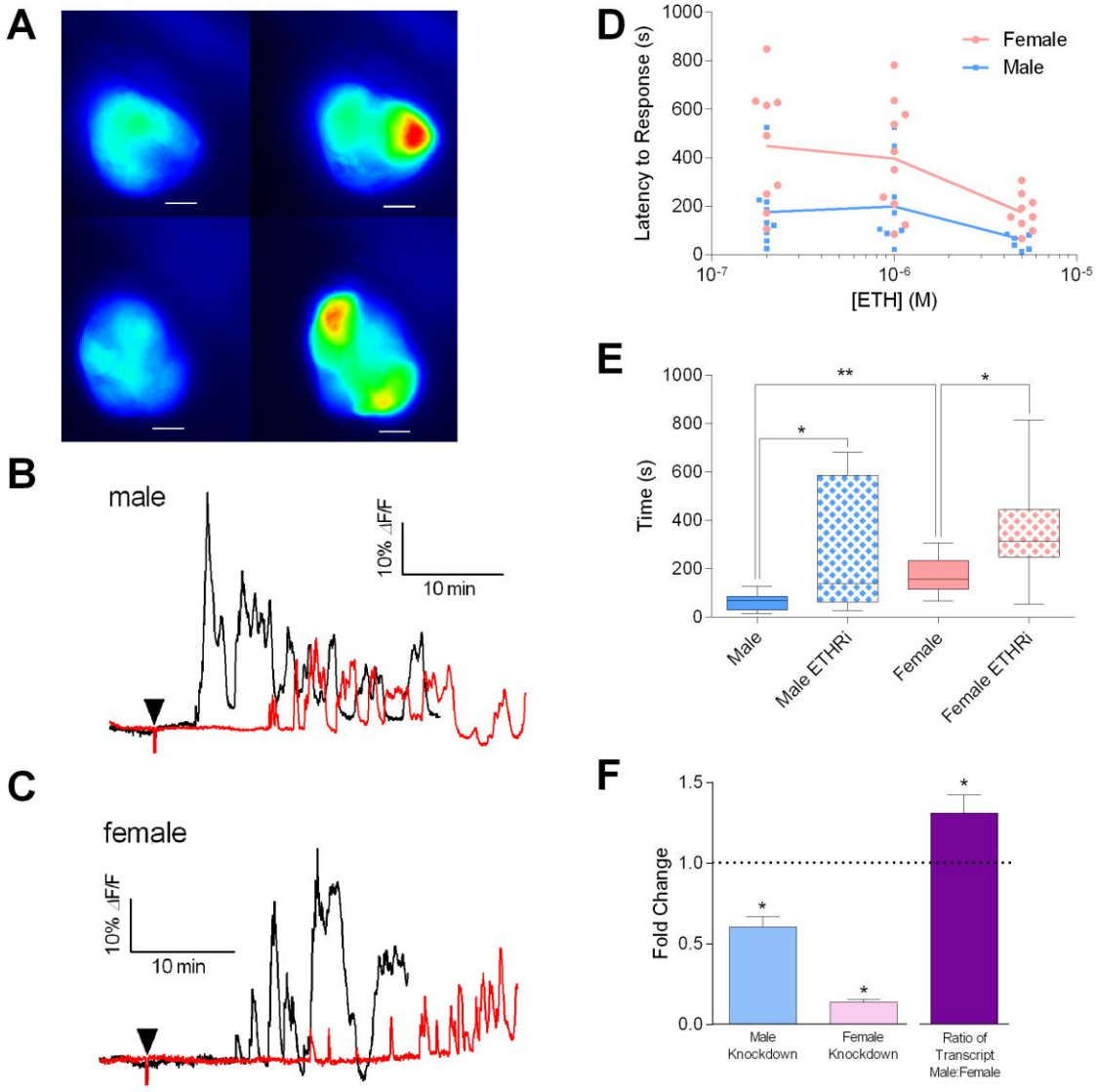


Figure 2.4. ETHR-Gal4 Labels the CA (A-B) Anti-JHAMT (red, A, B) and anti-GFP (green, A', B') of *ETHR-Gal4>UAS-MCD8-GFP* females (top) and males (middle) as well as costaining (A", B"). Anti-JHAMT (Green) staining of *ETHR-Gal4>UAS-RedStinger* males (C) and females (C'). (D) Zoomed out version of staining from C showing CA (red arrowhead) innervation continues to crop (yellow arrow), proventriculus , white dotted line.


A
B
male
10% ΔF/F
10 min
C
female
10% ΔF/F
10 min
D
Latency to Response (s)
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600
400
200
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Female
Male
10-7
10-6
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*
Male
Male ETHRi
Female
Female ETHRi
F
Fold Change
1.5
1.0
0.5
0.0
*
*
*
Male Knockdown
Female Knockdown
Ratio of Transcript Male:Female

Figure 2.5. ETH1 mobilizes calcium in adult CA in a dose-dependent manner

(A) CA from a female (top) and male (bottom) before (left) and after (right) exposure to 1 µM ETH1. **(**B-C) Time course of calcium mobilization in CA in response to 600 nM (red) and 2 µM (black) ETH1 exposure (*Aug21-Gal4>UAS-GCaMP3*). (D) Latencies to response for males and females following exposure to increasing concentrations of ETH1. Female latency was longer than male latency at the tested concentrations ($p <$ .0001) and latency was negatively correlated with ETH concentration ($p < 0.0001$), confirmed by factorial ANOVA (n=8-10). (E) Knockdown of ETHR (*JHAMT-Gal4>UAS-GCaMP5*;*UAS-ETHR-Sym*) decreased responsiveness to 5 µM ETH1 treatment, but among responders, both variance ($p < .01$) and mean ($p < 0.05$) latency were significantly increased in both sexes (F-Test and Mann-Whitney U test, respectively) (n=8-10). (F) ETHR transcript levels in males and females after silencing with *JHAMT-Gal4>UAS-ETHR-Sym* compared to genetic controls, as well as ETHR levels of control males compared to females (n=3). Error bars represent S.E.M. NS, $p >$ .05; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

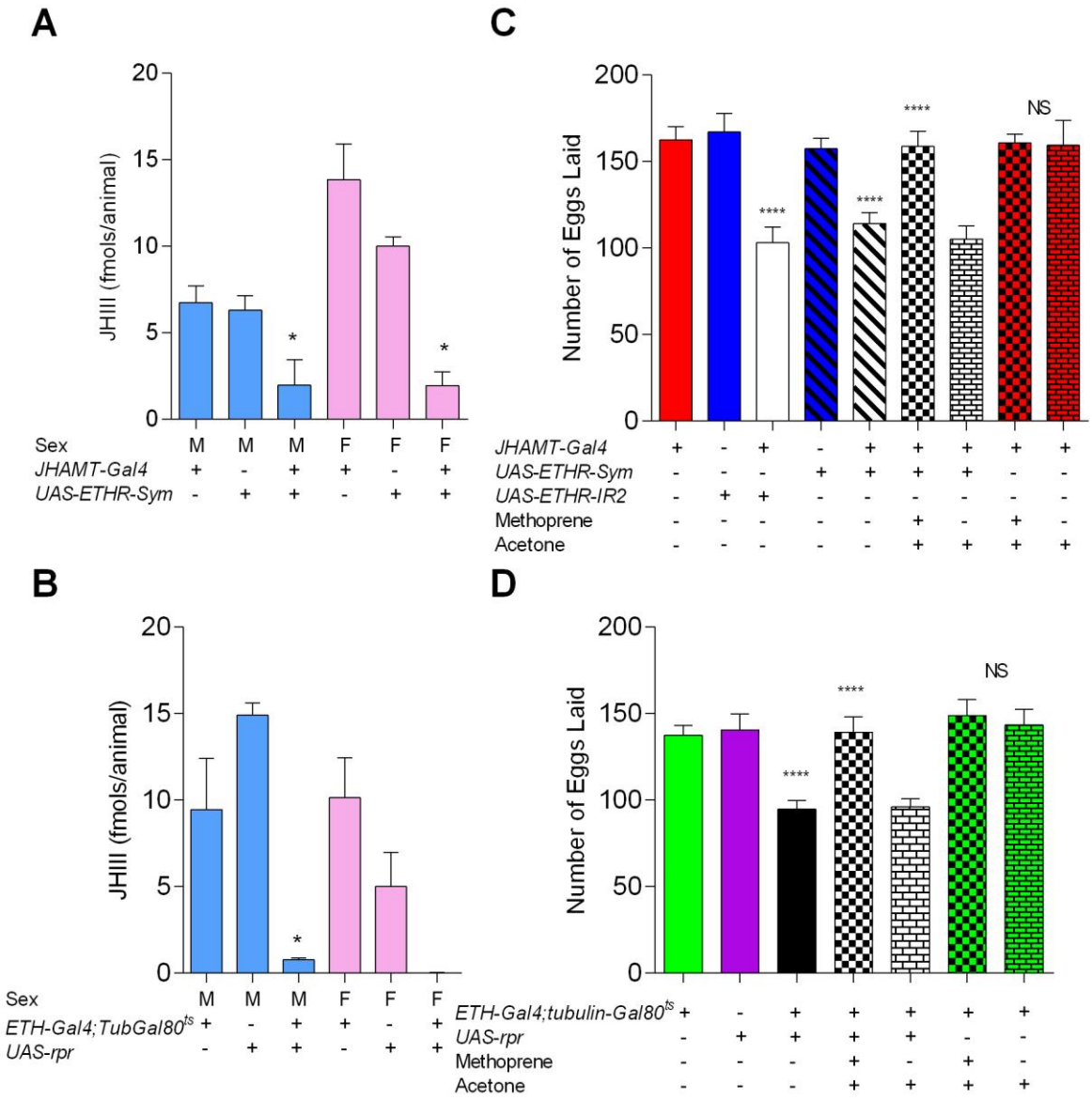
A
JHIII (fmols/animal)
20
15
10
5
0
*
*
Sex M M M F F F
JHAMT-Gal4 + - + + - +
UAS-ETHR-Sym - + + - + +
C
Number of Eggs Laid
200
150
100
50
0
****
****
****
NS
JHAMT-Gal4 + - + - + + + + +
UAS-ETHR-Sym - - - + + + + - -
UAS-ETHR-IR2 - + + - - - - - -
Methoprene - - - - - + - + -
Acetone - - - - - + + + +
B
JHIII (fmols/animal)
20
15
10
5
0
*
Sex M M M F F F
ETH-Gal4;TubGal80ts + - + + - +
UAS-rpr - + + - + +
D
Number of Eggs Laid
200
150
100
50
0
****
****
NS
ETH-Gal4;tubulin-Gal80ts + - + + + + +
UAS-rpr - + + + + - -
Methoprene - - - + - + -
Acetone - - - + + + +

Figure 2.6. Disruption of ETH signaling results in decreased JH levels and reduced reproductive success in females.

(A, B) Reduction of JH-III levels in both sexes following ETHR silencing in CA (*JHAMT-Gal4>UAS-ETHR-Sym*) or Inka cell-ablation (B) (*ETH-Gal4>tubulin-Gal80^{ts}/UAS-rpr*; *n*=100). (C, D). Reduced egg production by females following ETHR knockdown in the CA ((C); *JHAMT-Gal4>UAS-ETHR-Sym*) or Inka cell ablation ((D); *ETH-Gal4;tubulin-Gal80^{ts}/UAS-rpr*) is rescued by topical treatment with methoprene (*n*=15-25). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

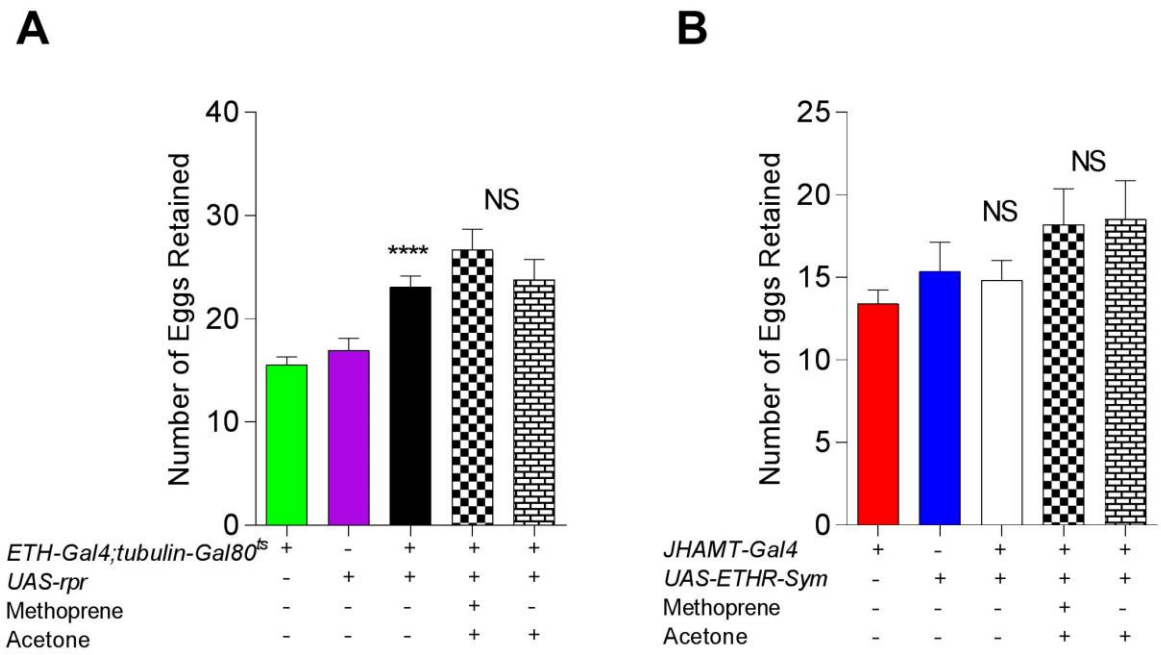

Figure 2.7. Increased egg retention in Inka cell ablated females. *ETH-Gal4>UAS-rpr* (Inka cell-ablated flies) (A) but not CA-ETHR-silenced (B) flies showed an increase in mature eggs (Stage 14) retained. Methoprene treatment had no effect on egg retention.

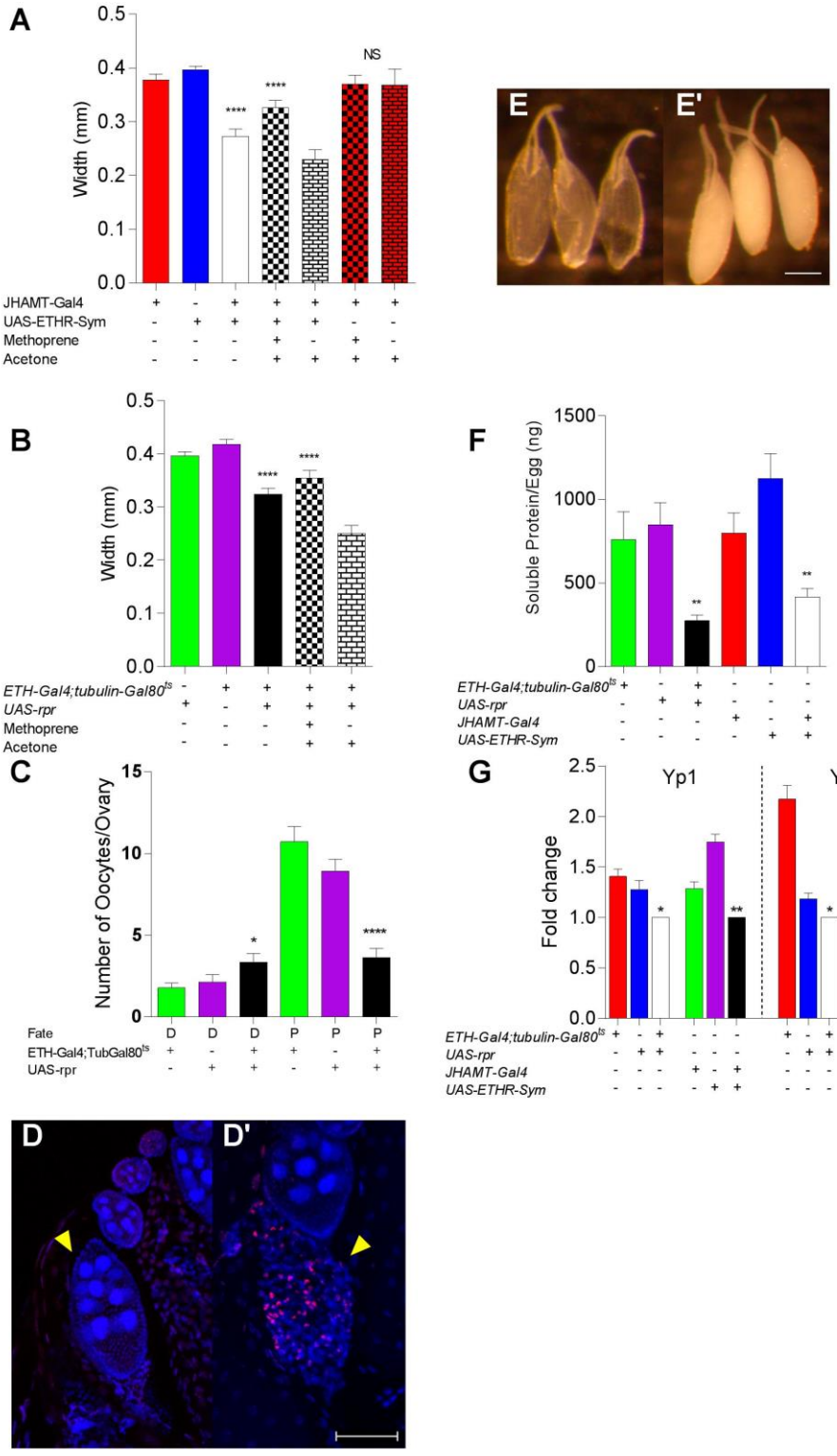
A
Width (mm)
0.5
0.4
0.3
0.2
0.1
0.0
NS
****
****
JHAMT-Gal4
UAS-ETHR-Sym
Methoprene
Acetone
E
E'
B
Width (mm)
****
****
ETH-Gal4;tubulin-Gal80ts
UAS-rpr
Methoprene
Acetone
F
Soluble Protein/Egg (ng)
1500
1000
500
0
**
**
ETH-Gal4;tubulin-Gal80ts
UAS-rpr
JHAMT-Gal4
UAS-ETHR-Sym
C
Number of Oocytes/Ovary
15
10
5
0
*
****
Fate
ETH-Gal4;TubGal80ts
UAS-rpr
D D D P P P
G
Yp1
Yp2
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
*
**
*
***
ETH-Gal4;tubulin-Gal80ts
UAS-rpr
JHAMT-Gal4
UAS-ETHR-Sym
D
D'

Figure 2.8. Disruption of ETH signaling leads to reduced ovary size, decreased yolk deposition, and altered egg development Reduction of ovary width following knockdown of ETHR in the CA (A) or ablation of Inka cells (B); one ovary per fly was examined (*n=35-55*). These effects are rescued by topical methoprene treatment at eclosion. (C-D) Ovarioles of day 5 females of Inka cell-ablated and control flies were separated from ovaries and DAPI and TUNEL stained. (C) Stage 8-13 eggs that were not undergoing apoptosis were classified as progressing (P), while those that were DAPI diffuse and TUNEL positive were labeled degenerating (D) . Example stage 9 eggs, progressing, taken from ovaries of control (D) and those undergoing apoptosis (indicated by TUNEL-positive red staining of DNA fragmentation), from Inka cell-ablated females (D') scale bar represents 50 μm. (D) Stage 9-13 eggs, including those degenerating at stage 8 and 9 (8D, 9D), from each ovary were totaled, defined as progressing (P) or degenerating (D) and compared (*n=15*). (E) Extreme example of low-yolk stage 14 eggs dissected from Inka cell-ablated females (E) compared to normal controls (E'). (F) Protein solubilized from 100 Stage 14 eggs (shown in E) following Inka cell ablation and ETHR knockdown in the CA (*n=6*). (G) QPCR of yolk protein mRNA compared with multiple t-tests, asterisk represents lowest significance value of comparisons to controls (*n=4-5*). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

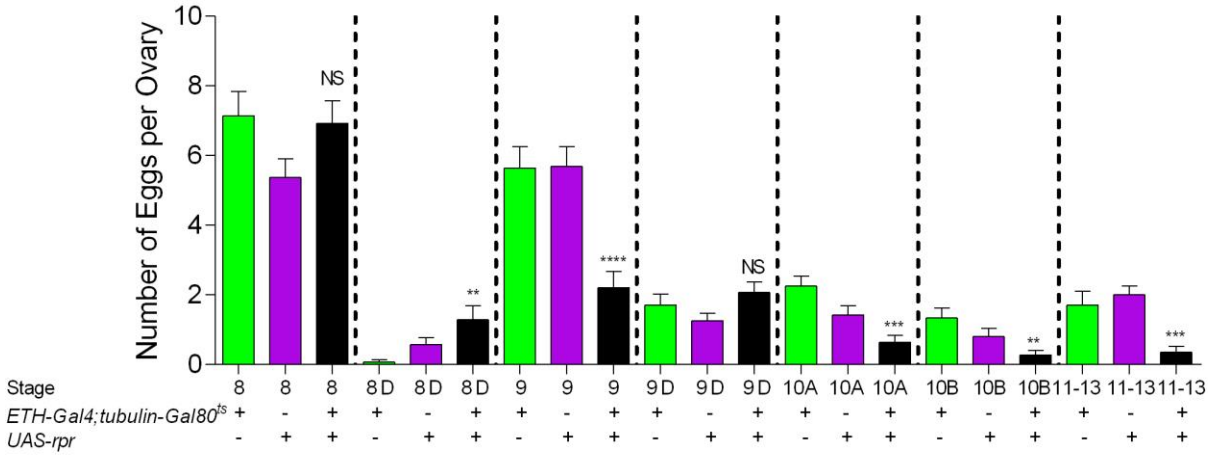

Figure 2.9. Ovariole profile of Inka cell ablated virgin females Ovarioles of day 5 females of Inka cell-ablated and control flies were separated from ovaries and DAPI stained. Stage 8-13 eggs including eggs degenerating at stage 8 and 9 (8D, 9D), from each ovary were totaled, defined as progressing (P) or degenerating (D) and compared (*n=15*). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

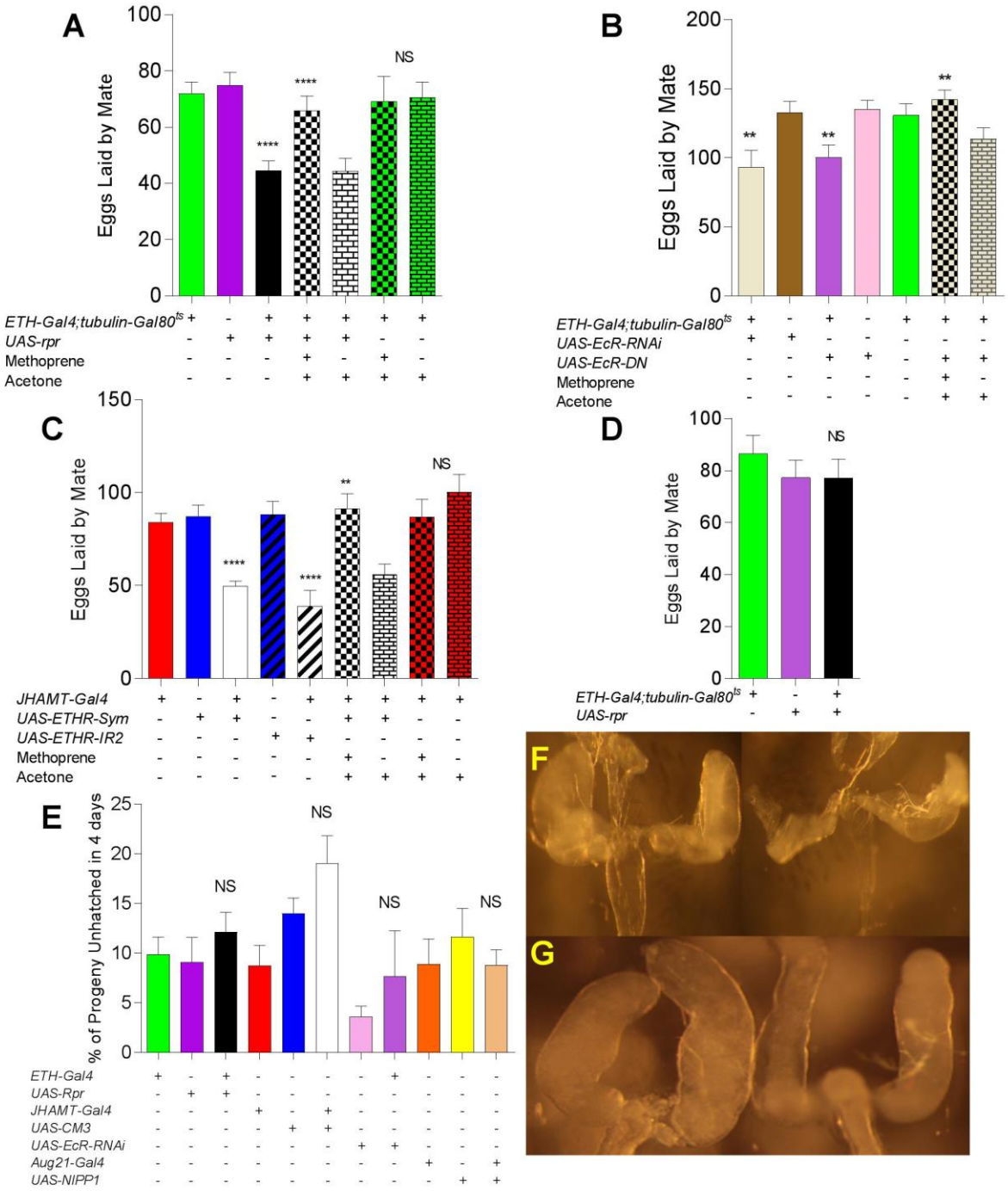
A
Eggs Laid by Mate
100
80
60
40
20
0
****
****
NS
ETH-Gal4;tubulin-Gal80ts + - + + + + +
UAS-rpr - + + + + - -
Methoprene - - - + - + -
Acetone - - - + + + +
B
Eggs Laid by Mate
200
150
100
50
0
**
**
**
ETH-Gal4;tubulin-Gal80ts + - + - + + +
UAS-EcR-RNAi + + - - - - -
UAS-EcR-DN - - + + - + +
Methoprene - - - - - + -
Acetone - - - - - + +
C
Eggs Laid by Mate
150
100
50
0
****
****
**
NS
JHAMT-Gal4 + - + - + + + + +
UAS-ETHR-Sym - + + - - + + - -
UAS-ETHR-IR2 - - - + + - - - -
Methoprene - - - - - + - + -
Acetone - - - - - + + + +
D
Eggs Laid by Mate
100
80
60
40
20
0
NS
ETH-Gal4;tubulin-Gal80ts + - +
UAS-rpr - + +
E
% of Progeny Unhatched in 4 days
25
20
15
10
5
0
NS
NS
NS
NS
ETH-Gal4 + - + - - - - + - - -
UAS-Rpr - + + - - - - - - - -
JHAMT-Gal4 - - - + - + - - - - -
UAS-CM3 - - - - + + - - - - -
UAS-EcR-RNAi - - - - - - + + - - -
Aug21-Gal4 - - - - - - - - + - +
UAS-NIPP1 - - - - - - - - - + +
F
G

Figure 2.10. Impaired ETH signaling reduces reproductive potential of males

Reduced egg production following mating of Canton-S females with males following Inka cell ablation ((A); *ETH-Gal4;tubulin-Gal80^{ts}/UAS-rpr*) or ETHR-knockdown in the CA ((B); *JHAMT-Gal4>UAS-ETHR-Sym or UAS-ETHR-IR2)* and rescue following topical treatment with methoprene (*n*=15-25). (C) Impairment of male reproductive potential following suppression of EcR expression in Inka cells (*n=20-25*). (D) Reproductive potential of Inka cell-ablated males returns to normal by day 10 (*n=10-15*). (E) Hatch rate of females mated to low JH males (*n=20-25*). (F-G) Accessory glands dissected from day 4 ETHR-knockdown in the CA or Inka cell-ablated males (*JHAMT-Gal4>UAS-IR2, ETH-Gal4;tubulin-Gal80^{ts}/UAS-rpr,* right) or controls (left)(scale bar 50 μm). Error bars represent S.E.M. NS, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

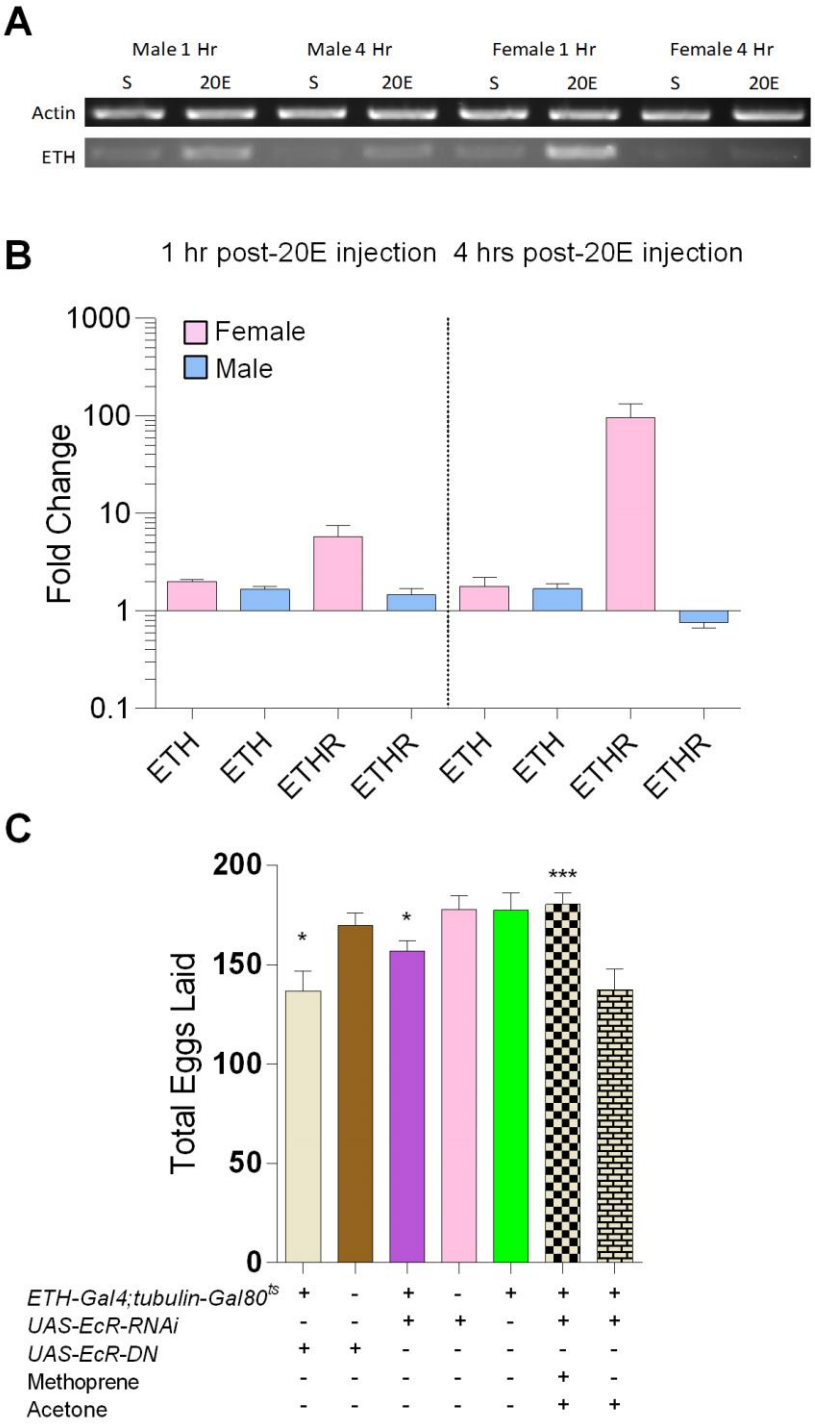
A
Male 1 Hr
Male 4 Hr
Female 1 Hr
Female 4 Hr
S
20E
Actin
ETH
B
1 hr post-20E injection
4 hrs post-20E injection
Female
Male
Fold Change
1000
100
10
1
0.1
ETH
ETHR
C
Total Eggs Laid
200
150
100
50
0
ETH-Gal4;tubulin-Gal80ts
UAS-EcR-RNAi
UAS-EcR-DN
Methoprene
Acetone

Figure 2.11. Impairment of 20E signaling in Inka cells reduces expression of ETH signaling genes and reproductive performance (A) RT-PCR of the ETH gene at 1 and 4 hours following saline (S) or 20E injection (*n=3-4*) (B) Fold-change in ETH and ETHR expression 1 hr and 4 hr after 20E injection measured by qPCR in females (pink bars) and males (blue bars) (*n=4-5*), statistical differences in gene expression between treated and control groups were significant at $p < 0.05$, assessed by Mann-Whitney test. (C) Fecundity is impaired following reduction of EcR expression in Inka cells following expression of EcR-RNAi or an EcR dominant negative (DN; *n=20-30*) and rescue with methoprene (*n=15-20*). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

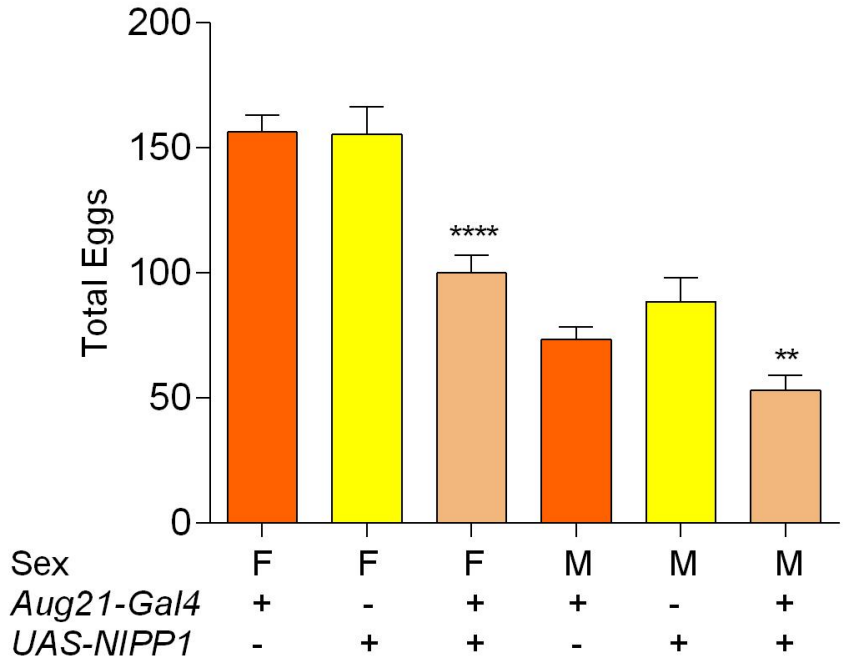

Figure 2.12. Reproductive potential of CA ablated flies *CA-ablated* flies show reproductive potentials reduced comparable to ETH-JH interrupted flies (see Fig. 3). Number of eggs produced by Canton-S females mated to males of the indicated genotype in the 72 hours following mating (left) for CA-ablated flies (*Aug21-Gal4>UAS-NIPP1*) as well as genetic controls (*n=15-25*). Number of eggs produced in the 72 hours following mating to a Canton-S male (right) by CA-ablated flies (*Aug21-Gal4>UAS-NIPP1*) and genetic controls (*n*=15-25). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

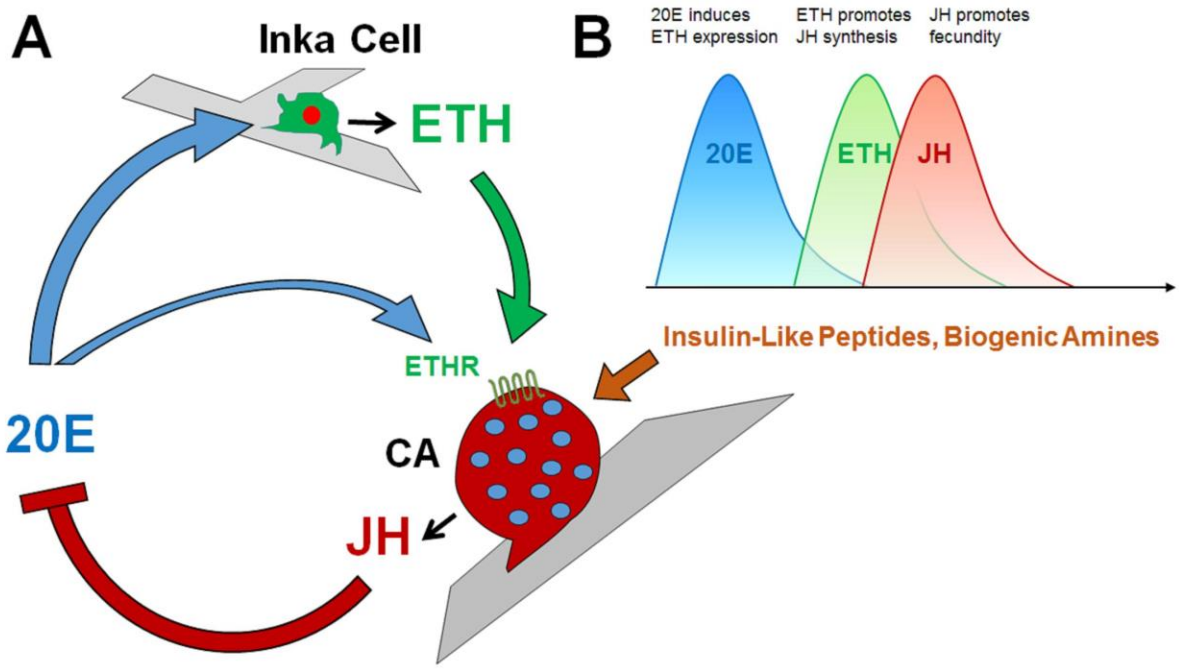

Figure 2.13. Model for gonadotropic coregulation by the hormonal network consisting of 20E, ETH, and JH in *Drosophila* adults (A) Ecdysone (20E) induces expression of ETH in Inka cells and ETHR in target tissues. The CA integrates ETH and other queues to determine JH level; high JH exerts negative feedback inhibition on 20E production. (B) Timing of 20E and JH release into the hemolymph. 20E induces ETH synthesis, but inhibits its release through inhibition of the secretory competence factor ßFTZ-F1. ETH release occurs as 20E levels decline.

Chapter 3

Altered Endocrine State Induced by Environmental Stressors Mediates Reproductive Arrest through Suppressed Oogenesis and Ovulation

Abstract

Environmental stressors induce changes in endocrine state, leading to re-allocation of energy from reproduction to survival. Female *Drosophila melanogaster* respond to thermal, nutrient, and olfactory stressors by arresting fecundity through elevation of the steroid hormone ecdysone. As a consequence, Ecdysis Triggering Hormone (ETH) levels decrease, resulting in arrested oogenesis, reduced octopaminergic input to the reproductive tract, and suppression of ovulation. ETH rescues heat or nutritional stress-induced attenuation of fecundity, suggesting its deficiency is critical to reproductive adaptability. Another olfactory stressor, carcass-derived scent, also elevates hemolymph ecdysone and reduces ovulation. Our findings indicate that, as a dual regulator of octopamine and juvenile hormone release, ETH signaling is crucial to oogenesis, ovulation, and normal levels of fecundity.

Introduction

Reproduction demands precise temporal and spatial coordination of energy resources in a dynamic environment. Under stressful conditions, organisms make critical metabolic decisions whether to opt for survival at the expense of reproduction or to bear the cost of reproduction at the possible expense of survival (Lack 1968; Schwenke et al. 2016). In

humans, chronic stress, malnutrition, or excessive exercise, all markers for conditions suboptimal for parturition, suppress fertility by changing endocrine state (Kalantaridou et al. 2004). This principle is also true for the fruit fly, *Drosophila melanogaster*, which offers opportunities for associating changes in gene expression and endocrine state with phenotypic changes in oogenesis and ovulation.

As arthropods are ectothermic, temperature is a critical factor in evaluating suitability of environmental conditions for procreation. Even brief exposures to cold or heat can deplete energy stores, resulting in attenuation of reproduction (Marshall and Sinclair 2010; Klepsatel et al. 2016; Gruntenko et al. 2003a). Mounting evidence suggests that the adult stress response of *Drosophila* is mediated, at least in part, by ecdysone signaling (Ishimoto and Kitamoto 2011). Ecdysone receptor mutants are more resistant to heat, starvation, and oxidative stress, and have a 50% longer lifespan (Simon et al. 2003), while heat and courtship stress elevate ecdysone levels (Hirashima et al. 2000; Ishimoto et al. 2009). Ecdysone also has a well-known direct role in regulating oogenesis and germ-line stem cell differentiation; more recently, it has been linked to follicle rupture (Uryu et al. 2015; Knapp and Sun 2017). Taken together, ecdysone signaling clearly mediates environmental stress responses leading to reproductive arrest. However, precise mechanisms through which it exerts this arrest remain undefined.

Since ecdysone facilitates reproductive activities, it is somewhat perplexing that its levels elevate under stressful conditions, thereby inhibiting reproduction. An intriguing explanation lies in the recent revelation that fecundity is critically influenced by ecdysis-triggering hormone (ETH) through its actions as an allatotropin (Meiselman et al. 2017).

ETH levels in turn are influenced by ecdysone. During the molt, high ecdysone levels induce expression of ETH and ETHR genes, but suppress Inka cell secretory competence by inhibiting expression of the transcription factor *βFTZ-F1*; ETH release is thereby blocked until ecdysone levels decline (Cho et al. 2014). Here we report that ETH signaling in adults is also influenced by steroid levels, with important ramifications for endocrine responses to stress.

Heat-stress induces impaired oogenesis, with fewer eggs in the later, vitellogenic stages of synthesis and a greater number of oocytes undergoing programmed cell death (Gruntenko et al. 2003a). Programmed cell death of nascent oocytes is a well-characterized mechanism for re-allocation of energy stores (Pritchett et al. 2009). Additionally, both heat-stressed and ETH-deficient flies show a paradoxical accumulation of mature oocytes, in spite of diminished oogenesis (Meiselman et al., 2017).

Accumulation of mature oocytes is a common phenomenon when local control over ovary and oviduct muscle by paracrine signals including proctolin, glutamate and octopamine (OA) is impaired (Ritsick et al. 2007; Kairamkonda and Nongthomba 2014; Lee et al. 2003; Rodríguez-Valentín et al. 2006). Since ETH is a prolific liberin during ecdysis, we asked whether it could modulate release of effectors in the reproductive tract. We report here that disruption of ETH signaling specifically in octopaminergic neurons innervating the female reproductive tract causes anovulation. Moreover, ETH treatment *in vitro* or *in vivo* stimulates ovulation.

Using heat and nutritional deprivation as stressors, we show that as circulating ecdysone changes in response to a stimulus, ETH levels do as well in an inverse manner. We also establish a new type of stressor for *Drosophila* females - presence of corpses – that is detected through olfaction. We describe a pattern of reproductive regulation, wherein altered endocrine state adjusts reproductive output in response to stressful environmental conditions.

Results

ETH deficiency-associated ovarian egg retention is phenocopied by ETHR knockdown in octopaminergic neurons

We showed previously that ETH deficiency causes increased ovarian retention of mature, stage 14 eggs (Meiselman et al. 2017). In this study, we investigated the cause of this egg retention phenotype and report that octopaminergic neurons innervating ovaries and oviduct are targets of ETH.

To further characterize physiological consequences of ETH deficiency on the reproductive tract, ETH release from Inka cells was disrupted using several experimental approaches. Reduction of ecdysone receptor expression selectively in Inka cells, the sole source of ETH, leads to significant increase in mature egg retention in ovaries of 4-day-old virgin females (Figure 1A). Likewise, Inka cell ablation or block of ETH release using temperature-sensitive *shibire* expression causes increased egg retention.

To identify cellular targets of ETH responsible for increased egg retention, we used the Trojan ETHR-Gal4 driver for GFP-mediated visualization and noticed labeling of octopaminergic neurons innervating the base of the ovary and oviduct (Middleton et al. 2006; Diao et al. 2016). These neurons express tyrosine decarboxylase 2, responsible for conversion of tyrosine to tyramine, a critical step in OA synthesis (Figure 1B)(Cole et al. 2005). As OA release and octopamine receptor activation are critical for ovulation in *Drosophila* (Lee et al. 2003; Lim et al. 2014; Sun et al. 2013), and ETH is known to target neuroendocrine cells (Kim et al. 2006; Roller et al. 2010), we examined whether disruption of ETH signaling in octopaminergic neurons influences ovulation. Indeed, ETHR knockdown in octopaminergic neurons of the female reproductive tract using two independent Gal4 drivers (*Tdc2-Gal4*, *Bwk-tqs-Gal4*) increases retention of mature eggs in the ovaries (Figure 1C) (Cole et al. 2005; Rodríguez-Valentín et al. 2006).

ETH induces ovarian contractions and ovulation via activation of octopamine neurons

Since ETH activates ETHR-expressing target cells through calcium elevation (Park et al. 2003; Kim et al. 2006), we asked whether ETH mobilizes calcium in octopaminergic neurons innervating the oviduct and ovaries. We exposed the reproductive tract of *Tdc2-Gal4/UAS-GCaMP6S* females to 10 µM ETH *in vitro* in presence of the OA antagonist 10 µM epinastine and 50 µM Mn^{2+} to suppress movement associated with muscle contractions and observed oscillatory fluorescence responses in OA neurons (Figure 2A-B).

OA amplifies contractions of the peritoneal sheath surrounding the ovaries (Middleton et al. 2006). We found that both OA and ETH treatment of excised ovaries evokes contractions of the peritoneal sheath in a concentration-dependent fashion (Figure 2C-D). Additionally, silencing ETHR expression in OA neurons eliminates contractile responses to 10 µM ETH (Figure 2E). Interestingly, constitutive, pre-treatment contraction frequency of the peritoneal sheath increased significantly in ETHR-silenced octopaminergic neurons ($P<0.05$). ETH-induced contractions are blocked by pre-treatment with epinastine, indicating that ETH-induced contractions occur via OA release.

Expression of the calcium reporter GCaMP6S in ovary epithelia using the 109-53-Gal4 driver (Figure 3A) allowed visualization of local calcium mobilization associated with ovarian contractions (Figure 2F). Both ETH and OA treatments (Figure 2G and 2H, respectively) evoke elevated calcium levels and bouts of strong contractions. Contractions occurring at the base of the ovary facilitate movement of stage 14 oocytes from the ovary into the oviduct (Video 3-4). In contrast, treatments with tyramine, glutamate, and proctolin all failed to stimulate ovary contractions (Figure 3B-D).

OA is necessary and sufficient for relaxation of the oviduct, which is required for ovulation (Rodríguez-Valentín et al. 2006). The relaxation response occurs via activation of two OA receptors (Octß2R and OAMB) in oviduct epithelium, resulting in calcium mobilization, induction of nitric oxide synthase, and NO release (Lim et al. 2014). Using the oviduct epithelium-specific OAMB-Gal4 driver, we examined oviduct calcium responses to ETH and OA exposure (Figure 4A-F). ETH and OA treatments stimulate

strong, sustained calcium responses in oviduct epithelium localized to the juncture of lateral and common oviducts (Figure 4A, D). In OA-treated females, the response is generally rapid and sustained for up to 20 minutes (Figure 4B). On the other hand, ETH treatment elicits transient, oscillatory calcium responses with longer latency (Figure 4G). Calcium responses to both ETH and OA are blocked by 1 μM epinastine (Figure 4C, F). Time to peak calcium levels in response to ETH treatment is significantly longer than OA-induced responses ($P<0.05$) (Figure 4G). When pretreated with OA, oviduct calcium responses to additional ETH treatment are undetectable (Figure 5A). As with ovary base contractions, tyramine, glutamate and proctolin elicit no calcium mobilization response from oviduct epithelium (Figure 5B-D).

We next examined whether injection of OA or ETH stimulates ovulation *in vivo*. Most *in vivo* studies of ovulation utilize a binary assay, whereby dissected ovaries are scored for presence or absence of an oocyte in the oviduct or uterus (Heifetz et al. 2005; Sun et al. 2013; Lim et al. 2014). In an attempt to measure ovulation rate, we scored position of the ovulating oocyte in the oviduct (Figure 6A) following injection of females with either saline, ETH, or OA. Injected individuals were flash-frozen 90 min post-injection. After freezing, females were dissected and examined for the position of the ovulating oocyte in the reproductive tract. Both ETH and OA injection induce *in vivo* ovulation, measured either by oocyte position (Figure 6B) or presence of an oocyte in the bursa (percent ovulated; Figure 6C). Mated females are more responsive than virgins to ETH, but not OA injection.

ETH rescues fecundity in heat stressed females

We observed decline in egg production following exposure of females to temperatures in the range 25-35^{o}C (Figure 7A), a known physiological response to heat stress in *Drosophila* (Dillon et al. 2009). At 30^{o}C, egg production is reduced three-fold, from 150 to 50 eggs/female collected over a 3-day period. We showed previously that yolk protein uptake, a critical component of mid-oogenesis, depends upon ETH levels; ETH-deficient females arrest oogenesis and exhibit impaired fecundity (Meiselman et al. 2017). Since ETH deficiency causes impairment of both oogenesis and ovulation, we expressed TRPA1 specifically in Inka cells to test whether *in vivo* ETH release could rescue the heat stress phenotype. Our results demonstrate significant rescue at 28, 30, and 32°C (Figure 7A).

We sought to confirm that ETH rescues the egg production phenotype by assessing fecundity following injection of ETH1 into unstressed and heat-stressed mated females. When mated females are heat-stressed, fecundity declines for a number of days, with duration depending on the length of the heat stress (Gruntenko et al. 2003c). We found that Canton-S females reduce egg production by ~80% following a 1 hour, 38°C heat stress. To avoid interfering with sperm storage, wild type females were mated on day 4, and either heat-stressed (1 hour in a water bath heated to 38°C) or faux stressed (1 hour in water bath at room temperature) exactly 24 hours later. When heat-stressed mated females were injected with 20 pmol ETH, we observed a significant rescue of this decline (Figure 7B). Faux heat-stressed females injected with ETH showed no difference in fecundity compared to controls (Figure 7C).

These data suggest that ETH rescues fecundity under stressful conditions. One possible explanation for these observations is that heat stress causes a decline in circulating ETH. As oogenesis and fecundity depend upon JH, and ETH is an obligatory allatotropin (Areiza et al. 2014; Meiselman et al. 2017), we sought to determine whether treatment with the juvenile hormone analog methoprene could rescue heat shock-induced repression of fecundity. Methoprene treatment did not increase fecundity (Figure 7C).

ETH rescues heat stress-induced arrest of oogenesis and egg retention

ETH stimulates fecundity by galvanizing progression of oogenesis via JH production (Meiselman et al. 2017). In this study, we have shown that ETH activates octopaminergic neurons to facilitate ovulation. Ovarioles of heat-stressed mated females exhibit reduction of vitellogenic oocytes and increased rates of mid-oogenesis apoptosis, which is rare in unstressed flies (Gruntenko et al. 2003a; Meiselman et al. 2017). As expected, ETH injection into heat-stressed females rescues oogenesis to unstressed levels (Figure 7D-E, Figure 8A-B).

Sugar starvation also is reported to promote mid-oogenesis apoptosis (Terashima 2004). We found that ETH injection into sugar-starved flies rescues oogenesis (S3C-S3D Fig). As mated females without an appropriate substrate on which to lay eggs rarely ovulate and oviposit (Yang et al. 2008), egg retention was not examined.

In the mated state, ovulation occurs soon after oogenesis is completed and egg retention is minimal. However, upon exposure to heat stress, females retain a much greater number of stage 14 oocytes, but no clear mechanistic explanation has been reported (Gruntenko et al. 2003a). We also observed that, while unstressed females retain very few eggs in their

ovaries, heat stress increases eggs retained by three-fold (Figure 7F). ETH injection reverses egg retention (Figure 7F), whereas treatment with methoprene increases the number of mature eggs retained, with increased egg production and sustained anovulation (Figure 7F). Taken together, our data suggest that heat stress depresses fecundity by simultaneously arresting both oogenesis and ovulation.

Heat and nutritional stress elevates ecdysone and lowers ETH

It is well-established that heat stress leads to elevated ecdysone levels (Hirashima et al. 2000; Gruntenko et al. 2003a). High ecdysone levels during molts block ETH release by suppressing expression of the competence factor *βftz-f1* (Cho et al. 2014). We have shown that heat-stressed females show hallmarks of ETH deficiency - arrested oogenesis and egg retention - and these phenotypes can be rescued by ETH treatment. We therefore asked whether elevated ecdysone under stressful conditions leads to ETH deficiency, which might explain oogenesis and ovulation phenotypes described above.

We found that heat stress or sugar starvation indeed elevates ecdysone levels (Figure 9A). We examined whether elevation of ecdysone under conditions of heat stress affects circulating ETH levels by performing enzyme immunoassay (EIA). To avoid rupturing Inka cells, we extracted hemolymph by making incisions in the ventral abdomen and dorsal thorax and bled the animals according to procedures described in the Methods section). The hemolymph extraction protocol was validated by injecting known quantities of ETH into mated females. We found that the quantity of ETH extracted increased in proportion to the dose injected (Figure 10A). This procedure also allowed us

to estimate the amount of hemolymph-borne ETH recovered (2.111±0.221%) and relative levels of circulating ETH in mated females (950 nM at time of extraction).

Circulating levels of ETH-like immunoreactivity are significantly reduced under heat stress conditions (Figure 9B). Likewise, sugar-starved females show a significant drop in ETH levels (Figure 9C). In contrast, wet starvation resulted in a decline of ecdysone levels (Figure 10B), but elevation of ETH (Figure 10C). Taken together, these data suggest an inverse relationship between levels of circulating ecdysone and ETH.

Ecdysone repression of ETH release is necessary and sufficient for heat stress-induced attenuation of oogenesis and ovulation

Elevated ecdysone levels are associated with activation of apoptosis during mid-oogenesis, leading to arrest of vitellogenesis, progression of post-stage 7 oocytes to latter stages of oocyte development, and decreased fecundity (Soller et al. 1999; Terashima et al. 2005; Wilson 1982; Gruntenko et al. 2003b). Recent evidence suggests that high ecdysone levels also may inhibit follicle rupture necessary for ovulation (Soller et al. 1999; Knapp and Sun 2017), but the mechanistic explanation for these phenomena remains lacking.

High ecdysone levels associated with molting induce ETH production, but block of release, causing Inka cells to increase in size and ETH-like immunoreactivity (Cho et al. 2014; Zitnan et al. 1996). To determine whether 20E has similar effects on Inka cells during the adult stage, we injected mated females with 20E (20 pmol) and found that Inka cells increased in volume and ETH-like immunoreactivity (Figure 9D-E).

These results suggest that high levels of ecdysone are associated with increased synthesis (Meiselman et al. 2017), but suppressed release of ETH during adulthood. Prior to ecdysis, high concentrations of ecdysone suppress expression of *βftz-f1*, which is required for secretory competence of Inka cells (Cho et al. 2014). We therefore examined whether we could rescue heat-stress-induced arrests in oogenesis, ovulation, and egg laying by disrupting ecdysone-mediated repression of Inka cell secretory competence. Using Inka cell-specific GAL4 drivers for: 1) EcR knockdown, 2) overexpression of an EcR dominant negative isoform, and 3) overexpression of *βFTZ-F1*, females were exposed to heat stress and tested for egg production and physiological markers of ETH-deficiency. Test females showed a clear increase in progression beyond mid-oogenesis (Figure 10E-F) and fewer mature eggs retained in their ovaries compared to controls (Figure 9G). Ovariole profiles thus suggest egg development and ovulation were rescued by these manipulations. Furthermore, expression of either dominant negative EcR or *βftz-f1* led to significant increases in egg production under heat stress conditions (Figure 9H). These findings confirm that stress-induced elevation of ecdysone levels reduces secretory competence of Inka cells, leading to ETH deficiency.

The Scent of Death Perturbs Endocrine State and Causes Anovulation

Besides heat, several stressors elevate hemolymph ecdysone levels, including sugar starvation and even chronic exposure to cocaine (Gruntenko et al. 2003a; Terashima et al. 2005; Sedore Willard et al. 2006). While examining egg retention in group-raised virgin females, we noticed each group had a common level of egg retention regardless of genetic background, and suspected a confounding variable. As adult crowding is a

reported stress (Joshi and Mueller 1997), we tested whether the number of individuals affected egg retention, but found no correlation between eggs retained and number of individuals housed in a vial. However, we noticed not all females placed in vials survived until the day of testing and therefore examined whether presence of dead animals influences egg retention. On day 0, female siblings were collected and half were pithed (brain destroyed) with a forceps tip. We found that females exposed to carcasses retained almost twice as many eggs as unexposed counterparts (Figure 11A).

We then sought to determine which sensory modality contributes to egg retention upon exposure to carcasses. When dead females were placed in a cheesecloth basket at the top of the vial (Fig 11B) to block visual or mechanosensory inputs, females still retained more eggs, suggesting a volatile cue (Fig 11C). We tested for olfactory and gustatory inputs by exposing *PoxN* and *Orco* mutant females to carcasses. The *PoxN* mutation re-specifies gustatory chemoreceptor neurons to mechanoreceptors(Awasaki and Kimura 1997), while *Orco* inactivates all olfactory receptor neurons(Larsson et al. 2004). Despite a surprisingly low number of eggs in controls, *PoxN* females had an increased number of eggs retained in the presence of corpses, whereas *Orco* mutants had no elevation in egg retention despite capacity to do so, demonstrated by increased retention after heat stress (Fig 11D). This suggests that olfaction is key to the "scent of death" response.

Since we have demonstrated here that egg retention can result from perturbation of endocrine state, we measured ecdysone levels of virgin females and males in vials with no carcasses and a number of carcasses equal to the number of live animals. As expected, we found ecdysone levels elevated in virgin females when incubated 24 hours

with corpses killed by either brain destruction (pithing) or freezing (Fig 11E). Males showed no clear change in steroid levels, suggesting this perturbation of endocrine state may be specific to females as a method to arrest reproductive activities (Fig 11E). These data suggest ecdysone elevation may be a common response to a variety of stressors, and a necessary step for stress response in reproductive arrest.

Discussion

Evidence presented here establishes a new paradigm for *Drosophila* reproduction, wherein stressful conditions are transduced via a hormonal cascade to divert energy flow away from egg production. This crucial adaptor links environmental conditions to reproductive output via synthesis and release of the command peptide ETH. As steroid levels fluctuate in response to stress, so too does ETH, a consequence of steroid-regulated changes in Inka cell secretory competence. ETH activates two downstream targets: JH-producing corpora allata and modulatory OA neurons innervating the ovary and oviducts. We characterize the nature of ETH dependence, and assign function and context to a newly recognized hormonal axis governing reproductive responses to stress.

Our previous report showed that ETH is an obligatory allatotropin, promoting oogenesis and fecundity through JH production; consequently ETH deficiency results in low JH levels and arrested oogenesis (Meiselman et al, 2017). In the present work we demonstrate for the first time that ovulation of stage 14 oocytes depends upon ETH activation of OA neurons innervating the ovary and oviduct. We also offer a comprehensive explanation for the change in distribution of vitellogenic oocytes reported in EcR mutants or under conditions of high or low ecdysone, depending on stress levels

(Soller et al. 1999; Carney and Bender 2000; Uryu et al. 2015; Gruntenko et al. 2003a). We show that ETH deficiency or ETHR knockdown results in accumulation of stage 14 oocytes in the ovary due to ovulation block (Lee et al. 2003; Lim et al. 2014; Rodríguez-Valentín et al. 2006) and provide for the first time a mechanistic link between altered endocrine state and ovulation.

ETH regulates OA signaling in the female reproductive tract

We have demonstrated that ETH promotes ovulation through activation OA neurons to induce contractions in the ovary and relaxation of the oviducts. It is interesting that ETH triggers calcium dynamics *in vitro* on distal axonal projections, suggesting ETH-stimulated OA release results from direct action of ETH on axons and/or nerve terminals. While ovary contractions in response to ETH exposure occur in both virgin and mated females, we chose virgin females for analysis due to higher spontaneous contractile activity in mated females. This is likely due to actions of ovulin after insemination, which stimulate outgrowth of octopaminergic neurons innervating the oviduct (Rubinstein and Wolfner 2013). In virgin females, concentration-dependent ETH actions on the ovary (low micromolar) are in the range predicted for activation of ETHR-A receptors (Park et al. 2003).

Acting through OA neurons, ETH mobilizes calcium in the epithelium enveloping the ovary, initiating bursts of contractions in the peritoneal sheath at the base of the ovary associated with ovulation. Although bath-applied ETH and OA are both sufficient to induce calcium mobilization in oviduct epithelium, they induce distinctive response patterns. OA causes a rapid, sustained calcium wave with a slowly waning plateau

following the peak response. ETH actions occur with longer latency and induce oscillatory calcium dynamics, which could be a consequence of periodic synaptic reuptake of OA by nerve terminals. No changes in intensity were observed between treatments or at different doses, suggesting a possible threshold effect. It is also interesting to note that calcium waves spread through the epithelial layer, suggesting that the epithelium is a functional syncytium, which undoubtedly aids in coordination of relaxation.

Injection of mated females with either ETH or OA induces ovulation *in vivo*, whereas injected virgin females respond much more weakly. In order for ovulation to occur, OA causes follicle rupture inside the ovaries, a process requiring one to several hours *ex vivo* (Deady and Sun 2015; Knapp and Sun 2017). We hypothesize that mated females are in the proper endocrine state for ovulation, and thus follicle rupture may already be in progress before application of ETH or OA. As follicle rupture is the critical first step for egg laying, this limiting factor would explain the length of time (up to 60 minutes) after physiological levels of ETH/OA are reached for *in vivo* ovulation to occur, given that ovary contraction and oviduct relaxation occur within seconds.

We also examined agents previously implicated in oviduct contractions, including tyramine, glutamate, and proctolin. While ineffectiveness of tyramine and glutamate is not surprising, the negative result with proctolin is at variance with prior literature (Ritsick et al. 2007). Examination of proctolin-induced contractions revealed that they are localized to the distal tip (germaria) of the ovaries. Moreover, proctolin does not

stimulate ovulation *in vitro*. It appears that the role of proctolin in *Drosophila* ovaries is more limited than in the well-studied locust oviduct (Belanger and Orchard 1993).

Stress (heat and sugar starvation) elevates ecdysone, creating ETH deficiency

We have shown that elevated ecdysone levels in response to heat and nutritional stress are associated with a drop in ETH levels. We previously hypothesized that the Inka cell secretory competence model governing ecdysis signaling during developmental stages may persist into adulthood (Meiselman et al. 2017). Results presented here support this hypothesis.

Both stress and ETH deficiency have similar consequences for reproduction, namely arrested oogenesis and reduced ovulation, resulting in increased stage 14 egg retention and lower egg production. Progression of mid-oogenetic oocytes is directly correlated with JH levels (Soller et al. 1999; Pritchett et al. 2009; Yamamoto et al. 1988), while OA release from reproductive tract neurons is necessary for ovulation (Cole et al. 2005; Lee et al. 2003). Here we show that arrested oogenesis and ovulation contributing to the ovariole profile observed in heat stressed flies (Gruntenko et al. 2003a) can be explained by ETH deficiency, which has a dual role in regulating JH levels and activity of OA neurons innervating ovaries and oviducts. Indeed, arrest of both oogenesis and ovulation deficiencies can be rescued by ETH, either through TRPA1 activation of Inka cells or direct injection of ETH1.

We examined the mechanism through which elevated ecdysone leads to ETH deficiency by performing rescue experiments designed to 1) suppress steroid signaling in Inka cells and 2) express the transcription factor ßFTZ-F1, which confers secretory competence of

Inka cells and is suppressed by high ecdysone levels (Cho et al., 2014). Although somewhat variable in their effectiveness, these manipulations resulted in clear rescue of oogenesis and ovulation in heat stressed females, confirming that the thermal stress response operates through the influence of ecdysone on Inka cell secretion.

Implications of the ecdysone/JH balance on oogenesis and ovulation

We found that methoprene treatment increases progression of oogenesis, but does not increase oviposition in stressed animals. These results are similar to those reported previously (Soller et al. 1999). In fact, we observed a significant increase in eggs retained after methoprene treatment, suggesting that synthesis of mature eggs resumes with JH treatment, but ovulation remains impaired under conditions of elevated ecdysone and ETH deficiency. This suggests that ovulation provides a gating mechanism under stressful conditions, limiting egg production while conditions are suboptimal (Figure 12). A recent report suggested that normal ecdysone levels stimulate follicle rupture and ovulation, but that elevated levels inhibit follicle rupture (Knapp and Sun 2017). The present work provides an additional mechanism for suppression of ovulation associated with elevated ecdysone levels: repression of ETH release leading to reduced OA neuron activity.

Drosophila responds to courtship, nutritional, chemical and thermal stress with elevation of ecdysone levels (Sedore Willard et al. 2006; Ishimoto et al. 2009; Bownes 1989; Hirashima et al. 2000). Here we show that sugar starvation and heat stress depresses ETH levels in the hemolymph. We only observed a relatively small reduction in ETH levels, but this could be due to two reasons. Our extraction method required dilution of the

hemolymph in order to minimize coagulation. This may pare any differences between samples in ETH immunoreactivity depending on success of the extraction. Second, we were operating at the limit of quantification (LOQ) for the ETH1 antiserum. Consequently, concentrations under 18 nM could not be accurately quantified and thus heat-stressed females may have even lower ETH than the EIA could indicate.

It is interesting to note that wet starvation reduces ecdysone levels and increases ETH levels, whereas sugar starvation increases ecdysone levels (Terashima et al. 2005), and, we show here, increases ETH levels. Wet-starved females were precisely synchronized in mating on day 4, and began starvation (no nutrient source, wet kimwipe) 24 hours later for an additional 24 hours. In order to mimic conditions described by Terashima et al., sugar-starved females were group raised until day 3, and groups were placed on agar+10% sucrose for 24 hours. Mating was not controlled in sugar-starved females, though it is known to influence ecdysone levels dramatically in the short term (Harshman et al. 1999). This in addition to our slightly different age (6 days after eclosion at time of measurement vs 4 days), and different method of starvation (wet starvation vs agar medium and 5% sucrose) may also change endocrine state. While these discrepancies may all be contributing factors, arguably the most interesting result is that ecdysone decrease led to elevated circulating ETH. This adds credence to the hypothesis that ETH and ecdysone levels are inversely correlated.

Unlike thermal and starvation stress, some stressors, like predators or unfamiliar xenotoxins, cannot be sensed. In such a case, the ability of an organism to sense recently deceased conspecifics could relay critical information that could be integrated into the

essential decision to reproduce or survive. We show here that in the absence of any other stress, an airborne scent released from conspecific carcasses can perturb endocrine state and arrest ovulation. Females exposed to carcasses change endocrine state to depress reproductive activities, and this state change depends on olfaction. Elevated ecdysone levels measured in exposed females suggest a conserved stress response of ecdysone elevation, which we show here causes depression of circulating ETH, deficiencies in oogenesis and in ovulation. It will be interesting to trace the pathway from olfaction to the state-change, as it will likely reveal a primitive, conserved network for coordination of brain and body.

Unique stresses may garner different endocrine responses because different types of cues require differential behavioral adaptation. The ability of a hormone to coordinate a wide variety of target tissues to change in state makes it a perfect tool for stress adaptation. As an organism encounters a new type of stress, they may adapt a new endocrine state to coordinate a tissue-wide response. Many hormones in closely related insects play markedly different roles, which evolve as rapidly as behavioral niches, but an endocrine core in E-ETH-JH is highly conserved, similar to the HPG axis among vertebrates. A hormonal network with competence to adjust reproductive output in response to environmental changes is undoubtedly a common phenomenon among multicellular organisms. The discovery of a stress response hormonal axis and, more aptly, a peptide hormone with the potential to alleviate stress-induced deficits in reproduction could be of particular relevance to the honey bee *Apis mellifera*. In recent years, *Apis* reproductives have been producing fewer progeny thanks to a variety of stressors, including

temperature extrema (Amiri et al. 2017; Pettis et al. 2016). While proctolin has already been found to be a short-term reproductive stimulant in *Apis* queens (Miranda et al. 2015), ETH is attractive as it can alter JH levels, which in turn may rescue poor pheromone production, the proximal cause of supersedure (Darrouzet et al. 2014; McQuillan et al. 2014; Winston et al. 1989).

In summary, we have demonstrated that the peptide hormone ETH is critical for proper functioning of octopaminergic neurons innervating the reproductive tract and, consequently, ovulation. Inka cells, the sole source of ETH, appear to be tightly regulated by hemolymph ecdysone levels. Stress-induced elevation of ecdysone levels leads to ETH deficiency, resulting in reduced reproductive output through arrest of oogenesis and ovulation. This provides the first mechanistic explanation connecting known endocrine perturbation and anovulation and sets the context for further examination of endocrine regulation of stress and reproduction in insects.

Materials and Methods

Flies

Flies used for immunohistochemistry, calcium imaging and CA ETHR silencing were raised at 23˚C on standard cornmeal-agar media under a 12:12 hr light:dark regimen. Inka cell ablated and EcR interrupted flies were raised as described previously (Meiselman et al. 2017). Inka cell blocked and conditional ETHR silenced or EcR-eliminated flies were raised at the $Gal80^{ts}$ permissive temperature (18°C) and moved to

the nonpermissive temperature (28°C) after eclosion until day 4. Use of double-stranded RNA constructs for silencing of ETHR (UAS-ETHR-Sym; UAS-ETHR-IR2 line (VDRC transformant ID dna697) were described recently (Kim et al. 2015). Bwq^{tqs}-Gal4 flies were obtained from Dr. Enrique Reynaud (Instituto de Biotecnología, Mexico City). OAMB-Gal4 was obtained from Dr. Kyung-An Han (University of Texas, El Paso). UAS-mCD8.mRFP, *Orco,*and *PoxN* flies were obtained from Dr. Anupama Dahanukar (University of California, Riverside). UAS-ßFTZ-F1 was described previously (Cho et al. 2014). ETHR-Gal4 was obtained from Dr. Ben White (National Institute of Mental Health, Silver Spring). All other fly lines were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN): Tdc2-Gal4 (BS no. 9313), UAS-TrpA1 (BS no. 26264), UAS-Red Stinger (BS no. 8574), UAS-mCD8-GFP (BS no. 5137), UAS-rpr (BS no. 8524), UAS-GCaMP6S (BS no. 42746), UAS-GCaMP5 (BS no. 42037), TubP-$Gal80^{ts}$ (BS no. 7017), ETH-Gal4 (BS no. 51982), UAS-EcR-RNAi (BS no. 37059), UAS-EcR.B1 (BS no. 6869).

Immunohistochemistry

Labeling of ETHR-expressing octopaminergic neurons

Ovaries were removed from group-raised females of the genotype Trojan ETHR-Gal4/UAS-mCD8-GFP and immediately placed in 4% paraformaldehyde for 55 minutes. Once fixation was complete, ovaries were rinsed briefly in PBS with 5% Triton-X (PTX) and 5 times for 10 minutes in 0.5% PTX. After washing, ovaries were blocked for 5

hours in 0.5% PTX+5% Normal Goat Serum (NGS), and then swapped for 0.5% PBST+5% NGS containing mouse anti-GFP primary (Invtirogen, 1:500) and rabbit anti-Tyrosine decarboxylase 2 (Covalab, 1:200). After two overnights, samples were washed 5X and Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen), Alexa Fluor555 goat anti-rabbit (Invitrogen) and 0.5 mg/ml DAPI in 0.5% PBST+5% NGS was added. After 4 days, samples were washed 5X, mounted in Aqua Poly/Mount (Polysciences Inc.), and imaged on a Leica SP5 confocal microscope.

Inka cell staining

Flies were mated on day 4, heat stressed or injected on day 5 and opened and fixed 24 hours later. Immunohistochemistry for visualization of the Inka cells was performed as described previously (Meiselman et al. 2017). After staining, we constructed image stacks using one micro sections. Volume of Inka cells was measured using the Volume Viewer and ROI tools in imageJ. ETH-IR volume is total volume minus the volume of the DAPI and Red-stinger labeled nucleus.

Treatments

Heat stress

Flies of indicated genotypes were mated on day 4 and 24 hours later were aspirated into an empty glass culture tube (no more than 5 females per tube) and submerged in a 38°C hot water bath for 1 hour. Flies were either injected first or placed on a food plate for egg laying immediately afterward.

Starvation

Wet starved females were mated on day 4. 24 hours later females were placed in a vial with a wet kim wipe for 24 hours before extreaction. Sugar starved females were group-raised until day 3 and placed on 3% agar+10% sucrose plates for 24 hours and extracted. For injections, sugar starved females were injected abdominally with either ETH or fly saline alone on day 3 and placed on 3% agar+10% sucrose plates for 48 hours before assessment of fecundity and oogenesis.

Injections/Topical application of methoprene

Injections or topical application of methoprene/acetone was performed immediately after heat shock. Females were anesthetized on ice and placed on an Echotherm chilling plate. For methoprene treatment. females were treated as described previously (Meiselman et al. 2017) before being given 72 hours for egg-laying. For injections, females were anaesthetized and injected with 50 nl of 100 μM ETH into the abdomen.

Egg Retention and Egg Laying

Female flies of the indicated genotype were isolated in 1 cm culture tubes with standard fly food at eclosion. For egg laying, after 4 days flies were mated with wild-type males and re-isolated for 24 hours to store sperm. After 24 hours, females were heat shocked, injected before being moved to apple juice-agar-yeast plates for three daysto lay eggs for three days as previously described, or (Meiselman et al. 2017). TRPA1 flies were raised at 23°C for four days and moved to an incubator of indicated temperature on an apple juice plate for three days. For egg retention, virgin females were dissected at 3PM, ovaries were opened up and mature, stage 14 oocytes in the ovaries were counted and compared.

Calcium Imaging

Octopaminergic Neurons

Group-raised females of the genotype Tdc2-Gal4/UAS-GCaMP6S;UAS-mCD8-RFP were reared at 28°C after eclosion until day 5. At day 5, females were cold anesthetized and ovaries were removed in fly saline and immediately added to 90µl of fly saline+10µM epinastine and 50µM Mn2+ to prevent contraction-associated movement of the sample. Sample was put on a glass cover slip and onto an SP5 inverted confocal microscope, recorded at 40x. After 100 seconds of habituation, ovaries were treated with 10µl of 100µM ETH1 (final conc. 10µM) and recorded for 10 minutes.

Epithelial response

Group-raised females of the genotype OAMB-Gal4/UAS-GCaMP6S (Oviduct epithelium) or 109-53-Gal4/UAS-GCaMP6S (Ovary epithelium) were reared at 28°C after eclosion until day 5. We used an imaging setup consisting of a Polychrome V monochromator (TILL Photonics/FEI) as light sources and a TILL Imago CCD camera. The microscope (Olympus Model BX51WI) was equipped with a 40x W NA 0.8 objective. Binning on the chip (8 X 8) was set to give a spatial sampling rate of 1 µm/pixel (image size 172 X 130 pixels, corresponding to 172 µm and 130 µm). Images were taken at a rate of 4 Hz. The excitation wavelength was 488 nm, and exposure time was 25 msec. Fluorescent light passing an excitation filter (370-510 nm) was directed

onto a 500 nm DCLP mirror followed by a 515 LP emission filter for EGFP. Timing of treatment was performed as above, bath was 180µl and 20µl treatment as indicated.

Ovary Contractions

For dose-response curves, ovaries were removed from cold-anesthetized, Canton-S virgin female flies and immediately added to 180µl of fly saline, and placed under a stereomicroscope with a Canon EOS Rebel T5i video camera. 20µl of 10 times the indicated dose of ETH or OA was added to the bath at 2 minutes after recording began and recording continued until 4 minutes. Videos were reviewed single blind and number of contractions during the two minutes prior to and after treatment was tallied. For OA neuron ETHR-silencing, videos were prepared in the same fashion, but contractions for each 30 second interval of the video was tallied, averaged, and graphed over time to analyze contractions over time. 200µM epinastine was added to the saline just before the ovaries when indicated.

Ovulation

In vivo ovulation was assessed by cold anesthetizing and injecting group raised day 4 females abdominally with 36nl of the indicated amount of ETH or OA dissolved in fly saline or saline alone. After 90 minutes of recovery time, flies were flash frozen with dry ice and cuticle was removed from around the ovaries to view the position of the egg. For each fly, the furthest ovary along the oviduct was scored for position and recorded.

Oogenesis

After three days of egg laying, ovaries were removed from flies of various treatments and DAPI/TUNEL stained using previously described protocol (Meiselman et al. 2017).

Enzyme Immunoassay (EIA)

ETH extraction

Canton-S females were mated on day 4, and 24 hr later were subjected to heat stress, wet or sugar starvation, or injected with ecdysone 24 hours later. After an additional 24 hours, groups of 8-12 females were cold-anesthetized on ice and placed in wells of a round-bottom glass slide on a chilling plate (Echotherm, Torrey Pines Scientific, Inc., San Diego) set to 2°C. Wings and tarsi were removed, and flies were moved to a dry well. To leach out the polar ETH from the hemolymph and prevent coagulation, 1µl of molecular grade water per fly was added to the bottom of the well and flies were opened on the dorsal thorax and ventral abdomen, taking care not to rupture tissue, including trachea. Flies were each gently depressed once to ensure mixing of hemolymph and water, removed and placed head down (to avoid genital tract contamination) in a 1 ml centrifuge tube, which was previously perforated several times at the bottom with a 27.5 gauge needle. Remaining hemolymph-water mixture was removed and added to the tube, and tube was centrifuged for 5 minutes at 5000 g. Flow through was collected and immediately moved to -80ºC. Once 50 flies were collected, the tube was spun at 16,000 g and supernatant was removed for analysis by EIA.

Ecdysone extraction

Females were prepared as above. Extraction differed in that free ecdysone was separated from its glandular source by removal of the ovaries and decapitation. Remaining body

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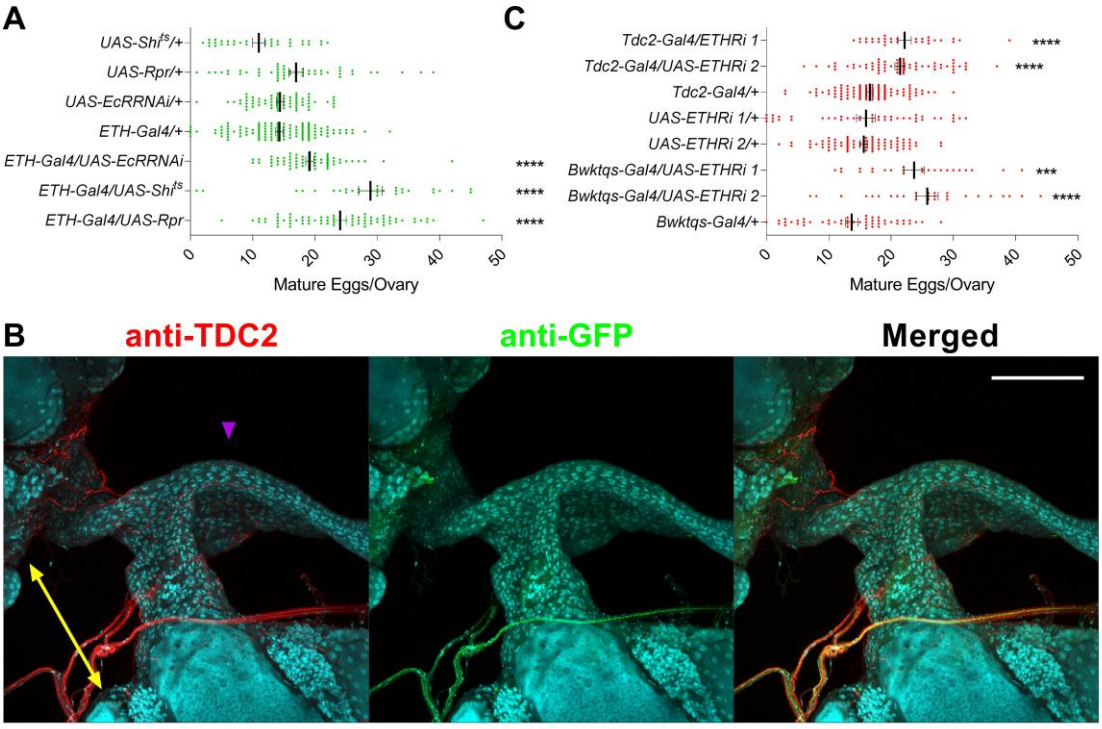

Figure 3.1. Disruption of ETH action on octopaminergic neurons causes egg retention in virgin females (**A**) Number of mature eggs retained in ovaries of isolated adult virgin females with Inka cells ablated (*ETH-Gal4;Tubulin-Gal80^{ts}/UAS-Reaper*), blocked (*ETH-Gal4/UAS-Shi^{ts}*), and EcR silenced (*ETH-Gal4;Tubulin-Gal80^{ts}/UAS-EcR-RNAi*) n=30-50. (**B**) Co-staining of ETHR positive neurons in the vicinity of the juncture of ovaries (yellow arrow) and oviduct (purple arrowhead) ETHR-Gal4/UAS-mCD8-GFP (Green), anti-TDC2 (red), DAPI (blue). Scale bars 100 µm. (**C**) Number of mature eggs retained in the ovaries of virgin females with ETHR silenced in all octopaminergic neurons (*TDC2-Gal4*) and in the neurons innervating the female reproductive tract (*Bwk^{tqs}-Gal4*) n=30-50. Error bars represent S.E.M. *** $p < .001$, **** $p < .0001$.

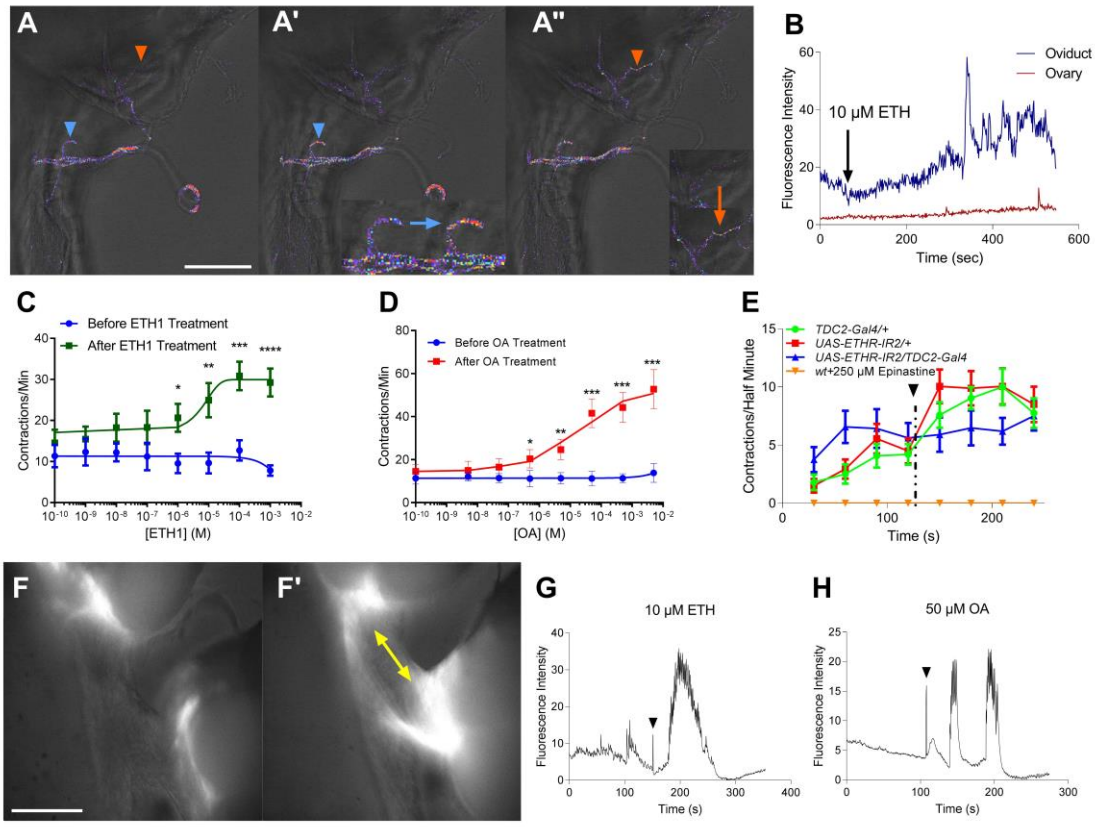
A
A'
A"
B
Oviduct
Ovary
10 μM ETH
Fluorescence Intensity
Time (sec)
C
Before ETH1 Treatment
After ETH1 Treatment
Contractions/Min
[ETH1] (M)
D
Before OA Treatment
After OA Treatment
Contractions/Min
[OA] (M)
E
TDC2-Gal4/+
UAS-ETHR-IR2/+
UAS-ETHR-IR2/TDC2-Gal4
wt+250 μM Epinastine
Contractions/Half Minute
Time (s)
F
F'
G
10 μM ETH
Fluorescence Intensity
Time (s)
H
50 μM OA
Fluorescence Intensity
Time (s)

Figure 3.2. ETH mobilizes calcium in octopaminergic neurons and evokes contractions of the ovarian peritoneal sheath (**A**) Calcium responses of excised ovaries from mated females of genotype *TDC2-Gal4*;*UAS-GCaMP6S* to ETH. Shortly after ETH1 exposure (10 μM), fluorescence responses are observed in octopaminergic innervation on the oviduct (**A'**, blue arrowhead, before/after blue arrow) and innervation at the base of the ovary (A", orange arrowhead, before/after orange arrow), consistent with calcium mobilization (scale bar = 100 μm). (**B**) Nerve terminal fluorescence intensity from (**A**) over time, before and after treatment. (**C, D**) Contractile responses of ovaries excised from Canton-S females before (Blue) and after ETH (Green, **C**) or octopamine (Red, **D**) treatment at various concentrations. Statistics indicate difference in frequency between the two (n=20). (**E**) Ovarian contractile responses following ETHR silencing in octopaminergic neurons (*TDC2-Gal4/ETHR-IR2*) and genetic controls before and after 10 μM ETH1 treatment indicated by the black arrowhead (n=25). (**F**) Calcium response in ovary epithelium (109-53-Gal4/UAS-GCaMP6S) before (**F**) and during (**F'**) contractions induced by ETH treatment (yellow arrows indicate calcium responses at the base of the ovary; scale bar = 100 μm). (**G-H**) Quantification of ovary fluorescence response to 10 μM ETH (**G,** black arrowhead) and 50 μM octopamine (**H,** black arrowhead) treatment. Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

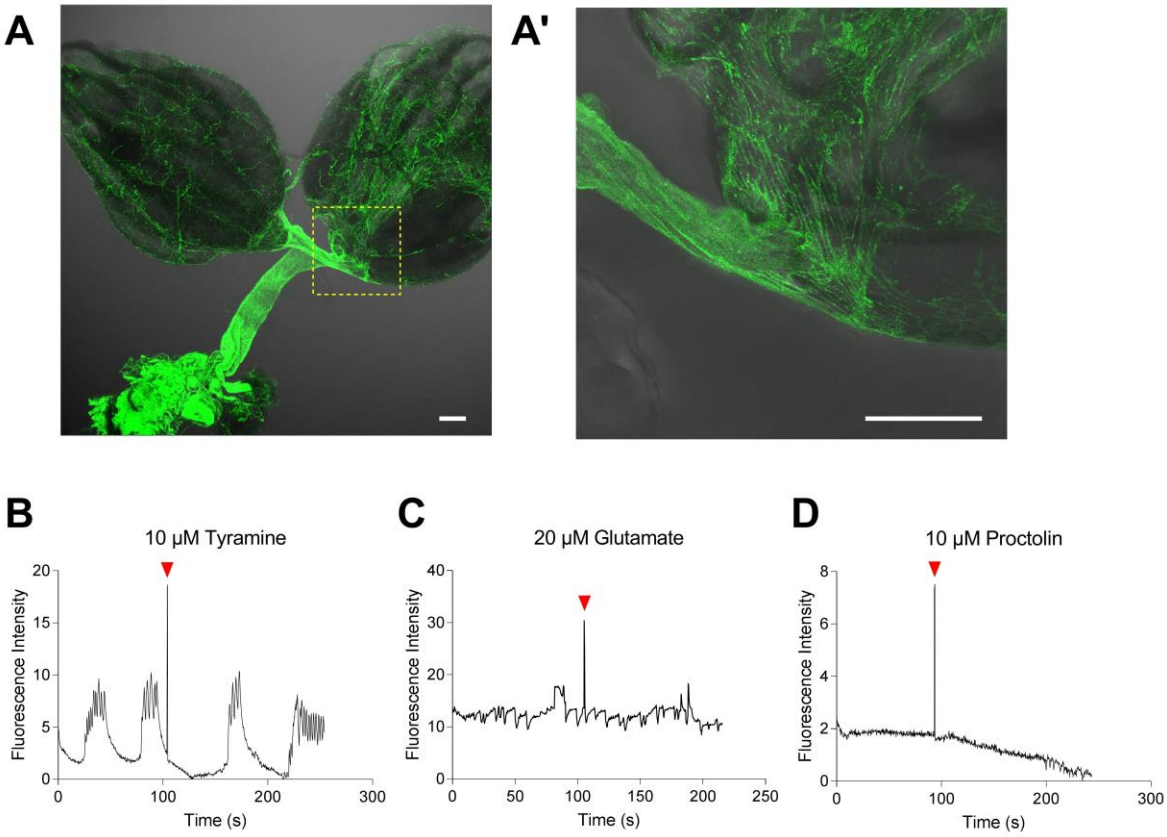

Figure 3.3. Tyramine, proctolin, and glutamate do not stimulate contractions at the base of the ovary (**A**) Expression pattern of 109-53-Gal4/UAS-mCD8-GFP in the female reproductive tract (**A**) and at the base of the ovary (**A'**) (scale bars = 100 µm). (**B-D**) Fluorescence intensity in basal ovary epithelium (*109-53-Gal4/UAS-GCaMP6S*) before and after tyramine (**B**), glutamate (**C**), and proctolin (**D**) at indicated doses (treatment at red arrowheads).

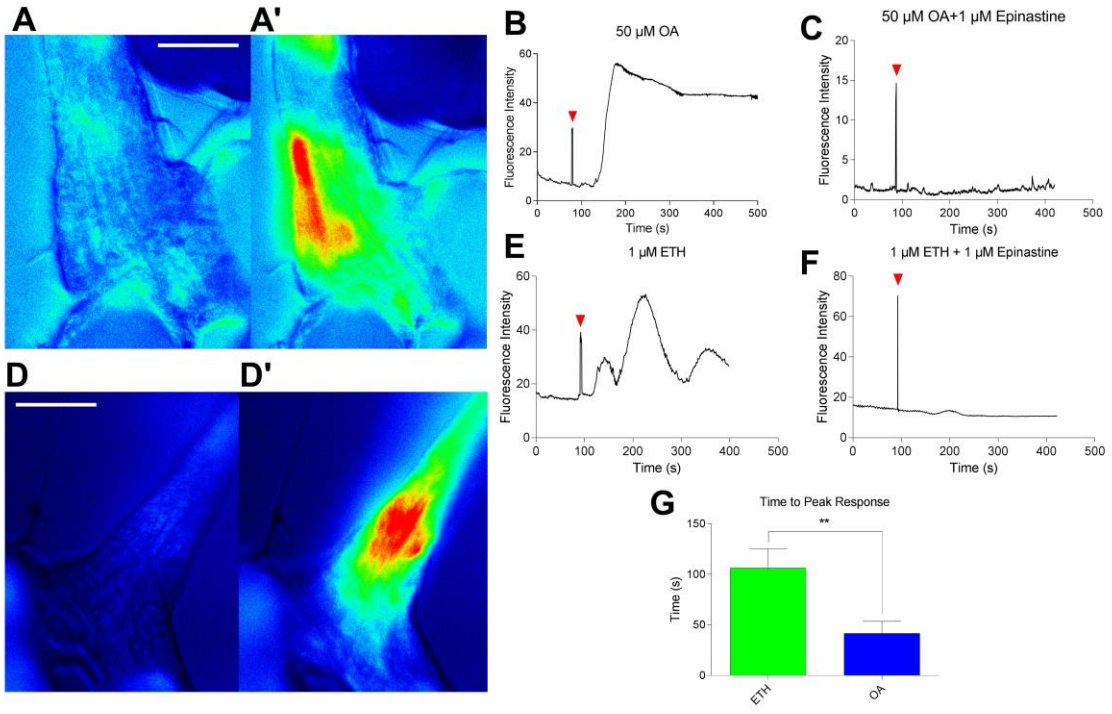

Figure 3.4. ETH mobilizes calcium in oviduct epithelium via octopamine release (**A, D**) Oviducts of *OAMB-Gal4/UAS-GCaMP5* females before (**A, D**) and after 50µM OA (**A'**) and 1 µM ETH (**D'**) treatment (Scale bars 100 µm). (**B, E**) Typical kinetics of the calcium responses to OA (**B**) and ETH (**E**). (**C**, **F**) Kinetics of oviduct calcium response to OA (**C**) and ETH (**F**) preincubated in 1 µM of the OA antagonist epinastine (treatment at red arrowheads). (**G**) Average latency to peak calcium response after ETH and OA treatment (n=10). Error bars represent S.E.M. * $p < .05$.

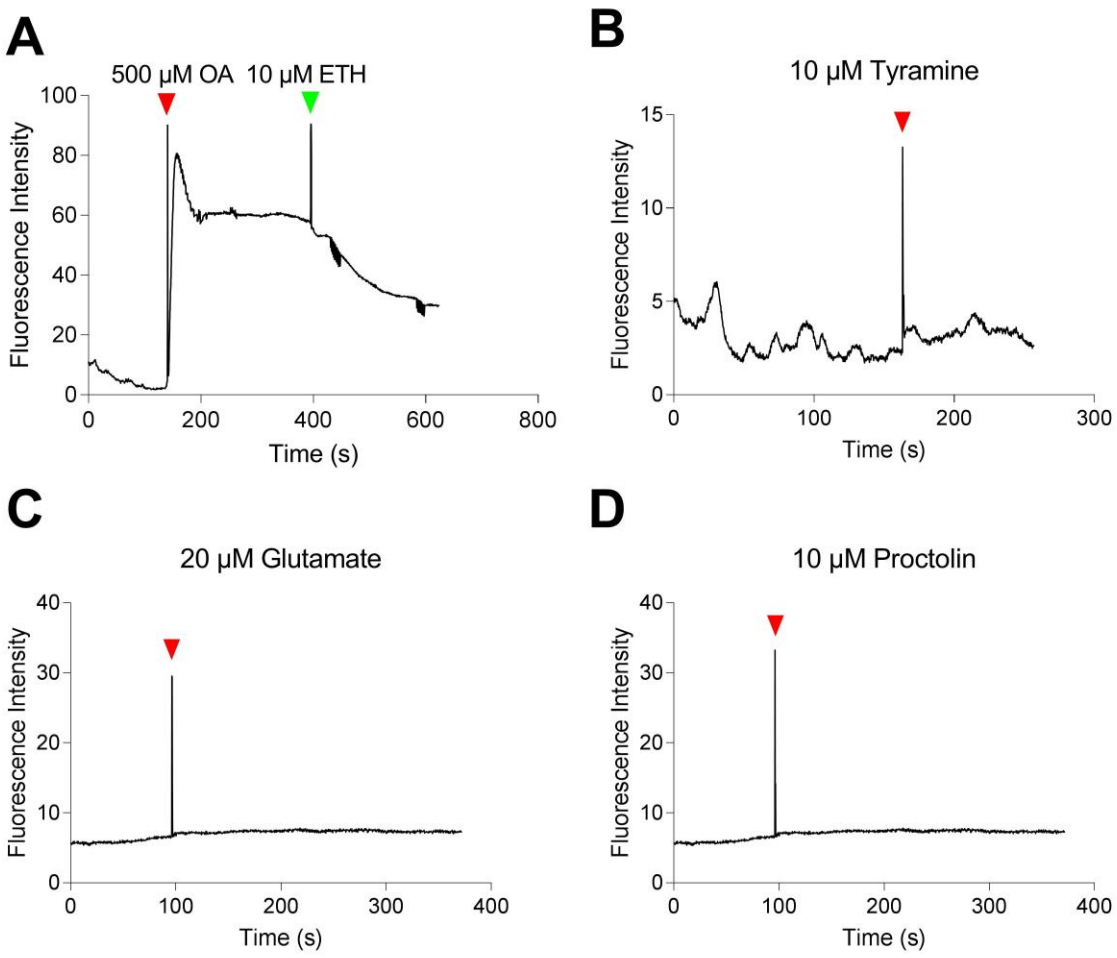

Figure 3.5. OA, but not tyramine glutamate or proctolin cause calcium release in the oviduct epithelium (**A**) Fluorescence intensity of oviduct epithelium (*OAMB-Gal4/UAS-GCaMP6S*) after saturating dose of octopamine (500 µM, red arrowhead) and subsequent ETH treatment (10 µM, green arrowhead). (**B-D**) Oviduct epithelium response to treatment (red arrowheads) with 10 µM Tyramine (**B**), 20 µM Glutamate (**C**), and 10 µM Proctolin (**D**).

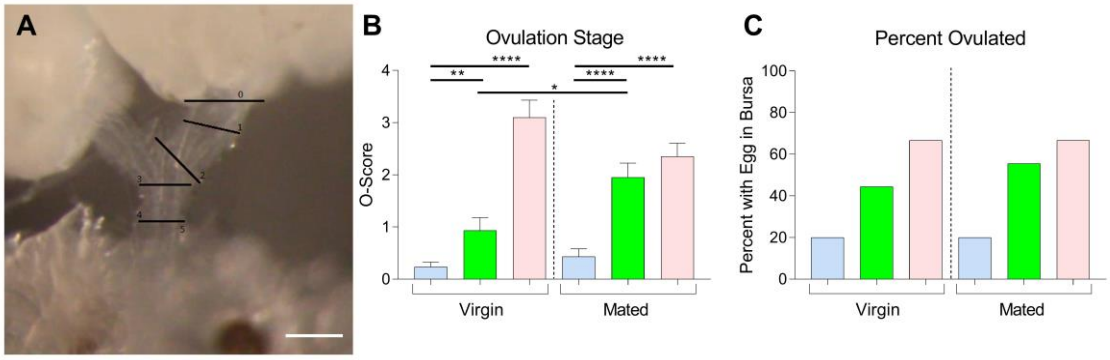

Figure 3.6. ETH activates ovulation *in vivo* (**A**) Scoring system for *in vivo* ovulation. Ovaries were dissected, and oocyte position was scored according to which physiological plane was broken by the tip of the furthest oocyte (scale bar = 100 µm). (**B**) Quantification of oocyte distance in oviducts of females 90 minutes after injection with saline (blue bars), ETH (green bars), or OA (pink bars) (n=20). (**C**) Percent of the same females with an egg present in their bursa (percent ovulated) (n=20). Error bars represent S.E.M. * $p < .05$, ** $p < .01$, **** $p < .0001$.

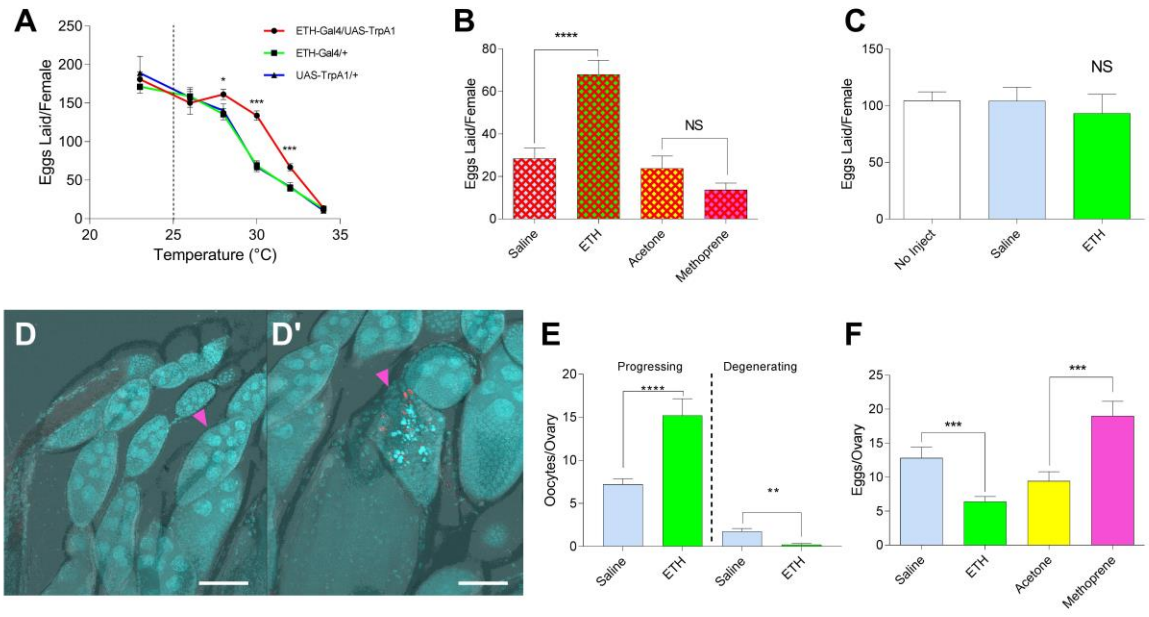

Figure 3.7. Heat stress attenuates fecundity, oogenesis and ovulation, and can be rescued by ETH injection (**A**) Number of eggs laid by *ETH-Gal4/UAS-TRPA1* females incubated at various temperatures for 3 days (n=20-25). (**B**) Mated females exposed for one hour to 38°C and treated immediately thereafter with ETH or methoprene (n=20-30). (**C**) Eggs laid by *Canton-S* females injected with ETH or saline at room temperature (n=10-15). (**D**) Stage 9 progressing (left, pink arrowhead) and degenerating (right, pink arrowhead) oocytes from heat stressed, ETH injected (**D**) or saline injected (**D'**) females, respectively, TUNEL (red) and DAPI (blue) stained (scale bars = 50 µm). (**E**) Quantification of progressing (stage 8/9 intact) and degenerating (stage 8/9 undergoing apoptosis) oocytes in saline and ETH injected females (n=15-20). (**F**) Number of mature eggs retained in ovaries of heat stressed females injected with saline or ETH, or treated topically with acetone alone or acetone and methoprene (n=15-20). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

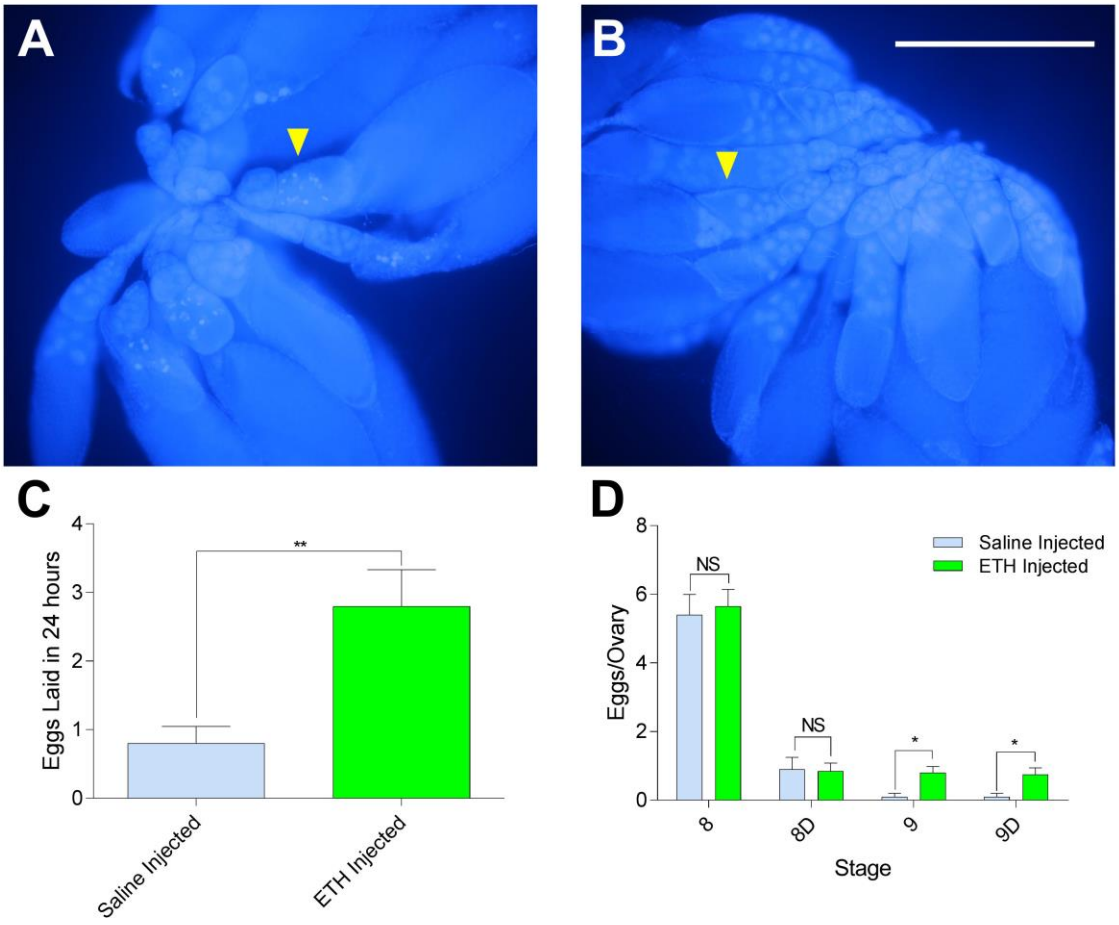

Figure 3.8. ETH injection rescues oogenesis in heat stressed and oogenesis and fecundity in sugar starved, mated females (**A, B**) DAPI-stained ovary from heat stressed, saline injected female (**A**) and one injected with ETH (**B**), mid-oogenetic oocytes, yellow arrowheads (scale bar = 500 µm). (**C**) Egg-laying of *Canton-S* females during 24 hours of sugar starvation after injection with saline or ETH just prior (n=15-25). (**D**) Staging of mid-oogenesis oocytes dissected from ETH or saline injected females after 24 hours of starvation (n=15-20). Error bars represent S.E.M. * $p < .05$, ** $p < .01$.

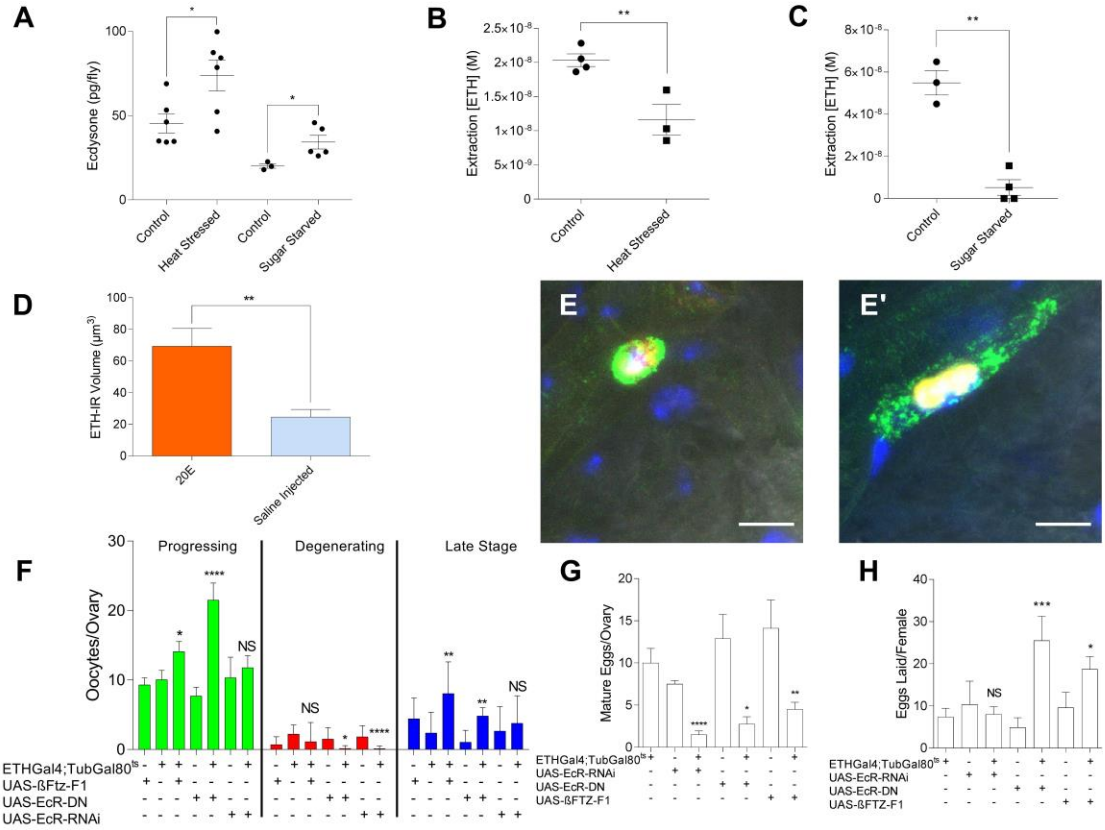
A
Ecdysone (pg/fly)
Control
Heat Stressed
Control
Sugar Starved
B
Extraction [ETH] (M)
Control
Heat Stressed
C
Extraction [ETH] (M)
Control
Sugar Starved
D
ETH-IR Volume (µm³)
20E
Saline Injected
E
E'
F
Oocytes/Ovary
Progressing
Degenerating
Late Stage
NS
ETHGal4;TubGal80ts
UAS-ßFtz-F1
UAS-EcR-DN
UAS-EcR-RNAi
G
Mature Eggs/Ovary
ETHGal4;TubGal80ts
UAS-EcR-RNAi
UAS-EcR-DN
UAS-ßFTZ-F1
H
Eggs Laid/Female
NS
ETHGal4;TubGal80ts
UAS-EcR-RNAi
UAS-EcR-DN
UAS-ßFTZ-F1

Figure 3.9. Ecdysone suppression of Inka cell secretory competence reduces ETH Levels in hemolymph of stressed flies (**A**) Ecdysone levels determined from unstressed/fed flies 24 hours after heat shock, or 24 hours after starvation determined by EIA (n=15 flies per point). (**B, C**) ETH levels determined from hemolymph extractions of unstressed mated females, 24 hours after heat shock (**B,** see Figure 6 Supplement E), and after 24 hours of sugar starvation (**C,** see Fig. 6 Supplement F) determined by enzyme immunoassay (EIA) (n=50 flies per point). (**D**) Volume of ETH immunoreactivity in A4 Inka cells from saline, 20E injected, or heat-stressed females (n=10-20). (**E**) ETH immunoreactivity of Inka cells (green) from *ETH-Gal4/UAS-Redstinger* females 24 hours after injection with saline (**E**) or 36 ng 20-hydroxyecdysone (**E'**)(scale bars = 10 µm). (**F-H**) Heat-stressed females of the genotypes *ETH-Gal4;Tubulin-Gal80^{ts}/UAS-ßFTZ-F1*, *ETH-Gal4;Tubulin-Gal80^{ts}/UAS-EcR-DN*, *ETH-Gal4;Tubulin-Gal80^{ts}/UAS-EcR-RNAi*, and genetic controls scored for oogenesis (**E**) stage 8-9 progressing (green), degenerating (red), and late stage (10-13) oocytes (blue), (**F**) egg retention and (**G)** eggs laid during the three days after heat shock (n=15-30). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

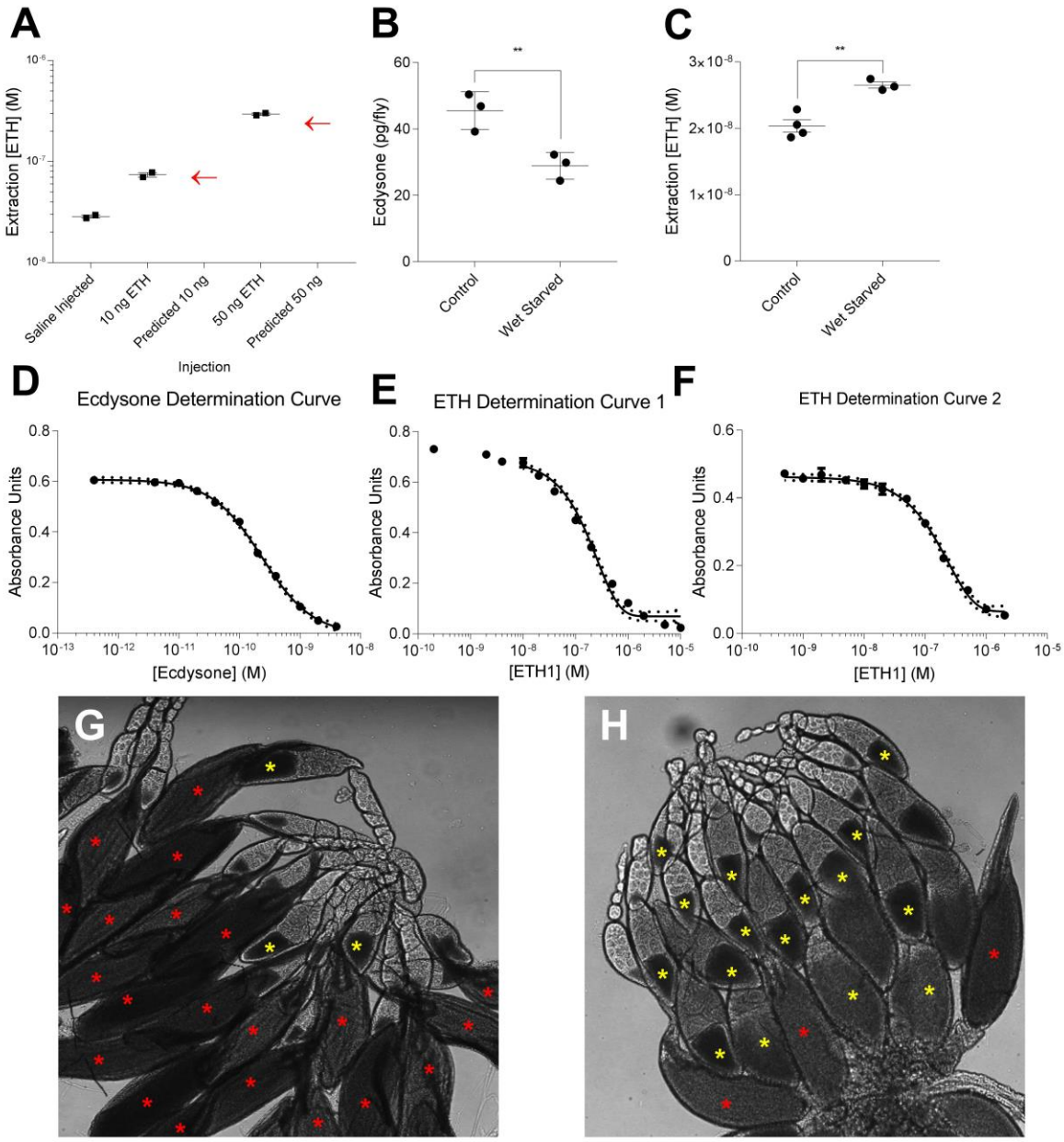
A
Extraction [ETH] (M)
Saline Injected
10 ng ETH
Predicted 10 ng
50 ng ETH
Predicted 50 ng
Injection
B
Ecdysone (pg/fly)
Control
Wet Starved
C
Extraction [ETH] (M)
Control
Wet Starved
D
Ecdysone Determination Curve
Absorbance Units
[Ecdysone] (M)
E
ETH Determination Curve 1
Absorbance Units
[ETH1] (M)
F
ETH Determination Curve 2
Absorbance Units
[ETH1] (M)
G
H

Figure 3.10. Enzyme immunoassay validation, hormonal state change with wet starvation, and genetic rescue of heat stress-induced oogenesis arrest (A) EIA after injection of saline, 10 ng or 50 ng ETH, along with predicted concentrations based on relative quantity injected (red arrows). Recovery was found to be ~2% of the predicted ETH concentration in the hemolymph, assuming a total blood volume of 1 μl. (**B-C**) Hemolymph ecdysone **(B)** and ETH **(C,** ETH determination Curve 1) levels in unstressed and wet starved females. **(D-F)** EIA standard curves used for quantification of ecdysone (**D**) and ETH (**E-F**) levels from Figure 6 A-C, respectively (r square > 0.99). (**G-H**) Example ovaries from heat stressed females of the genotype *UAS-ßFTZ-F*1 (**G**) and *ETH-Gal4;TubulinGal80^{ts}/UAS-ßFTZ-F1*(**H**). Red arrowheads indicate mature eggs retained, while yellow arrowheads indicate vitellogenic, progressing oocytes (scale bars = 500 μm).

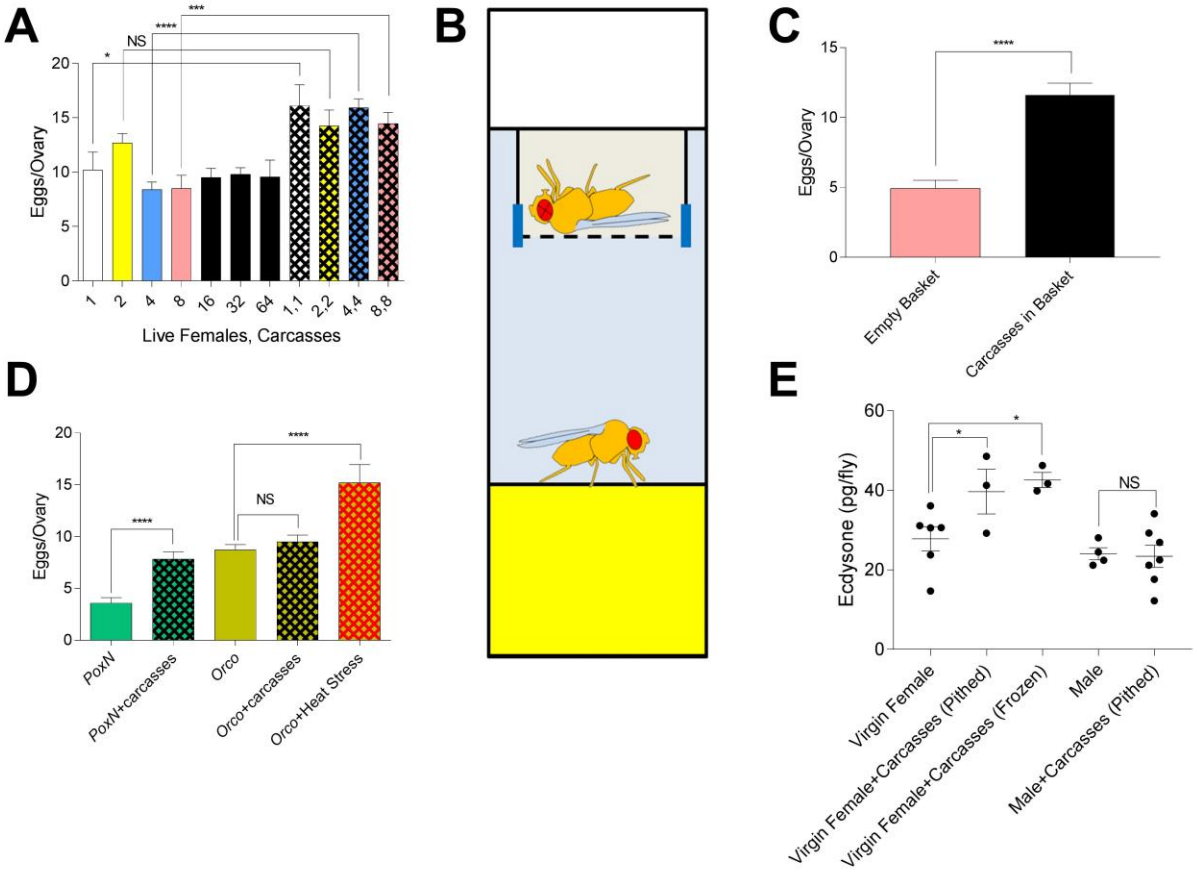
A
B
C
D
E
Eggs/Ovary
Live Females, Carcasses
1
2
4
8
16
32
64
1,1
2,2
4,4
8,8
NS
*
****
***
Empty Basket
Carcasses in Basket
PoxN
PoxN+carcasses
Orco
Orco+carcasses
Orco+Heat Stress
Ecdysone (pg/fly)
Virgin Female
Virgin Female+Carcasses (Pithed)
Virgin Female+Carcasses (Frozen)
Male
Male+Carcasses (Pithed)

Figure 3.11. Carcass-derived scent causes an increase in ecdysone levels and in egg retention

(**A**) Number of mature eggs in the ovaries of virgin females kept in a 9.25cm height 2.2cm diameter food vial with the indicated number of live females or live females, female carcasses. (**B**) Schematic of vials with separation baskets to isolate live females from tactile, gustatory, and visual sensing of carcasses. (**C**) Egg retention in females raised in a vial with carcasses separated by a cheesecloth basket, or the basket alone. (**D**) Egg retention from females deficient in gustation (Δ*PoxN*) and olfaction (Δ*Orco*) (n=20). (**E**) Ecdysone levels from virgin females and males exposed to carcasses for 24 hours prior to determination (n=15 per point). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, *** $p < .001$, **** $p < .0001$.

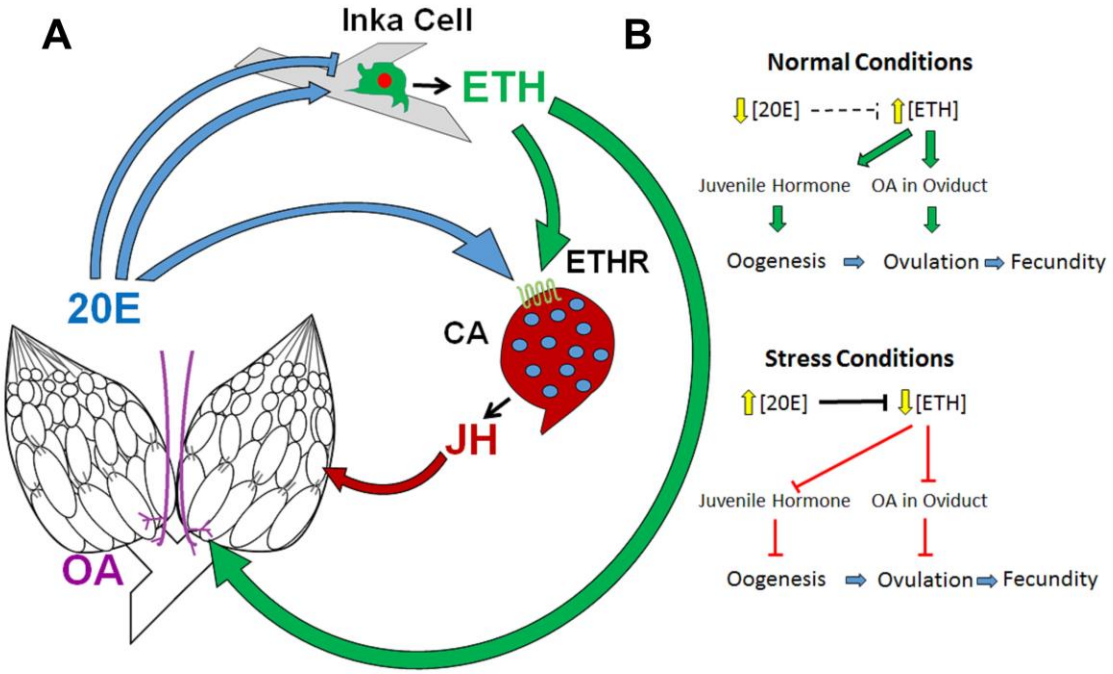

Figure 3.12. Model depicting *Drosophila melanogaster* adult endocrine axis

(**A**) Map showing the direct influences of 20E, ETH, JH and OA on one another and on the female reproductive system. (**B**) A model depicting how circulating ecdysone can regulate fecundity via ETH and its targets. Fecundity depends hierarchically on oogenesis and ovulation.

Chapter 4

Courtship Discrimination Depends Upon Ecdysis Triggering Hormone Signaling

Abstract

The endocrine system allows distal tissue subtypes to respond to global cues transducing information relevant to their function. An individual's decision to engage in courtship is modulated both by external cues from potential mates and internal cues including maturation, health, and experience. Here we show primary sensory neurons critical for mate discrimination in Drosophila melanogaster express the receptor for, and depend on, the peptide hormone Ecdysis Triggering Hormone (ETH). Elimination of the glandular source of ETH, the Inka cells, leads to increased courtship toward both male and female conspecifics. Additionally, Inka cell-ablation completely eliminated post-copulation courtship inhibition (PCCI). Silencing ETHR in several neurons critical for aversive pheromone detection, including OR67D olfactory sensory neurons, GR32A gustatory sensory neurons, and GABAergic antennal lobe interneurons caused context-specific courtship disinhibition. Interestingly, PCCI was predominately dependent on OR67D. In sum, we present evidence that ETH activates a variety of neuronal targets for modulation of adult male courtship behavior.

Introduction

Modulation of sensory perception is critical for prioritization of appropriate

behaviors under varying physiological conditions. In the model organism *Drosophila melanogaster*, a growing body of evidence suggests a variety of internal signals triggered by starvation have been shown to enhance detection of appetitive gustatory(Inagaki, Panse, & Anderson, 2014; Kain & Dahanukar, 2015; Marella, Mann, & Scott, 2012; Y. Wang, Pu, & Shen, 2013), and olfactory (Farhan et al., 2013; Root, Ko, Jafari, & Wang, 2011), ligands. It seems that the other appetitive desire, sexual satiation, is no exception. In mice and in *Drosophila* pheromone receptors depend on hormonal stimulation for timing of activation of receptivity-mediating neurons (Haga-Yamanaka, Ma, He, Qiu, & Lavis, 2014; Lin et al., 2016). Understanding these modulators is a critical foundation for contextualizing drive pathways, whose study has been a cornerstone of modern basic behavioral neuroscience.

Drosophila courtship begins with a series of innately programmed, stereotypical behaviors. Male orientation, chasing, tapping, licking, and singing (one wing extension) are all necessary for a male to detect fitness of his mate and to advertise his own (D. Yamamoto & Koganezawa, 2013). If a female is appropriately stimulated, she will respond by pausing more frequently and allowing a male to attempt copulation. The male steps are critical for interpretation of species and sex conveyance signals, which are largely pheromonal. Two of these pheromones, cis-vaccenyl acetate (cVA) and 7-tricosene (7-T) are present on males and mated females and are critical for the avoidance of each (Dickson, 2008; D. Scott, 1986; L. Wang et al., 2011). In males, these pheromones are sensed by the olfactory receptor OR67D and the gustatory receptor GR32A, respectively. cVA can also be sensed by OR65A, which inhibits OR67D

activity after long-term activation via GABAergic interneurons (Liu et al., 2011).

Here we show that ETH deficiency causes a major perturbation of courtship behavior, marked by general disinhibition of courtship toward both sexes. We identify ETHR-expressing neurons that have critical roles in courtship, focusing on courtship repression, and show the sexual dimorphism of ETHR expression. A subset of these include primary sensory neurons necessary for sensing these pheromones and the interneurons modulating the cVA pathway depend upon Ecdysis triggering hormone (ETH) and its receptor (ETHR). Elimination of either ETH or ETHR specifically in GABAergic interneurons, OR67D, or GR32A-expressing primary sensory neurons led to derepression of general courtship. Further, we show that repression of post-copulation courtship depends upon ETH release at copulation, and more specifically ETH-activation of OR67D neurons.

Results

ETH Deficiency Disinhibits Male Courtship Behvaior

We've recently shown that ETH has a critical role in reproductive behavior of females and the physiology of males during adulthood, and that normal Juvenile Hormone (JH) depend upon ETH and ETHR (Meiselman et al., 2017). As male-female courtship relies depends upon ETH (Wijesekera, Saurabh, & Dauwalder, 2016), we sought to determine whether ETH-deficient males were impaired in mating efficacy. Contrary to expectation, we found ETH deficiency, brought on by either Inka cell ablation or prevention of ETH release with the dynamin mutant *shibire* caused an

increase in male-female courtship, leading to increased copulation success (Figure 1A-B). We found that courtship ETH-deficient males toward conspecific males increased more than five-fold (Figure 1C-D), similar to numbers reported in ecdysone receptor mutant flies (Ganter et al., 2007). Finally, and perhaps most interestingly, we found that courtship toward females did not terminate after successful mating (Figure 1E-F). Courtship of ETH-deficient males appears to be broadly disinhibited.

Courtship inhibition depends upon a variety of cues, including visual, tactile and pheromonal (Krstic, Boll, & Noll, 2009). We sought to determine whether ETH-deficiency caused inability to distinguish conspecifics. We placed Inka cell-ablated males in a 1.5cm diameter courtship chamber with decapitated males, females and virgin females to determine if preference for virgin females was still present in ETH-deficient flies. Interestingly, while the total time performing courtship behaviors toward any subject was significantly elevated in ETH-deficient males (Figure 2A), they still spent more than half of total time courting in pursuit of the decapitated virgin females, which was not significantly different from controls (Figure 2B-C). Taken together, this suggests ETH-deficiency results in a general increase in courtship drive.

ETHR in Antennal Lobe Interneurons Suppresses Male-Male Courtship

In order to determine how ETH was influencing male courtship behavior, we assessed neuronal and glandular targets expressing the ETH receptor. We found that silencing the ETH receptor with the Aug21-Gal4 driver, a label for the source of JH, the corpus allatum (Mirth, Truman, & Riddiford, 2005), resulted in high male-male courtship (Figure 3A). As ETHR silencing in the CA reduces JH levels (Meiselman et al., 2017),

we attempted to rescue JH deficiency with methoprene treatment, but found it insufficient to return male courtship to normal levels (Figure 3A). Next, we tried silencing the ETH receptor with the more-specific JHAMT-Gal4, and eliminating the CA completely by overexpressing the proapoptotic gene *reaper* (Figure 3B). Neither treatment perturbed male-male courtship, suggesting JH levels were unrelated to the observed abnormality.

An examination of the nervous system expression of Aug21-Gal4 showed strong labeling of antennal lobe interneurons (Figure 3C). We noticed that the Trojan ETHR-Gal4 also labeled AL interneurons (Figure 3D), which appeared to be GABAergic local interneurons neurons necessary for tuning olfactory response to certain odors (Chou et al., 2010). We screened several morphologically similar Gal4 lines, and found silencing ETHR in GMR46E11, Krasavietz and NP3056-Gal4 lines caused strong male-male courtship (Figure 3E-F). These LNs are necessary for habituation to a critical male pheromone, cis-Vaccenyl Acetate (cVA) (Hong & Wilson, 2015; Liu et al., 2011; Tachibana, Touhara, & Ejima, 2015).

We also found evidence that JH has a modulatory influence over male-male courtship. While methoprene rescue of Aug21-Gal4/UAS-ETHRi males didn't resolve in an appreciable decline in overall male-male courtship, it did modulate the courtship profile (Figure 4A). Aug21 and AL LN driven ETHR-silencing resulted in strong courtship during the first three minutes of encountering another male, but while GMR46E11-ETHR-silenced males declined courtship over the course of ten minutes, Aug21-Gal4/UAS-ETHR-RNAi males continued elevated courtship, resulting in an overall higher CI (Figure 4A-B). Comparing the first three minutes of courtship toward

other males (initial courtship) to minutes 7-10 (terminal courtship), R46E11 (local interneuron specific for the AL from the rubin collection) driven ETHR-silenced males displayed learning not observed in Aug21 ETHR-silenced males. However, methoprene treatment elevated initial courtship and slightly diminished terminal courtship (Figure 4C). This modulation may be related to JH's role in courtship initiation and in short-term memory (Wijesekera et al., 2016)(Lee, Accepted, Current Biology).

Sexually Dimorphic ETHR Expression in Primary Sensory Neurons

The Trojan ETHR-Gal4 is a specific driver to ETHR expressing neurons; at least 72% of ETHR-positive neurons respond to ETH treatment with calcium oscillation (Diao et al., 2016). Using this driver, we can visualize and manipulate ETHR-expressing tissue. We labeled ETHR-Gal4 neurons with UAS-mCD8 and noticed labeling of primary sensory neurons, including some known to be sensitive to pheromones. We also noticed clear sexual dimorphism in expression pattern in olfactory and gustatory sensory neurons (Figure 5-6).

The Drosophila antennal lobe contains 43 glomeruli which form one to one connections with olfactory sensory neurons (Laissue & Vosshall, 2008). As a result, and because of the various broad odorant screens that have been performed, it is possible to associate morphology of expression not only with odorants, but with behavioral responses (Hallem & Carlson, 2006). Similarly, gustatory receptors also have unique patterns of expression, and neurons expressing particular GRs can be associated with ligands and function (Kunio Isono, 2010). We focus here on those neurons associated with pheromone sensing and courtship drive (Dahanukar & Ray, 2011).

ETHR-Gal4 Expression in Male Antennal Lobes and Taste Organs

In the female, we find OSNs innervating six glomeruli labeled (Figure 5A-D): OR47B-VA1v, a sensor of female pheromones including methyl laurate (Dweck et al., 2015; Lin et al., 2016), OR67D-DA1 a sensor of the male pheromone cis-Vaccenyl Acetate (cVA) (Kurtovic, Widmer, & Dickson, 2007), OR82A-VA6, a sensor of the dipteran pheromone geranyl acetate (Schlief & Wilson, 2007), OR2A-DA4m, unknown ligand (Laissue & Vosshall, 2008), OR19A/B-DC1, a sensor for valencene, a citrus borne attractant or kairomone that may aid in oviposition site selection (Kohl, Huoviala, & Jefferis, 2015), OR85E/OR33C-VC1, which respond to Ethyl acetate, cyclohexanone and fenchone, plant odors (Laissue & Vosshall, 2008). We also see some antennal lobe interneuron expression (roughly X cell bodies) very little expression in female tarsi, and broad expression in labellar taste neurons (Figure 5D-E).

ETHR-Gal4 Expression in Male Antennal Lobes and Taste Organs

In the male, we also identify 6 OSNs (Figure 6A-C): As in the female, we see OR47B, OR67D, OR82A, OR2A and OR19A/B positive OSNs, though it should be mentioned that OR2A expression is much stronger in the male, but instead of the kairomone receptor OR19A, we find OR42A-VM7, which responds to ethyl butyrate most strongly, but is also involved in gain control of other OSNs in response to a variety of odors that generate weaker responses (Grewal et al., 2014). Note that DL3, black arrow, is not labeled. ETHR-Gal4 also expressed in IR75D neurons which innervate the VL1 glomerulus. We see almost twice the number of AL interneuron cell bodies (Figure 6A), several gustatory receptor neurons in the male forelegs (Figure 6D)(medial and

posterior tarsi had no expression), and labellar expression (Figure 6E).

Pheromone-sensing Primary Sensory Neurons Depend upon ETH Signaling

ETHR is a Gq GPCR whose cellular response is elevated calcium signaling(Park, Kim, Dupriez, & Adams, 2003). As such, in order to find neuronal targets responsible for courtship disinhibition observed in ETH-deficient males, we looked for neurons whose activity suppresses courtship. As ETHR expresses in primary sensory neurons, we silenced ETHR OR67D and GR32A-expressing neurons and examined courtship behavior.

ETHR-silencing in GR32A Gustatory Receptor Neurons Elevates Male-Male Courtship

The two dominant male pheromones, 7-Tricosene and cVA, are sensed by GR32A-expressing taste neurons and by OR67D-expressing OSNs, respectively (Mucignat-Caretta, 2014). We found that males with ETHR silenced specifically in OR67D neurons had normal courtship toward other males (Figure 7A), whereas males with ETHR-silenced using GR32A-Gal4 driver, as well as overlapping bitter drivers GR33A and GR89A-Gal4s, showed a three-fold increase in male-male courtship (Figure 7B). We used the LexA-AOP system to express RFP in GR32A neurons and GFP with the ETHR-Gal4 and examined taste organs to determine if ETHR-expressed in GR32A neurons. We found no overlap in male tarsi (Figure 7C), but were able to detect cell bodies expressing both GR32A and ETHR in the male labellum (Figure 7D). GR32A positive neurons stimulate an aversive response to a variety of bitter tastants, 7-Tricosene in particular has been shown to elicit an electrophysiological response in short hairs of the labellum, including S6 (Lacaille et al., 2007). We tested S6 in genetic control males, and those

with ETHR-silenced, and found several concentrations of denatonium, one such bitter compound, elicited fewer action potentials with a longer latency to response (Figure 7E-F). This suggests ETHR deficiency results in diminished sensitivity to ligands in primary sensory neurons, likely including pheromones.

ETHR Silencing in OR67D and GR32A-expressing neurons elevates Post-Copulatory Courtship Behavior

One of the most intriguing behaviors of ETH deficient males was a complete absence of post-mating refractoriness (Figure 8A). After dismounting, males typically court much less than prior to mating, with less exploratory behavior, and only short (<5 seconds) bouts of courtship concluded with a termination of chasing, followed quickly by grooming of the sense organs. ETH deficient males continue progression through stereotypical courtship behavior at a rate similar to that shown prior to copulation, with long courtship bouts, uninterrupted by sensory organ grooming (Figure 8B). Drosophila males typically detect matedness by the presence and absence of cVA, a volatile, male-specific pheromone synthesized in ejaculatory bulbs and deposited onto females during mating (Butterworth, 1969; Jallon, Antony, & Benamar, 1981). 7-Tricosene is canonically present on males, but is transferred to males during mating(D. Scott, 1986). To determine whether sensation of either pheromones played a critical role in this behavior, we examined GR33A and OR67D null mutants for post-copulation courtship index (PCCI) (Figure 8C)(Kurtovic et al., 2007; Moon, Lee, Jiao, & Montell, 2009). We found that OR67D^{-} mutant showed a strong and significant increase in post-copulation

courtship behavior. Next, we silenced ETHR in GR32A and OR67D-expressing neurons and pan-neuronally and found that ETHR-silencing in each region causes increases in both post-copulation courtship (Figure 8D) and in extended courtship bouts (>5 seconds) (Figure 8E), OR67D-driven silencing having the strongest effect. Additionally, we found that treatment of nerve terminals innervating the DA1 glomerulus responded to 10μM ETH treatment with a rapid, sustained elevation of calcium levels (Figure 8F).

Discussion

We show here that the peptide hormone ETH is necessary for courtship inhibition and detection of multiple aversive pheromones, suggesting a broad role for the peptide in males as a courtship regulator. Our data support the model that normal courtship depends upon ETH levels in the hemolymph. At low levels of ETH or with specific inactivation of the ETH receptor, males are less responsive to courtship inhibitory pheromones, both at the level of the primary sensory neuron response and in behavior.

ETH deficiency causes a general elevation of male courtship behavior, while not perturbing ability to differentiate between sexes. It was curious discrimination was unimpeded in ETH deficient males, though it is possible that visual cues may be a stronger determinant of courtship targets at close range when volatile cues are mostly shared by decapitated targets. While ETHR does express in primary photoreceptors, no role has yet been attributed to this expression, and total dependency is unlikely. General courtship could be elevated by hyperactivity, and ETH deficient flies are hyperactive, but we found that JH-deficient flies were as well, yet showed no increase in

male-male courtship (Meiselman, unpublished).

ETHR expression and apparent antagonistic influences over courtship behavior

We find ETHR in a variety of pheromone-sensing neurons, which, interestingly, detect both male and female pheromones. Pheromones are critical for distinguishing males from females, young from old, and mated from virgins(Kuo et al., 2012; Mucignat-Caretta, 2014). It is therefore counter-intuitive to find that critical tools for making these assessments respond similarly to the same endocrine cue. We find ETHR in GR32A and OR67D, sensors of pheromones present on males and mated females, and inhibitors of courtship behavior when their ligands are present. We also find ETHR in OR47B, a sensor of female pheromones and a courtship stimulator, whose signaling is necessary for the courtship advantage of older males over younger. Moreover, we find that within a critical pathway for cVA sensing, ETHR expresses on neurons that have opposing roles on behavior.

cVA is a Drosophila male pheromone that can be sensed by both OR67D and OR65A-positive OSNs (Ha & Smith, 2006; Kurtovic et al., 2007; van der Goes van Naters & Carlson, 2007). OR65A-expressing neurons are responsible for initial courtship response to cVA and act via lateral inhibition of OR67D neurons via NP3056 and Krasavietz positive antennal lobe interneurons (Ejima et al., 2007; Liu et al., 2011). Our expression of ETHR in both OR67D and in GABAergic neurons that inhibit OR67D in habituation is, again, seemingly conflicting.

Additionally of interest, we found ETHR to express in GRNs of male forelegs, critical for courtship behavior, though expression did not overlap with GR32A neurons. The next most likely candidates are GR68A and ppk23 (Bray & Amrein, 2003; Lu, LaMora, Sun, Welsh, & Ben-Shahar, 2012), the latter being more likely than the former as ETHR overlaps with fruitless expression in neurons including OR67D and OR47B, and GR68A does not overlap with fruitless (Shankar, Chua, Tan, Calvert, & Weng, 2015).

As the role of ETH in the female, as a facilitator of egg-laying, is clearer than in the male, it would be interesting to test whether ETHR expression in OSNs that sense fruity odors is a critical component of oviposition site-seeking behavior.

ETHR is a Gq-coupled GPCR, which stimulates calcium release in receptor-expressing tissue. It it possible that elevated calcium concentration may decrease the ionic gradient across neuron membranes and make action potentials more likely to fire. In primary sensory neurons, it is therefore no surprise that ETHR deficiency increases latency to firing and decreases spike count in GR32A neurons. Assuming this principle extends to all primary sensory neurons in which ETHR expresses, ETH could cause hypersensitivity (or maintain normal sensitivity) to pheromone ligands. However, preliminary data from OR67D neurons does not agree with this hypothesis, despite their ability to respond to ETH. Context may suggest different modulatory dynamics in the OSNs and gustatory receptors.

Post-copulation courtship inhibition

Here we describe courtship behavior immediately following copulation behavior for the first time. Males typically avoid mated females and are slightly hypoactive. ETH signaling impairment ablates this behavior, and males continue to court as if they had not mated. We show that this behavior predominantly depends upon OR67D, the male OSN necessary for sensing cVA. OR67D null mutants and OR67D-ETHR-silenced males show greatly increased duration and frequency of courtship bouts during the 10 minute window immediately following copulation. It is interesting that males show no male-male courtship occurs in OR67D-ETHR-silenced males, which would be expected in flies in which OR67D neurons did not function (Kurtovic et al., 2007). As the absence of ETHR in OR67D neurons is only functionally significant after mating, it suggests that the neurons are intact and functional, but a post-copulation change in ETH signaling is critically missed. This may suggest a post-copulatory release of ETH, but this remains to be elucidated.

The context of male ETH signaling

ETH deficiency results in broadly elevated courtship, as does broad ETHR silencing. ETH generally seems to have a courtship inhibitory role, but injection of ETH or stimulation of ETH release from Inka cells with either TrpA1 or ReAChR did not significantly depress courtship behavior (data not shown). We found that chronic and acute heat stress, known to depress ETH levels in the female (Meiselman, Unpublished), had no effect on male-male courtship or PCCI. The implication is that the male role for ETH is that it does not activate tissues in an acute fashion as we see in female ovulation.

Another suggestion is that ETH is a facilitator of development of the male courtship systems, indeed, JH, which depends upon ETH levels is necessary for the maturation of OR47B (Lin et al., 2016). However, we found that injection of high levels of ETH into day 1-2 males did not accelerate courtship competence (Data not shown). Moreover, we found that male mating changed transcript levels of ETH genes. We expect the ETH signaling cascade acts as a "readiness" signal. Though radically different in structure and signaling pathways, testosterone in humans may have a similar role. We found that isolated males continuously increase expression of all ETH genes until roughly the period of sexual maturity (Day 10).

Androgen receptor is present in mammalian neurons for the execution of sexual behaviors, and those behaviors are dependent upon them (Juntti, Tollkuhn, Wu, Fraser, & Soderborg, 2010). However, exogenous testosterone to eugonadal mice has only subtle effects, with no perturbation of courtship behavior (Celec, Ostatníková, & Hodosy, 2015). Also similar to ETH medium term abstinence from ejaculation increases testosterone levels (M. Jiang, Jiang, Zou, & Shen, 2003), whereas long term abstinence suppresses testosterone (Jannini et al., 1999), phenomena we observe with the ETH signaling genes (observed in Meiselman et al and Figure 8F).

The Role of ETH in Courtship Regulation

We've previously shown that ETH is a status quo signal: Circulating ETH levels in the female are depressed when steroid levels elevate in response to various environmental stressors to coordinate egg production with environment. We were unable to perturb courtship behavior with several stresses, including chronic or acute heat stress, or wet

starvation (data not included). Indeed, the suggestion that a drop in ETH levels could occur with stress would by extension elevate courtship toward conspecifics, a seemingly inappropriate response, as in many other species, stress has an inhibitory effect on male libido (Chenoweth, 1981; Hanafy & Khalil, 2015; Lenzi, Lombardo, Salacone, Gandini, & Jannini, 2003; Marai, Habeeb, & Gad, 2002).

One intriguing role for ETH to manipulate target courtship regulatory systems is at the conclusion of mating. While it is unclear whether or on what time scale endocrine state is perturbed following mating, we show here that ETH signaling interruption causes a severe disruption of post-mating behavior. While the male bears less cost of reproduction, mating does deplete stores of sperm and accessory gland proteins. Accessory gland proteins are quite notable, as their rate of synthesis depends upon JH, which in turn relies upon ETH signaling (Meiselman et al., 2017; K. Yamamoto, Chadarevian, & Pellegrini, 1988). Indeed, in the closely related male carribean fruit fly *Anastrepha suspensa*, JH levels increase after mating (Teal, Gomez-Simuta, & Proveaux, 2000). While JH levels increase in females as well (Sugime et al., 2017), male increases occur without the aid of sex peptide, the only other allatotropin known to influence CA activity (Areiza, Nouzova, Rivera-Perez, & Noriega, 2014; Moshitzky et al., 1996). As we've detected steroid levels increase immediately after mating in males (Figure 8F) this may suggest a direct relationship between 20E and ETH levels in adult males, which suggest sexually dimorphic dynamics of ETH release. The regulators of ETH release, ecdysone genes and eclosion hormone (Dalton, Lebo, Sanders, Sun, & Arbeitman, 2009), and the ETH genes exhibit strong sexual dimorphism (Meiselman et al., 2017), so it is

likely principles of dynamics discovered in the female do not translate across sex lines, but these principles remain untested.

We demonstrate here that courtship behavior is critically dependent on ETH signaling, and suggest multiple targets for this dependency. Further work will be necessary to determine the contextual relevance of these findings.

Materials and Methods

Fly Strains

Flies used for immunohistochemistry were raised at 23˚C on standard cornmeal-agar media under a 12:12 hr light:dark regimen. Inka cell and corpus allatum- ablated flies were raised at the Gal80^{ts} permissive temperature (18°C). Following eclosion, they were moved to the nonpermissive temperature (29°C) for 24 hours, then moved to 23°C until day 4. Inka cell-blocked flies were raised at 18ºC until eclosion, and transferred to 28ºC until day 4. Corpora allata-ablated flies were reared as described previously (R. Yamamoto, Bai, Dolezal, Amdam, & Tatar, 2013) at 29°C, isolated prior to eclosion, and transferred to isolated chambers held at 23°C prior to mating and fecundity analysis. The JHAMT-Gal4 fly line has been described recently (Wijesekera et al., 2016). Use of double-stranded RNA constructs for silencing of ETHR (UAS-ETHR-Sym; UAS-ETHR-IR2 line (VDRC transformant ID dna697) were described recently (D.-H. Kim et al., 2015). Aug21-Gal4 flies were obtained from Dr. Korge (Freie Universität Berlin). Interneuron Gal4 lines (LCCH-Gal4, NP3056-Gal4, Krasavietz-Gal4, NP6277-Gal4, and

HB4-93-Gal4) were obtained from Dr. Liqun Luo (Stanford University). ETHR-Gal4 was obtained from Dr. Ben White (National Institute of Mental Health, Silver Spring). GR32A-LexA, GR33A^{-}, and AOP-mCherry were obtained from Dr. Anupama Dahanukar (University of California-Riverside). OR67D^{-} was obtained from Dr. Anandasankar Ray (University of California-Riverside). All other fly lines were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN): UAS-Red Stinger (BS no. 8574), UAS-mCD8-GFP (BS no. 5137), UAS-CD4-tdGFP (BS no. 35836), UAS-rpr (BS no. 8524), UAS-GCaMP6S (BS no. 42746), TubP-Gal80^{ts} (BS no. 7017), ETH-Gal4 (BS no. 51982), GMR46E11-Gal4 (BS no. 50272). All flies used for behavior experiments were backcrossed for at least five generations into the Canton-S background.

Courtship Assays

Male-male and male-female courtship

For all behavior experiments, males were isolated prior to eclosion in culture tubes with food. These naive day 5 adult male flies were put in a 1cm in diameter courtship chamber with a wild type Canton-S male or virgin female subject and observed. In the case of male-male courtship, test males were scored for stereotypical courtship behaviors and index was created as a measure of time performing those behaviors out of 10 minutes. For male female courtship, time of copulation was recorded, and percentage copulated was assessed for each minute in the first ten minute interval. Those flies that did copulate were allowed to dismount, and then the male was observed and scored for courtship for the ten minutes immediately following the dismount. Extended courtship

bouts measures only the bouts lasting greater than 5 seconds in length. All scoring was performed single blind.

Competition Assay

Males were placed in a 1.5 cm mating chamber with a decapitated mated female, a male, and a virgin female and recorded for 20 minutes. Time spent performing courtship behaviors toward each individual was tabulated and represented both as a sum and a percentage of total time spent courting.

Immunohistochemistry

We crossed *ETHR-Gal4* transgenic flies with *UAS-mCD8-GFP* flies to produce progeny expressing GFP in ETHR-expressing cells for immunohistochemical staining. Day 4 adults were dissected in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS overnight at 4˚C. After washing with PBS-0.5% Triton X-100 (PBST) five times and blocking in 3% normal goat serum in PBST for 30 minutes at room temperature, samples were incubated with anti-NC82 (Sigma, 1:1000) anti-GFP antiserum (Invitrogen, 1:1000) dilution in PBST for 2 days at 4˚C. Tissues were washed with PBST three times, incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen), Alexa Fluor 635-labeled Goat anti-mouse (Life Technologies), 0.5 mg/ml DAPI, and 2% NGS overnight, and washed 4 times for 10 minutes each in PBST before imaging. Immunofluorescence was recorded using a confocal microscope (Leica model

SP5) with FITC filter in the Institute of Integrative Genome Biology core facility at UC Riverside.

Electrophysiology

Single-sensillum recordings were performed using the tip-recording method(Benton & Dahanukar, 2011) with 30 mM tricholine citrate as the electrolyte(Wieczorek & Wolff, 1989). Recordings were obtained from male flies aged 4-5 days. Neuronal responses were quantified by doubling the number of spikes in the 0–500 ms window upon contact with the stimulus.

Calcium Imaging

Whole brains from group-raised, mature (day 4-6) males were dissected and immediately placed in 180 µl of fly saline with 50mM Glucose and 50 µM Zn^{2+}. After 100 seconds, 20 µl of 50 µM ETH was added to the bath. We used an imaging setup consisting of a Polychrome V monochromator (TILL Photonics/FEI) as light sources and a TILL Imago CCD camera. The microscope (Olympus Model BX51WI) was equipped with a 40x W NA 0.8 objective. Binning on the chip (8 X 8) was set to give a spatial sampling rate of 1 µm/pixel (image size 172 X 130 pixels, corresponding to 172 µm and 130 µm). Images were taken at a rate of 4 Hz. The excitation wavelength was 488 nm, and exposure time was 25 msec. Fluorescent light passing an excitation filter (370-510 nm) was directed onto a 500 nm DCLP mirror followed by a 515 LP emission filter for EGFP. 5 minutes of continuous images were acquired from each CNS preparation and ETH was applied into a bathing media ~5 min after imaging onset.

Methoprene Treatment

Within 24 hours of eclosion, adult males or females were cold anesthetized and treated topically on the dorsal side of the abdomen with 72 (females) or 36 (males) nl of either acetone, or 0.01% methoprene dissolved in acetone (~300 nM). The entire procedure took under 20 minutes, after which flies were returned to their housing.

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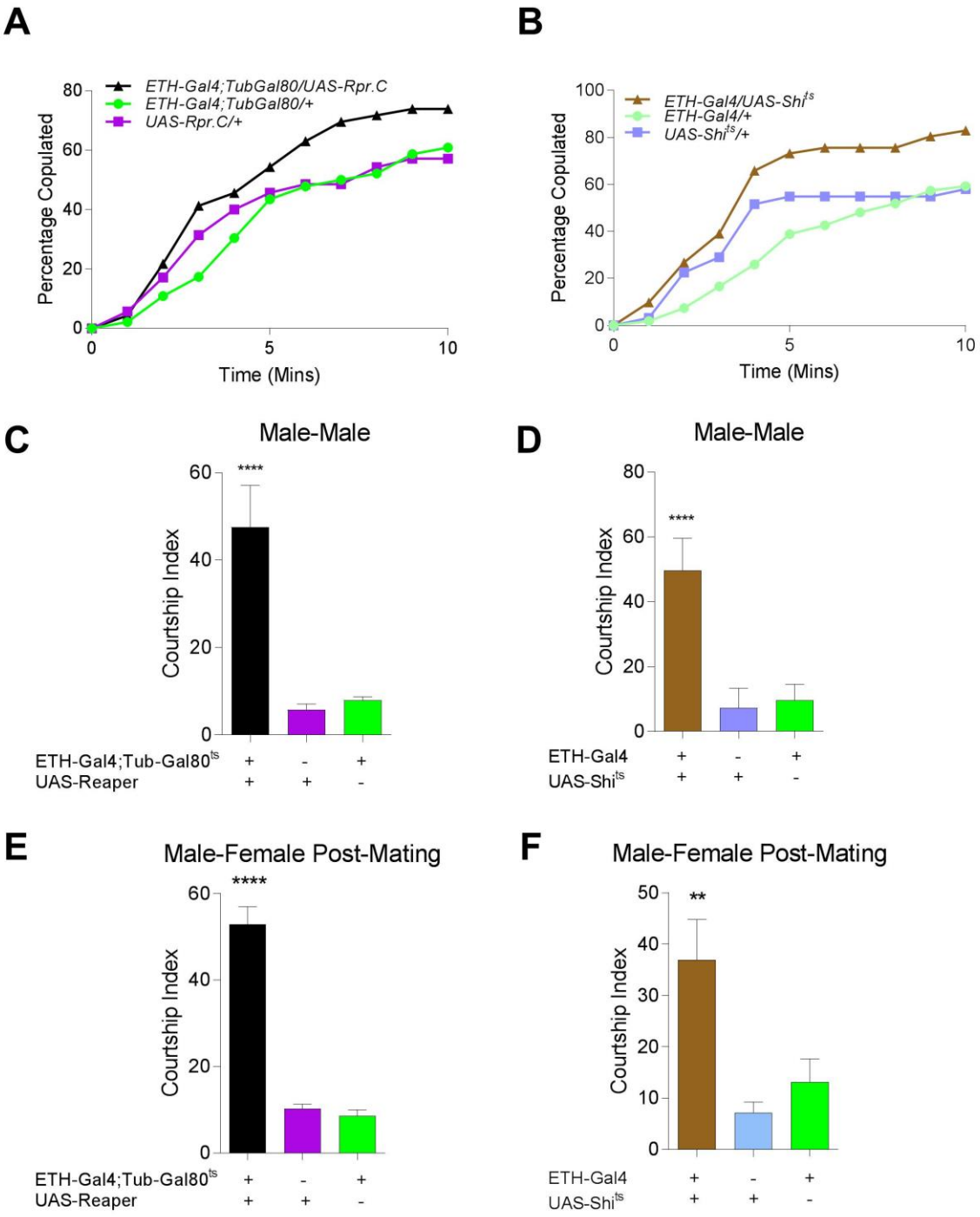
A
ETH-Gal4;TubGal80/UAS-Rpr.C
ETH-Gal4;TubGal80/+
UAS-Rpr.C/+
Percentage Copulated
Time (Mins)
B
ETH-Gal4/UAS-Shi^ts
ETH-Gal4/+
UAS-Shi^ts/+
Percentage Copulated
Time (Mins)
C
Male-Male
Courtship Index
****
ETH-Gal4;Tub-Gal80^ts
UAS-Reaper
D
Male-Male
Courtship Index
****
ETH-Gal4
UAS-Shi^ts
E
Male-Female Post-Mating
Courtship Index
****
ETH-Gal4;Tub-Gal80^ts
UAS-Reaper
F
Male-Female Post-Mating
Courtship Index
**
ETH-Gal4
UAS-Shi^ts

Figure 4.1. ETH deficiency elevates male courtship behavior toward virgin females, males, and mated females (A-B) Minute by minute cumulative totals of males of indicated genotypes (A) Inka cell-ablated, *ETH-Gal4;TubulinGal80^{ts}/UAS-Reaper*, (B) Inka cell-Blocked, *ETH-Gal4/UAS-Shibire^{ts}*) and genetic controls with wild-type females (n=50-60). (C-D) Male courtship index (time spent courting over total time, 600s) toward a wild-type males for Inka cell-ablated (C), Inka cell blocked (D), and genetic controls (n=15-20). (E-F) Post-mating courtship index (time spent courting over total time starting at dismounting, 600s) toward a wild-type females for Inka cell-ablated (C), Inka cell blocked (D), and genetic controls (n=20-30). Error bars represent S.E.M. NS, ** $p < .01$, **** $p < .0001$.

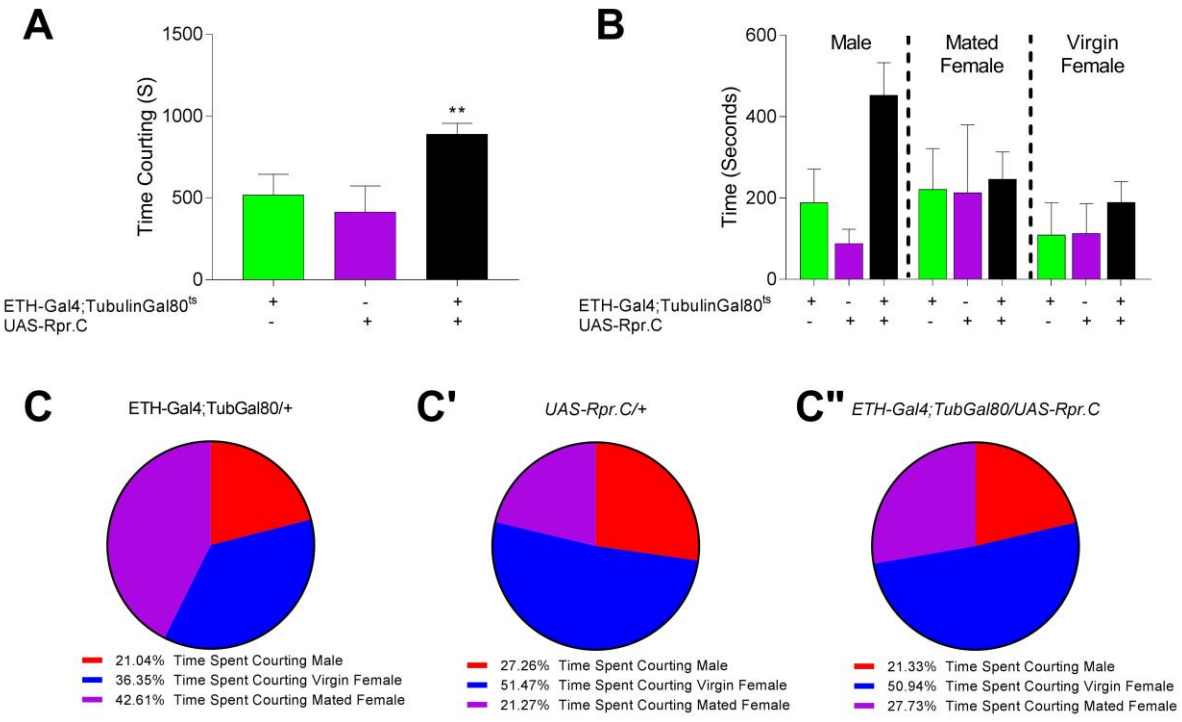

Figure 4.2. Distribution of courtship behavior in a competition assay (A) Total time spent performing courtship behaviors toward any subject for Inka cell-ablated (ETH-Gal4;Tubulin-Gal80^{ts}/UAS-Reaper) males and genetic controls (n=10-25). (B) A representation of the data from (A) showing time spent courting indicated subject. (C) Pie graph representation of total time spent courting for Gal4 control (C), UAS control (C'), and Inka cell-ablated males. Error bars represent S.E.M. ** $p < .01$.

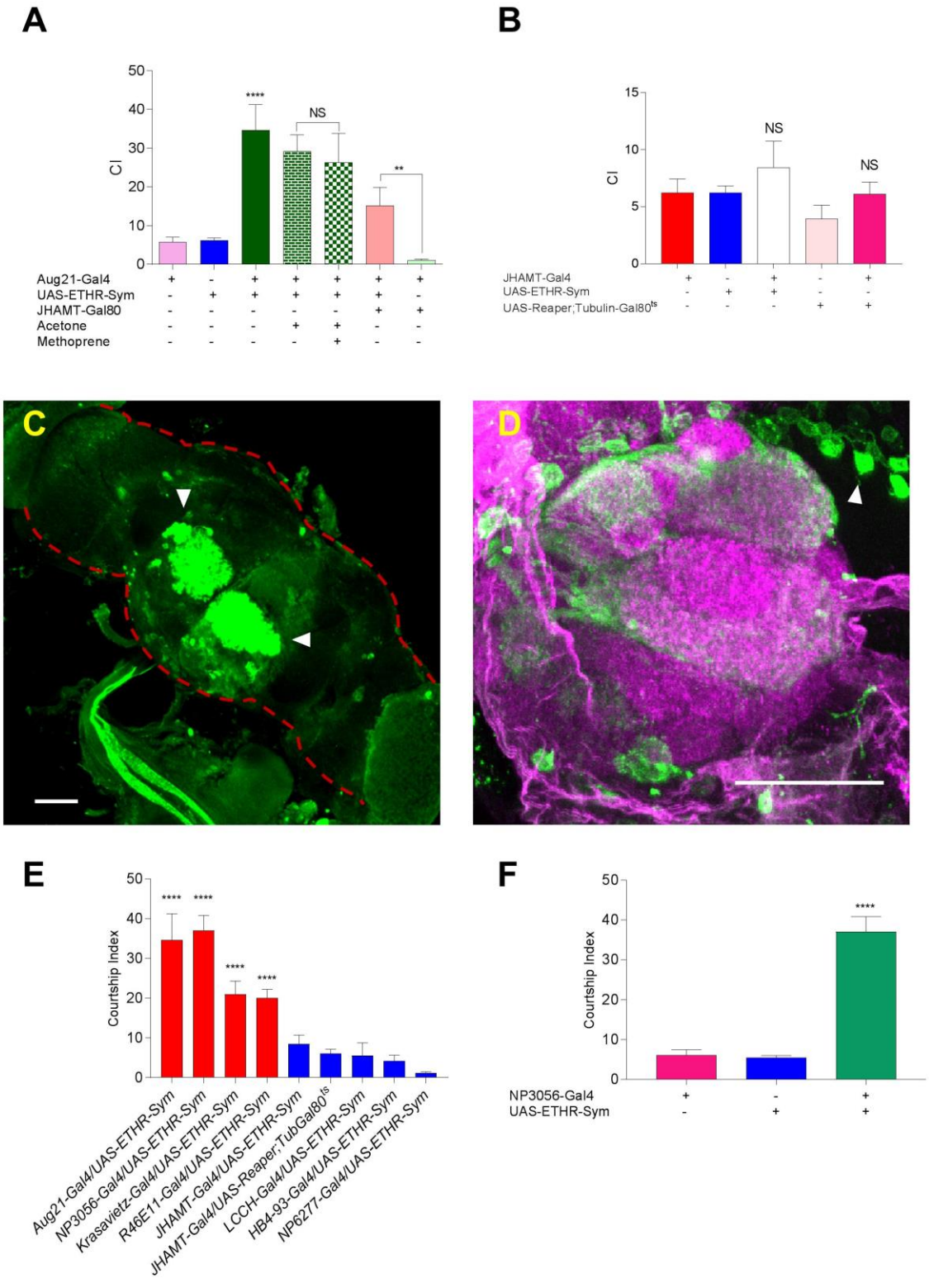
A
CI
****
NS
**
Aug21-Gal4
UAS-ETHR-Sym
JHAMT-Gal80
Acetone
Methoprene
B
CI
NS
NS
JHAMT-Gal4
UAS-ETHR-Sym
UAS-Reaper;Tubulin-Gal80ts
C
D
E
Courtship Index
Aug21-Gal4/UAS-ETHR-Sym
NP3056-Gal4/UAS-ETHR-Sym
Krasavietz-Gal4/UAS-ETHR-Sym
R46E11-Gal4/UAS-ETHR-Sym
JHAMT-Gal4/UAS-Reaper;TubGal80ts
JHAMT-Gal4/UAS-ETHR-Sym
LCCH-Gal4/UAS-ETHR-Sym
HB4-93-Gal4/UAS-ETHR-Sym
NP6277-Gal4/UAS-ETHR-Sym
F
Courtship Index
NP3056-Gal4
UAS-ETHR-Sym

Figure 4.3. ETHR in Aug21-labeled antennal lobe interneurons inhibits male-male courtship (A) Male courtship index toward wild-type males for Aug21-Gal4/UAS-ETHR-Sym and genetic controls, as well as courtship indices from methoprene and acetone and acetone alone treated Aug21-Gal4/UAS-ETHR-Sym males (n=10-20). (B) Male courtship index for JHAMT-Gal4/ETHR-Sym and CA-ablated (JHAMT-Gal4;TubulinGal80^{ts}/UAS-Reaper)(n=10-15). (C) CNS expression of Aug21-Gal4/UAS-mCD8-GFP. White arrowheads indicate antennal lobe, brain outlined by red-dashed lines, scale bar 50 µm. (D) ETHR-Gal4/UAS-MCD8 expression in the antennal lobe, white arrowhead indicates interneuron cell body, scale bar 20 µm. (E) Male courtship index for screen of AL interneuron Gal4 lines morphologically similar to Aug21-Gal4. Red bar indicates significantly different from respective genetic controls, blue, not significant (n=10-15). (F) Strongest hit (NP3056-Gal4/UAS-ETHR-Sym) from E represented with genetic controls (n=12-15). Error bars represent S.E.M. NS, **** $p <$ .0001.

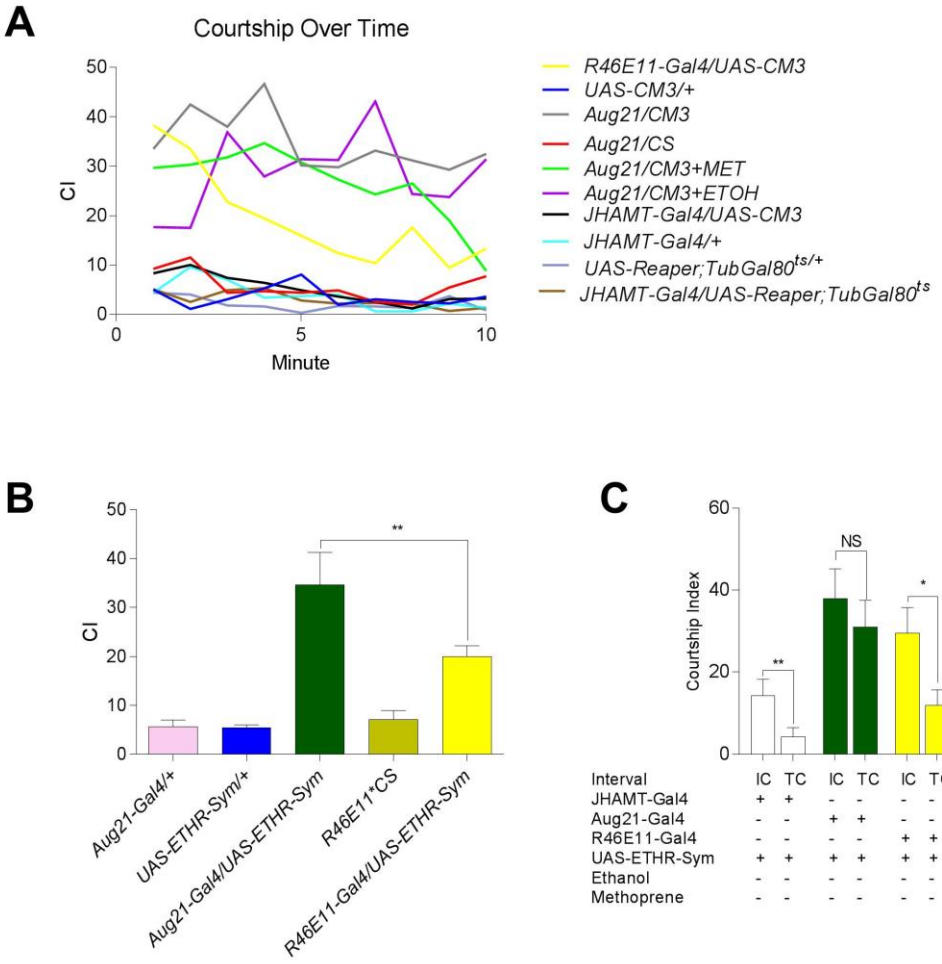
A
Courtship Over Time
CI
Minute
R46E11-Gal4/UAS-CM3
UAS-CM3/+
Aug21/CM3
Aug21/CS
Aug21/CM3+MET
Aug21/CM3+ETOH
JHAMT-Gal4/UAS-CM3
JHAMT-Gal4/+
UAS-Reaper;TubGal80ts/+
JHAMT-Gal4/UAS-Reaper;TubGal80ts
B
CI
Aug21-Gal4/+
UAS-ETHR-Sym/+
Aug21-Gal4/UAS-ETHR-Sym
R46E11*CS
R46E11-Gal4/UAS-ETHR-Sym
C
Courtship Index
NS
Interval
JHAMT-Gal4
Aug21-Gal4
R46E11-Gal4
UAS-ETHR-Sym
Ethanol
Methoprene
IC
TC

Figure 4.4. Examination of courtship profile of Aug21-drive ETHRi (A) Courtship index for every minute during a 10 minute window for flies of indicated genotypes/treatments (n=10-25). (B) Male courtship index for Aug21/UAS-ETHR-Sym and genetic controls compared to GMR46E11-Gal4/UAS-ETHR-Sym (n=15-25). (C) Initial courtship (IC, minutes 0-2) and terminal courtship (TC, 8-10) compared for JHAMT, Aug21, and GMR46E11-Gal4 ETHR-silenced flies and methoprene/ethanol treated Aug21-Gal4/UAS-ETHR-Sym (n=12-15). Error bars represent S.E.M. NS, * $p <$ .05, ** $p <$ .01.

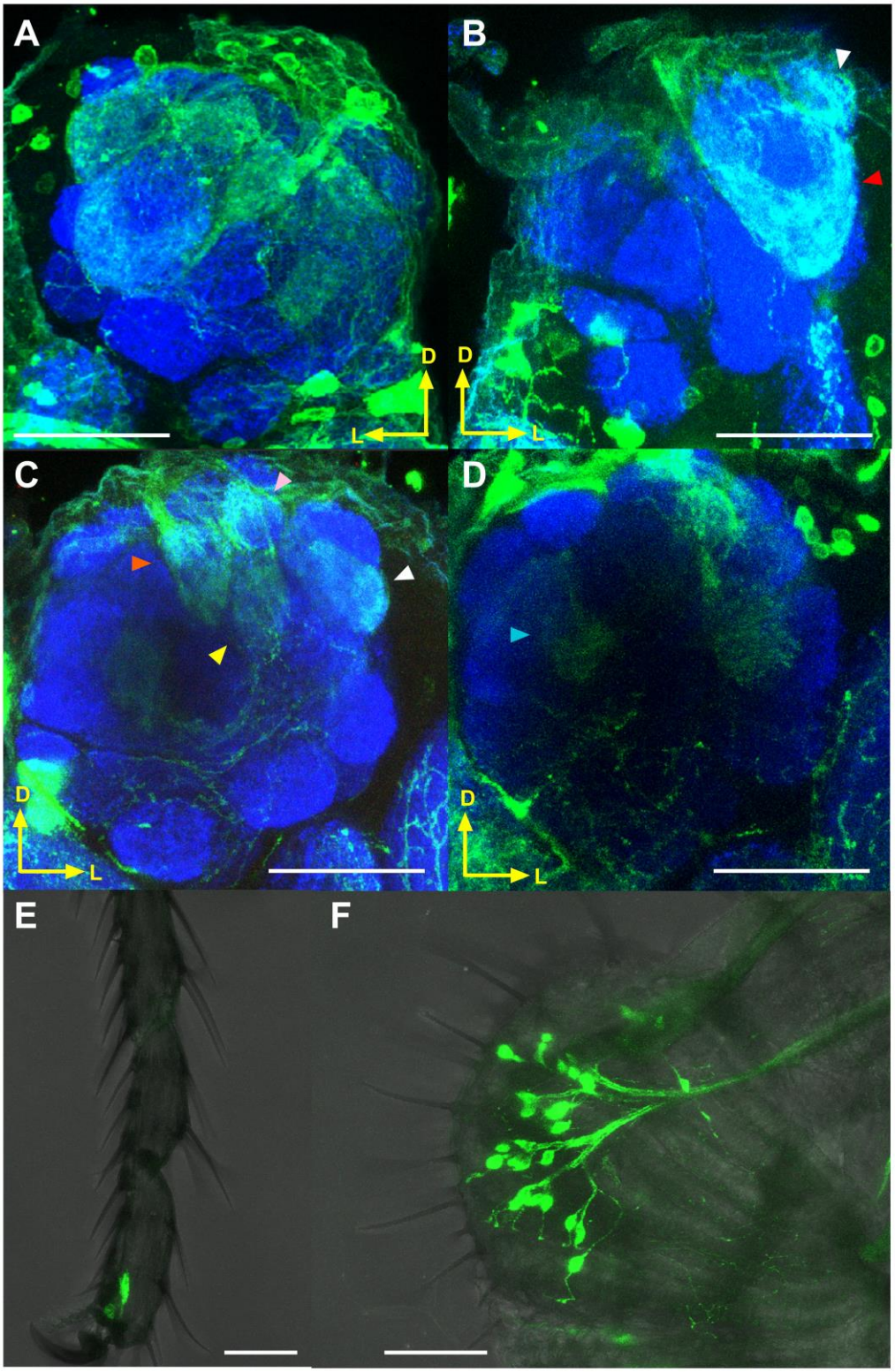
A
B
C
D
E
F
D
L

Figure 4.5. Female expression pattern of ETHR-Gal4 (A-D) Antennal lobe expression of ETHR-Gal4/UAS-mCD8 in females. OR67D-DA1 white arrowhead, OR47B-Va1v red arrowhead, OR2A-DA4m pink arrowhead, OR82A-VA6 yellow arrowhead, OR19A-DC1 orange arrowhead, OR85E/OR33C-VC1 blue arrowhead (yellow arrows indicate dorsal/lateral, scale bar 20 µm). (E-F) Taste organ expression of ETHR-Gal4 in prothoracic tarsus (E, scale bar 50 µm) and labellum (F, scale bar 50 µm).

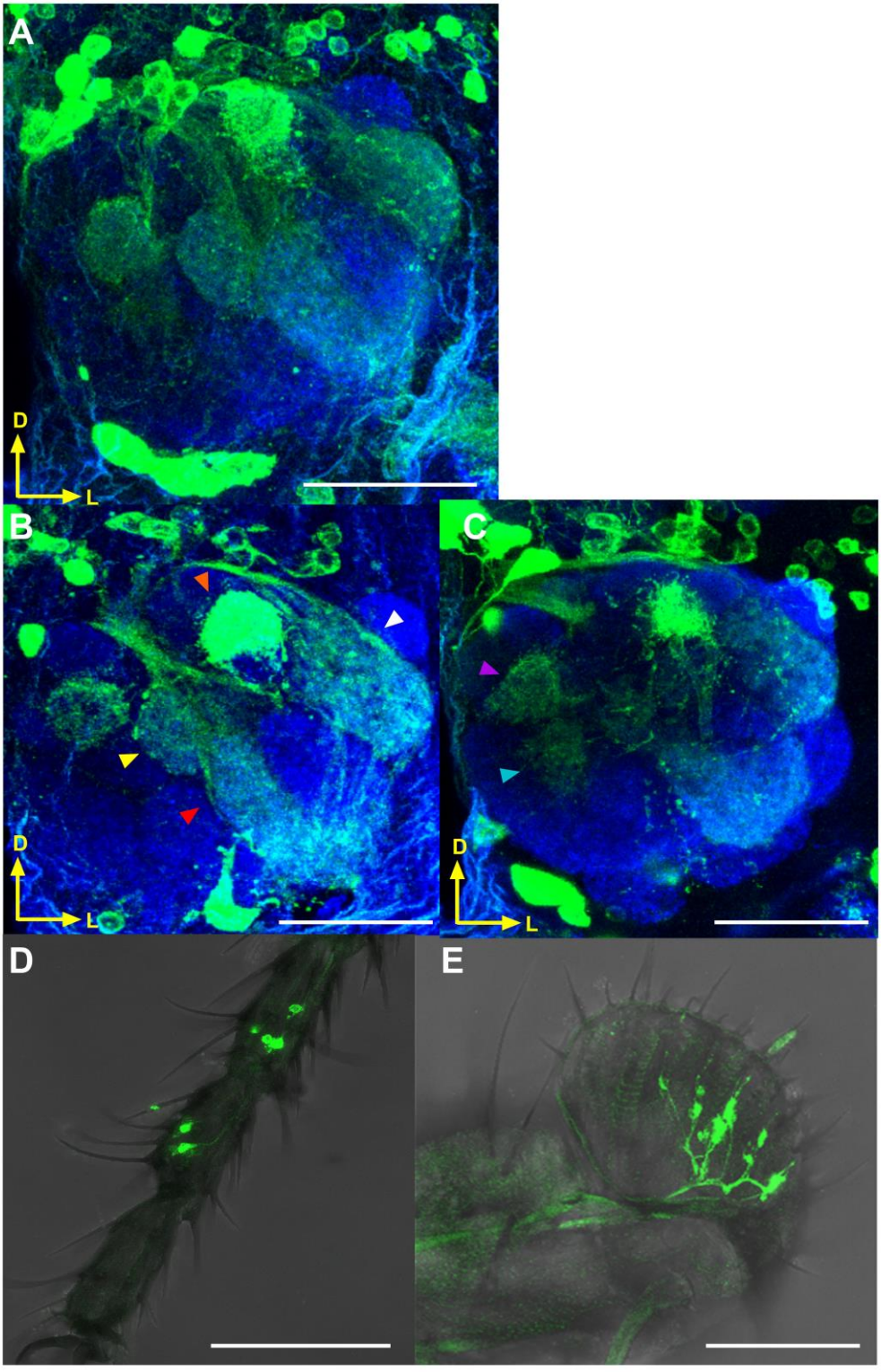
A
D
L
B
D
L
C
D
L
D
E

Figure 4.6. Male expression pattern of ETHR-Gal4 (A-C) Antennal lobe expression of ETHR-Gal4/UAS-mCD8 in females. OR67D-DA1 white arrowhead, OR47B-Va1v red arrowhead, OR2A-DA4m orange arrowhead, OR82A-VA6 yellow arrowhead, OR42A-VM7 purple arrowhead, OR85E/OR33C-VC1 blue arrowhead (yellow arrows indicate dorsal/lateral, scale bars 20 μm). (D-E) Taste organ expression of ETHR-Gal4 in prothoracic tarsus (D, scale bar 100 μm) and labellum (E, scale bar 100 μm).

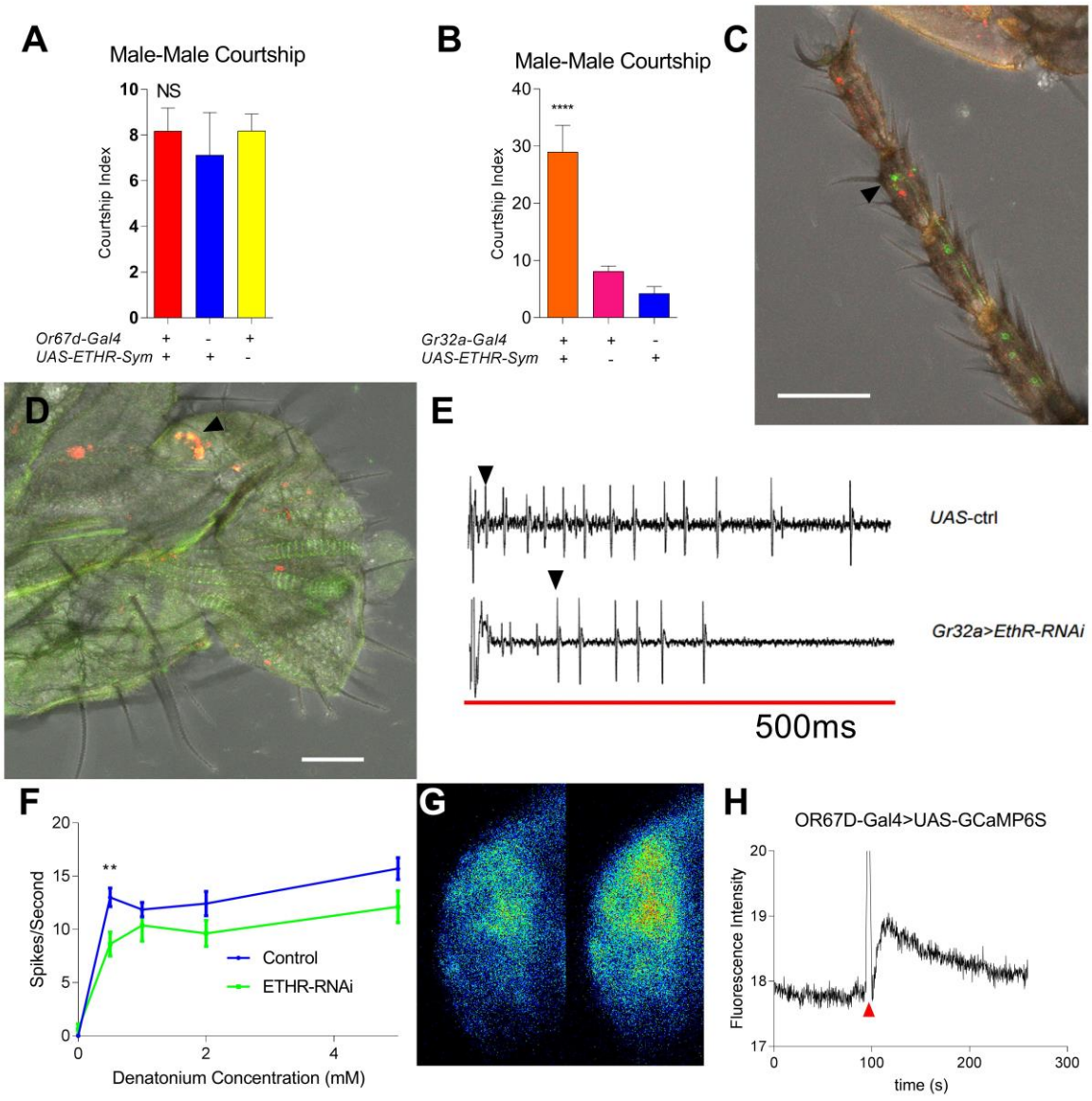
A
Male-Male Courtship
NS
Courtship Index
10
8
6
4
2
0
Or67d-Gal4 + - +
UAS-ETHR-Sym + + -
B
Male-Male Courtship
****
Courtship Index
40
30
20
10
0
Gr32a-Gal4 + + -
UAS-ETHR-Sym + - +
C
D
E
UAS-ctrl
Gr32a>EthR-RNAi
500ms
F
**
Spikes/Second
20
15
10
5
0
Control
ETHR-RNAi
0
2
4
Denatonium Concentration (mM)
G
H
OR67D-Gal4>UAS-GCaMP6S
Fluorescence Intensity
20
19
18
17
0
100
200
300
time (s)

Figure 4.7. Functional dependency of primary sensory neurons on ETHR (A-B) Male-male courtship index of males with ETHR silenced in aversive pheromone sensor neurons OR67D (A) and GR32A (B)(n=15-20). (C-D) UAS-mCD8;ETHR-Gal4/Aop-mCherry;GR32A-LexA non-overlapping expression in tarsi (C, scale bar 100 µm) and overlapping expression in labella (D, scale bar 25 µm). (E) GR32A-Gal4/UAS-ETHR-IR2 electrophysiological response of S6 to 1mM denatonium for 500 ms after contact black arrowheads indicate latency to spike and example spike for counts. (F) Average spikes/second after contact with denatonium of indicated concentrations (n=8). Error bars represent S.E.M. NS, $p > .05$; * $p < .05$, ** $p < .01$, **** $p < .0001$.

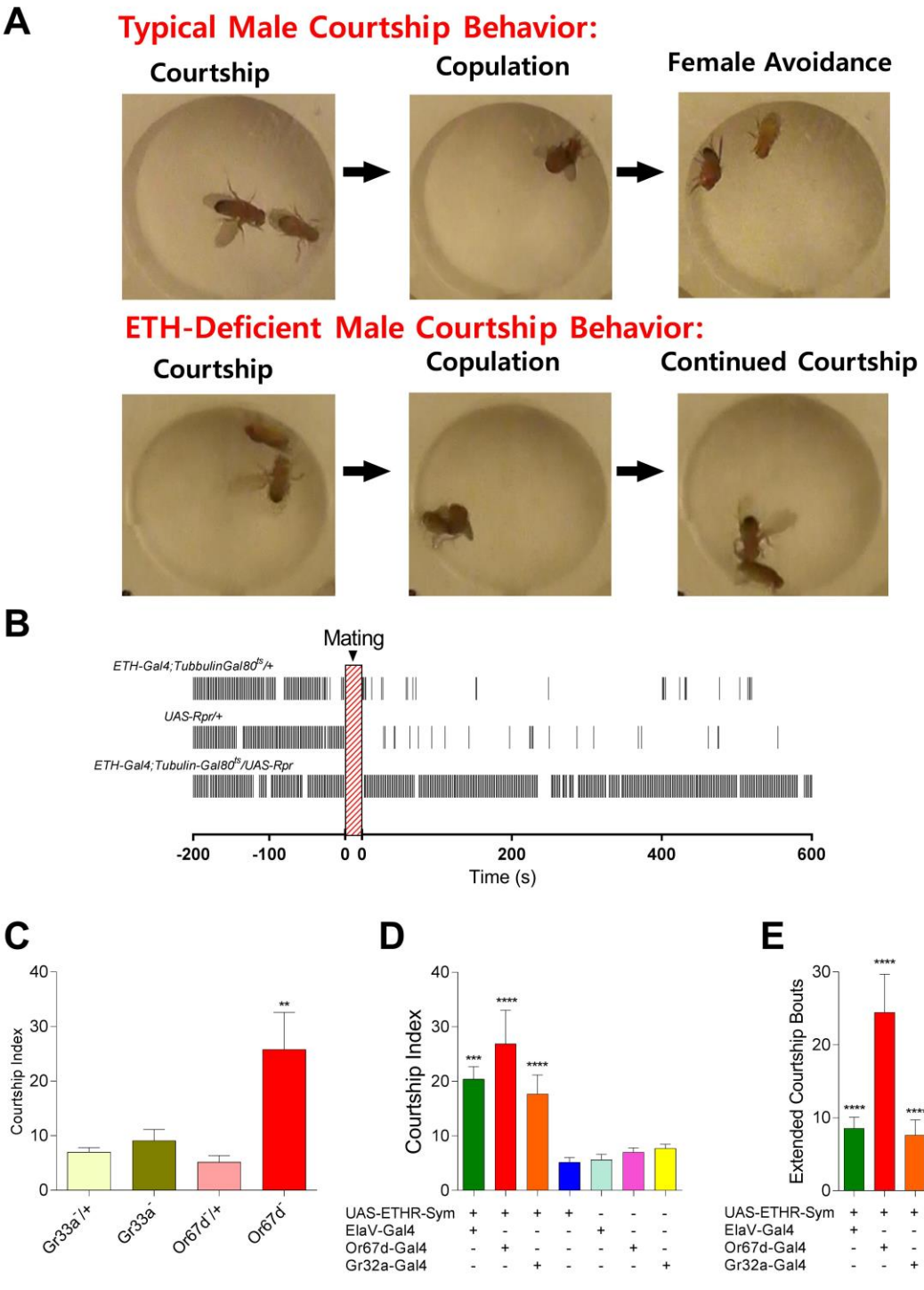
A
Typical Male Courtship Behavior:
Courtship
Copulation
Female Avoidance
ETH-Deficient Male Courtship Behavior:
Courtship
Copulation
Continued Courtship
B
Mating
ETH-Gal4;TubbulinGal80ts/+
UAS-Rpr/+
ETH-Gal4;Tubulin-Gal80ts/UAS-Rpr
-200
-100
0 0
200
400
600
Time (s)
C
Courtship Index
Gr33a-/+
Gr33a-
Or67d-/+
Or67d-
D
Courtship Index
UAS-ETHR-Sym
ElaV-Gal4
Or67d-Gal4
Gr32a-Gal4
E
Extended Courtship Bouts
UAS-ETHR-Sym
ElaV-Gal4
Or67d-Gal4
Gr32a-Gal4

Figure 4.8. Post-copulation courtship inhibition depends upon OR67D (A) Description of post copulation courtship behavior in typical males, characterized by avoidance of the now mated female, and ETH-deificient males which continue to court after mating. (B) Seconds spent courting 200 seconds before mating and 600 seconds after dismount for Inka cell-ablated males and genetic controls. Black bars represent seconds performing courtship behavior. (C) Courtship index of GR33A null mutants and OR67D null mutants as well as heterozygous controls during the 10 minutes immediately following copulation (n=10-20). (D) Raw post-copulation courtship index for males with ETHR silenced pan-neuronally (Elav-Gal4) or in GR32A and OR67D-Gal4 (n=10-20). (E) Extended courtship bouts as a percentage of the 10 minutes after copulation from (D).

Chapter 5

Ancillary Consequences of ETH deficiency

Abstract

Ecdysis triggering hormone is a critical regulator of adult reproductive behaviors. With a depth of understanding of its targets and the effects of excess and deficient ETH signaling, we can begin to unravel the purpose of the hormone, as well as conserved principles in endocrinology. Elimination of the Inka cells specifically in the adult stage causes several phenomena beyond deficient oogenesis, ovulation, and male courtship behavior. In addition to the previously described deficiencies, hyperactivity, a drop in hatch rate or egg viability in females, and an increase in virgin female egg laying were all observed in ETH deficient flies, and each warranted investigation.

Introduction

Hyperactivity in ETH deficient flies as a human disease model for ADHD

Diagnosis of attention deficit hyperactivity disorder (ADHD) is on the rise, with an estimated 5% of American children showing symptoms. Thyroid abnormalities and thyroid resistance have been associated with ADHD-like symptoms (Kuppili, Pattanayak, Sagar, Mehta, & Vivekanandhan, 2017). Indeed, nongenetic mouse models of ADHD utilize Polychlorinated biphenyl (PCB) 153 treatment, and prenatal PCB exposure is a predictor of ADHD diagnosis (Eubig, Aguiar, & Schantz, 2010; Johansen et al., 2014). PCBs are also a known endocrine disruptor, specifically permuting thyroid signaling

(Brown, 2005). Mothers with hypothyroidism were more likely to have children with ADHD (Modesto et al., 2015). The establishment of a disease model in the genetically tractable fruit fly, *Drosophila melanogaster,* could lead to new drugs and therapies applicable to the ever-increasing number of diagnosed children, especially given that long term treatment with amphetamines causes neurotoxicity and the recent revelation that methylphenidate causes changes in brain-wide neurotransmission (Advokat, 2016; Solleveld, Schrantee, Puts, Reneman, & Lucassen, 2017).

Thyroid hormone is attractive for system modeling not only due to its correlation with the disease, but because of its broad role in modulating aminergic synthesis in dopaminergic, seratonergic and norepinehprinergic neurons, all of which are attenuated in children with ADHD (O. M. Ahmed, El-Gareib, El-Bakry, Abd El-Tawab, & Ahmed, 2008; Blum et al., 2008). Current genetic models in both mouse and fly are limited to dopamine synthesis and receptor mutants, and proteins of unknown function (Lebestky et al., 2009; Leo & Gainetdinov, 2013; van Swinderen & Brembs, 2010). JH and thyroid hormones are both lipophilic, and carry a large number of functional similarities (Flatt, Moroz, & Tatar, 2006). Thyroid hormone even causes pleiotropic, JH-like effects in metabolism and immunity when applied to insects. Like TH, JH both stimulates synthesis of and depends on feedback from dopamine and octopamine (insect version of norepinephrine) for internal homeostasis (Gruntenko et al., 2005). Several studies have examined hyperacivity of JH-perturbed flies, with mixed results (Argue, Yun, & Neckameyer, 2013; Z. Liu, Li, Prasifka, Jurenka, & Bonning, 2008; Wijesekera, Saurabh, & Dauwalder, 2016). As none of these flies have actually had JH levels determined, I

therefore examined whether our ETH and CA-ETHR-silenced flies, with measured JH decrease were hyperactive. We were able to consistently induce hyperactivity, a critical component of ADHD, by interrupting JH homeostasis during a critical period of adult brain maturation.

Here, we demonstrate that ETH and subsequent JH deficiency during a critical period of adult brain maturation induces hyperactivity. ETHR silencing with one CA Gal4 but not another, weaker Gal4 driver induces hyperactivity. I go on to show that injection of ETH causes transient hypoactivity. These data suggest ETH or JH deficient flies are an intriguing model for ADHD and thyroid disorders.

Female hatch rate declines after Inka cell ablation

In nature, the organism able to use resources most efficiently is most likely to survive. Even behaviors with minute differences in energy expenditure can be amplified over generational time. Here we show that *Drosophila melanogaster* females reduce there fertility during periods of stress not only by reducing egg synthesis and release, but by withholding sperm from gametes.

Drosophila melanogaster females typically lay at least 140 eggs during the week following mating, after which sperm supply begins to depelete and unfertilized eggs are laid more frequently as time progresses (Wilson, DeMoor, & Lei, 2003). We found that elimination of the Inka cells led to an immediate drop in the percentage of eggs laid that hatched. Silencing ETHR ubiquitously with the Tubulin-Gal4 driver also caused this increase. We investigated the eggs laid by affected females and found that they do not carry paternal genes and have not progressed into embryogenesis, suggesting they had

not been fertilized. After a directed Gal4 screen we were able to identify an enhancer trap line in which ETHR silencing with two different RNAi constructs caused inviability. Iterestingly, EcR-RNAi in the Inka cells, which should limit the amount of ETH gene produced, do not cause a significant increase in inviability. While it is currently unclear what the exact target-tissue of ETH is that stimulates inviability, or whether another Inka cell component is responsible for sperm mobilization, we present evidence here that Inka cells and possibly ETH are necessary for fertilization of mature oocytes.

ppk neurons depend upon ETHR signaling before mating

An animal's behavioral choices depend upon not just neuronal wiring but upon endocrine state. The internal state of an organism provides a melieu that can coordinate distal tissues with environmental influence, including critical decision-making neurons. *Drosophila melanogaster* sex peptide has received a great deal of attention since its discovery, as it is an external cue, depositied in females by male mating partners, that causes drastic neuronal and endocrine changes after mating (Aigaki, Fleischmann, Chen, & Kubli, 1991; Chapman et al., 2003; Chen et al., 1988; H. Liu & Kubli, 2003; Moshitzky et al., 1996; Yapici, Kim, Ribeiro, & Dickson, 2008). One neuronal subset critical for translating the sex peptide signal into mated-like behavior are the *ppk* neurons (Häsemeyer, Yapici, Heberlein, & Dickson, 2009). When sex peptide's $G_{i/o}$ GPCR is activated in *ppk* neurons, it inhibits them, causing derepression of oviposition, refractory mating behavior, and ecdysteroid synthesis (Ameku & Niwa, 2016; Häsemeyer et al., 2009). I found that this neuronal subset also expresses ETHR, a $G_{\alpha q}$ GPCR, and ETHR-silencing causes mated-like egg laying in virgin females. As *ppk* neurons have been

shown to regulate 20E levels, which I have shown regulate ETH production in females (Meiselman et al., 2017), this may prove to be a critical component of the adult female endocrine network, and suggests an interesting feedback loop for endocrine state change associated with mating.

In this chapter, I will explore the implications of reliance of certain neurons, tissues and glands upon ETH. I demonstrate clearly that ETH and subsequent JH deficiency causes hyperactivity in *Drosophila melanogaster* adults, which I believe presents and excellent model for thyroid hormone-derived attention deficit hyperactivity disorder. I show that Inka cell killing and possibly ETH deficiency causes a reduction in egg fertilization, adding to the principle established in Chapter 2; stressed females arrest fecundity by lowering ETH levels. Finally, I demonstrate that *ppk* neurons depend upon ETH signaling in the virgin female, suggesting a novel feedback loop of involved the E-ETH-JH axis. These not only improve our understanding of the critical peptide hormone, ETH, and the adult endocrine system, they provide a foundation for exciting new paradigms in endocrinology.

Results

JH deficiency in Inka cell-killed flies causes hyperactivity

While performing courtship assays (Chapter 3) I noticed male courtship intensity was much higher than controls. I sought to determine whether this was due to elevated courtship motivation or general hyperactivity. Flies have an innate reflex to climb after

being knocked down, and hyperactivity can be assessed with a simple geotaxis assay (Bainton et al., 2000). I killed the Inka cells specifically in the adult stage using ETH-Gal4;tubulin-Gal80^{ts}/UAS-Reaper, and compared geotaxis to genetic controls (Figure 1A). ETH-deficient flies move roughly 45% faster than do genetic controls. Likewise, blocking ETH release from Inka cells (ETH-Gal4;UAS-Shi^{ts}) causes a similar increase in geotaxis, though not to the degree observed in Inka cell killing (Figure 1B). Next, we tested whether elevation of ETH levels in Canton-S wild-type flies had any effect on motility. Interestingly, flies injected with 20ng of ETH were hypoactive one hour after injection, taking more than twice as much time to climb 4cm (Figure 1C).

As ETH has a critical role as an allatotropin (Meiselman et al., 2017), we examined whether ETHR silencing in the corpus allatum (CA) induced hyperactivity. We used two different drivers, JHAMT-Gal4 and Aug21-Gal4, reported to drive to the CA. JHAMT-Gal4, ETHR-silenced flies have a faster average velocity but are not significantly different from controls within the n-range of our experiment (n=50) (Figure 2A). Aug21-Gal4, ETHR-silenced flies phenocopy ETH-deficient flies, with an approximately 35% increase in velocity (Figure 2B). As we found Aug21 drives to antennal lobe interneurons as well as neurons in the central complex in addition to the CA, we examined whether pan-neuronal ETHR silencing had an effect on geotaxis. We found that Nrv-Gal4/UAS-ETHR-RNAi had no effect on geotaxis (Figure 2C). Finally, CA-ablated flies (JHAMT-Gal4;Tubulin-Gal80^{ts}/UAS-Reaper) showed hyperactivity similar to ETH-deficient flies, approximately 45% faster than controls (Figure 2D), suggesting JH deficiency is solely responsible for the increased velocity of Inka cell-killed flies.

Elimination of the Inka cells impairs fertilization

Fecundity examinations of females with adult-specific ablation of the Inka cells shows that in addition to impaired oogenesis and ovulation, a large portion of eggs laid do not develop into larvae. At 25°C, eggs laid by recently mated females take approximately 12-15 hours to hatch (Ashburner & Thompson, 1978). We allowed females 3 days to lay eggs on an apple juice plate, removed them, and gave deposited eggs 24 hours to hatch before counting the total number of larvae and unhatched eggs. Generally about 5% of eggs laid by wild-type and control flies fail to hatch during this period, while roughly 40% of eggs laid by Inka cell-ablated females do not hatch (Figure 1A). Genetic controls not exposed to the permissive temperature (25°C) do not lay inviable eggs, showing that the genetic background does not effect hatch rate (Figure 3B). Additionally, blocking Inka cell release with tetanus toxin or using the temperature sensitive dynamin mutant Shibire^{ts} causes a similar effect (Figure 3C). Pretreatment of Inka cell-ablated females with the JH analog methoprene or the 20E analog RH5992 does not affect hatch rate (Figure 3D). I also eliminated EcR in the Inka cells, as the ETH gene depends upon 20E for expression. Interestingly, there was no change in the number of unhatched eggs after 4 days (Figure 3E).

To determine at what stage of embryogenesis eggs were arresting development, I collected unhatched eggs laid by Inka cell-ablated females and controls left over after the three day counting. Eggs were dechorionated, fixed and stained for DNA with DAPI. While unhatched eggs from controls usually arrested in late embryogenesis (Figure 4A), eggs taken from Inka cell killed were mostly DAPI negative (Figure 4A', B), suggesting

gamete fusion had not occurred. I next looked for expression of a paternal gene. After mating Inka cell-ablated females to a ubiquitin-GFP fusion mutant, I looked for GFP expression in progeny. While unhatched eggs from controls fluoresced approximately 90% of the time, only 15% of eggs from Inka cell ablated females had any green fluorescence (Figure 4C).

I also used the heat shock paradigm to investigate fertilization. I show in Chapter 2 that heat shocking attenuates reproduction and ETH levels go down after heat shock. This being the case, low ETH levels should mean heat-shocked females will have low fertilization rate. I found that almost all eggs laid by mated, heat shocked females are unfertilized, and this can be rescued to some extent by ETH injection, but not saline injection (Figure 5A). I also noticed that heat-shocked, methoprene rescued females had "small eggs" in their ovaries, a phenotype observed in kep1 mutants, which have limited late stage nurse cell dumping (Di Fruscio et al., 2003). Intriguingly, kep1 mutants were also reported to have a 45% reduction in hatch rate, very similar to that of ETH deficient females.

Next, I silenced ETHR ubiquitously during the adult stage (Tubulin-Gal4/UAS-CM3;TubulinGal80^{ts}). I found hatch rate declined marginally (Figure 6A), suggesting ETH activation of ETHR in a target tissue is likely the cause of decreased egg viability. I performed a directed Gal4 screen, silencing ETHR with 46 Gal4 drivers to determine where ETHR Several reports have suggested poor hatch rate has been associated with aminergic/glutamatergic neuron disfunction in the vicinity of the female reproductive tract(Kairamkonda & Nongthomba, 2014; Rodríguez-Valentín et al., 2006), but ETHR

silencing in the region had no effect on fertilization. Indeed, silencing ETHR neuronally, in germaria, and in spermathecae did not affect hatch rate with two notable exceptions (Figure 6B). ETHR silencing with either 109-53-Gal4 and OAMB-Gal4, which both express in oviduct epithelium, depressed hatch rate (Lee, Rohila, & Han, 2009). However, this may be a false positive as poor hatch rate varied considerably with each cross for OAMB-Gal4 but not 109-53-Gal4. Interestingly, ETHR-Gal4 did not express in the oviduct epithelium.

An endocrine feedback system regulating egg laying

The virgin female endocrine state is drastically different than mated females due to sex peptide's modulation of 20E and JH levels (Ameku & Niwa, 2016; Moshitzky et al., 1996). I investigated whether ETH deficiency impacted virgin female egg laying due to its reported role in oogenesis through JH (Meiselman et al., 2017). Contrary to expectations, I found virgin female egg production was slightly elevated but highly variable in Inka cell-ablated females (Figure 7A). I next determined the degree to which silencing ETHR specifically in the CA impacted virgin female egg laying. CA-ETHR-silenced flies laid approximately 35% fewer eggs than did controls, similar to the discrepancy seen by the same genotypes in the mated female (Figure 7B) (Meiselman et al., 2017). This suggested a confounding factor in a potentially non-JH target, so I silenced ETHR pan-neuronally with ElaV-Gal4, as ETHR expresses in neurons (Diao et al., 2016). Pan-neuronal silencing resulted in a doubling in the total number of eggs laid (Figure 7C). It has been reported that inactivation of fru-positive, ppk-positive neurons in

the female reproductive tract induces mated-like egg laying in virgin females (Häsemeyer et al., 2009). ETHR-Gal4 espressed in the characteristic ppk^{+}, fru^{+} neuronal subset in the uterus, but, interestingly, not the ppk^{+} fru^{-} region on the lateral oviduct (Figure 7D). We found ETHR silencing with the ppk-gal4 elevated virgin female egg laying, whether silencing occurred through the whole life of the animal or specifically during the adult stage (Figure 7E). These data suggest ppk neurons depend upon ETH for function in adult virgins.

Discussion

In addition to its well-described role as a command peptide of developmental transition, ecdysis-triggering hormone has proven to be critical for regulation of adult behaviors (Kim, Zitnan, Galizia, Cho, & Adams, 2006; Meiselman et al., 2017; Park, Filippov, Gill, & Adams, 2002; Zitnan, Kingan, Hermesman, & Adams, 1996). *Drosophila melanogaster* uses the E-ETH-JH axis to activate adaptive behaviors in response to environmental changes. We show here several processes that depend upon ETH in activity, fertilization, and maintenance of virgin female egg laying.

Hyperactivity is a well-known starvation response in humans, mammals, and in *Drosophila melanogaster*, as it is thought to stimulate food-seeking behavior in times of nutritional stress (M, C, & JD, 2004; Pirke, Broocks, Wilckens, Marquard, & Schweiger, 1993; Yang et al., 2015; Yu et al., 2016). It is also known that starvation is accompanied by high ecdsyone, which may suppress secretory competence of Inka cells leading to low ETH (Terashima, Takaki, Sakurai, & Bownes, 2005). Given this and the suggested role

of ETH as an anti-stress hormone, it is not surprising that ETH deficiency leads to hyperactivity.

We show here that ETH deficiency causes hyperactivity, and injection of ETH peptide into the hemolymph causes acute hypoactivity. The ability of ETH injection to cause hypoactivity within one hour is particularly interesting as it suggests the endocrine deficiencies we observe result in a physiological, but not developmental impairment. ETH activates octopamine release in the vicinity of the female reproductive tract and likely does so in the brain as well, given that elimination of its receptor in the brain causes hyperactivity (Yu et al., 2016). We show here that JH is most responsible for hyperactivity, as CA ablation, but not panneuronal ETHR silencing causes climbing deficiency. Using a less crude tool, it has been shown that ETH activation of the brain prevents hyperactivity, suggesting redundancy. ETH also stimulates the release of JH, on which dopamine homeostasis depends (Argue et al., 2013; Gruntenko, Karpova, & Alekseev, 2007). We show that Aug21-Gal4 but not JHAMT-Gal4 driven silencing of the ETH receptor in the CA causes hyperactivity. The discrepancy between the two is very likely related to the strength of the drivers, as Aug21 expression in the CA is stronger. However, due to Aug21's broad expression in the nervous system, silencing in AKHR neurons that cause hyperactivity or another target being the culprit, or even an additive effect cannot be ruled out. It is, however, clear that in ETH deficient flies, catecholamine levels and reward pathways are likely to be severely impaired beyond just dopamine deficiency, which would be a model which may be a more effectively mimic ADHD than current model flies.

We found that Inka cell ablation or blocking of Inka cell release causes a drop in the proportion of eggs laid that do not hatch. We've shown that ETH release is impaired by high 20E associated with stress. It is interesting that stored sperm is conserved during periods of stress-induced reproductive arrest in a similar manner to protein or glycogen reserves.

Elimination of the ecdysone receptor, on which the ETH gene depends, had no effect on egg viability. This could be due to the relatively weak dependency of ETH on EcR in the female (Meiselman et al., 2017), but this is also the first suggestion that a non-ETH signaling molecule could be released from the Inka cells. Eggs laid by Inka cell-ablated don't carry paternal genes nor do they condense their nuclei, which occurs with gamete fusion. Unhatched eggs taken from controls were general later stage embryos, suggesting the bulk of the extra unhatched eggs are unfertilized.

The low fertilization rate of heat-shocked females and the ability of ETH injection to partially restore hatch rate is a strong argument in favor of ETH playing an important role in mediation of fertilization. A possible explanation for the low hatch rate was found in the methoprene rescue of heat-shocked females. ETH-deficient, methoprene rescued females developed "small eggs" suggesting a defect in late oogenesis vitellogenesis. An interruption in the kep1 pathway would also explain why ETH deficient female oocytes are exceptionally low in yolk protein (Meiselman et al., 2017), ETH and JH have partially redundant roles in oogenesis. Unfortunately, ETHR-Gal4 did not label the region, nor did any germarium driven silencing of ETHR cause a decline in hatch rate and it is thus still unclear whether this hypothesis is correct.

When we silenced ETHR in ubiquitously with the tubulin driver, I observed a significant decrease in hatch rate, though only around half that of Inka cell killing. As tubulin-Gal4 drives unevenly to most tissues, I proceeded with a directed Gal4 screen. Three of the 46 lines tested had a significant increase in unhatched eggs laid when compared to UAS-CM3, but only two of those were elevated when compared to Gal4 controls; 109-53-Gal4 and OAMB-Gal4. While 109-53-Gal4 driven silencing of the ETHR decreased hatch rate in every cross, whereas OAMB-Gal4-driven silencing often produced flies with normal hatch rates, unlike Inka cell killing. It remains unclear whether this is due to the driver or an unknown confound.

Both of these lines express in the oviduct epithelium and in follicle cells of the germaria, but I was unable to see any staining in the region with ETHR-Gal4. The oviduct epithelium is an intriguing target, as it is responsible for hydration of the oviductal lumen, and egg activation (Sartain & Wolfner, 2013). Egg activation includes both resumption of meiosis and the crosslinking of the vitelline membrane, a critical step for formation of the micropyle and thus fertilization (Karr, 1991). I also believe there is a possibility that neurons facilitating sperm transport are impaired, though ETHR silencing in implicated neurons and pan-neuronally had no effect. It is also worth considering that ETH releases another signaling molecule, like proctolin, which may be produced in the Inka cells and is known to target the female reproductive system (Ritsick, Edens, Finnerty, & Lambeth, 2007; Taylor et al., 2004).

I also found that ETH may be necessary for maintenance of virgin female egg laying. ETH deficiency produced high variability in virgin female egg laying, which

could be due to the opposing influences of females producing fewer eggs due to JH deficiency and mated-level release of mature oocytes. Indeed, when we silenced ETHR specifically in the CA, virgins laid fewer eggs, while pan-neuronal or ppk-driven silencing elevated virgin egg-laying. ETHR-Gal4 expression seemed to be limited to ppk-positive and fru-positive neurons in the abdominal nerve to the uterus, while not labeling fru-negative neurons on the base of the oviduct. ETHR-silencing, which causes the elimination of a Gq-coupled GPCR, had the same effect on ppk signaling as silencing the neurons with the dynamin mutant *shibire* (Häsemeyer et al., 2009). This seems logical as ETHR signaling appears to be a necessary homeostasis receptor as we find several neurons described in this dissertation and in unpublished work from our lab. Indeed, several of the well-studied mammalian homologs of the ETH receptor activate their tissues through constituitive activity (Pantel, 2006).

The likelihood that ETH also plays an active role in regulation of the ppk neurons also suggests an intriguing paradigm for endocrine changes between virgin and mated females (Figure 8A-B). In virgin females, JH levels are totally dependent upon ETH (Meiselman et al., 2017). During development, 20E levels and JH levels are always opposite one another, while one is high, the other is suppressed (Dubrovsky, Dubrovskaya, & Berger, 2004). This changes after mating, however, when 20E and likely JH both elevate to stimulate oogenesis (Harshman, Loeb, & Johnson, 1999; Teal, Gomez-Simuta, & Proveaux, 2000). Though we show 20E and ETH have an opposing relationship in titer, once a female has mated, the sex peptide deposited in her system by the male obviates ETH's role and stimulates the CA (Moshitzky et al., 1996).

Meanwhile, sex peptide receptor, a $G_{i/o}$-coupled GPCR, inhibits ppk neurons and elevates 20E levels, again obviating ETH signaling to the region. This elevated 20Einhibits ETH release, further transforming endocrine state (Ameku & Niwa, 2016). When females use up available sperm, sex peptide levels decrease, leading to a state change and resumption of virgin-like behaviors, including receptivity.

Despite lingering questions remaining with this work, ETH clearly has an interesting diversity of roles in the adult stage.

Materials and Methods

Fly Strains

Flies used for immunohistochemistry, calcium imaging and CA ETHR silencing were raised at 23˚C on standard cornmeal-agar media under a 12:12 hr light:dark regimen. CA and Inka cell-ablated flies were raised at the $Gal80^{ts}$ permissive temperature (18°C). Following eclosion, they were moved to the nonpermissive temperature (29°C) for 24 hours, then moved to 23°C until day 4. Inka cell blocked and conditional ETHR silenced or EcR-eliminated flies were raised at the $Gal80^{ts}$ permissive temperature (18°C) and moved to the nonpermissive temperature (28°C) after eclosion until day 4. The JHAMT-Gal4 fly line has been described recently (Wijesekera, Saurabh, & Dauwalder, 2016). Use of double-stranded RNA constructs for silencing of ETHR (UAS-ETHR-Sym; UAS-ETHR-IR2 line (VDRC transformant ID dna697) were described recently (D.-H. Kim et al., 2015). Aug21-Gal4 flies were obtained from Dr. Korge (Freie Universität Berlin). Syt12-Gal4 and Send1-Gal4 were provided by Dr. Jianjun Sun (University of

Connecticut). Ppk-Gal4 was provided by Dr. Wes Grueber (Columbia University). Information on Gal4 lines used in screens available on request. All other fly lines were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN): UAS-Red Stinger (BS no. 8574), UAS-mCD8-GFP (BS no. 5137), UAS-CD4-tdGFP (BS no. 35836), UAS-rpr (BS no. 8524), Nrv-Gal4 (BS no. 6799), UAS-TNT (BS no. 28837), UAS TNTimp (BS no. 28844), Elav-Gal4 (BS no. 458), TubP-Gal80^{ts} (BS no. 7017), ETH-Gal4 (BS no. 51982), UAS-EcR-RNAi (BS no. 37059), UAS-EcR.B1 (BS no. 6869). All flies used for behavior experiments were backcrossed for at least five generations into the Canton-S background.

Geotaxis Assay

4-day old virgin males were placed in vials with 5 total males. Vials were tapped down once on a greenstone lab bench top. Males were videotaped to time how long it took them to climb 4 cm.

Egg Production

Newly eclosed females of noted genotype were kept in incubators at 50% humidity in isolation vials until day 4, at which point they were paired with a wild type *Canton-S* male in courtship chambers. Following mating, females were isolated in 10x35mm dishes filled with 4 ml of apple juice diet supplemented with 0.5 g of yeast and allowed to lay eggs for three days at 23°C. Flies were then discarded and progeny were tallied. Larvae were counted immediately after removal of the female, and remaining eggs were given 24 hours to hatch, after which all eggs, hatched and unhatched, were counted. Virgin

females were given the same conditions sans mating, and all laid eggs were tallied immediately after the three day period.

Treatments

Injections

Wild-type males were cold anesthetized on an Echotherm chilling plate and either faux injected, injected with 50 nl fly saline, or 20ng ETH in 50 nl fly saline. After 1 hour recovery time, they were assayed as described above.

Methoprene Treatment

Within 24 hours of eclosion, adult males or females were cold anesthetized and treated topically on the dorsal side of the abdomen with 72 (females) or 36 (males) nl of either acetone, or 0.01% methoprene dissolved in acetone (~300 nM). The entire procedure took under 20 minutes, after which flies were returned to their housing.

Tebufenazide Feeding

Flies of indicated genotype were raised according to the Inka cell ablated protocol. After eclosion, flies were reared on standard diet plus 0.05% ethanol or 0.05% ethanol and 1.5 ng/µl RH-5992 until mating, after which they were put on standard apple juice plates.

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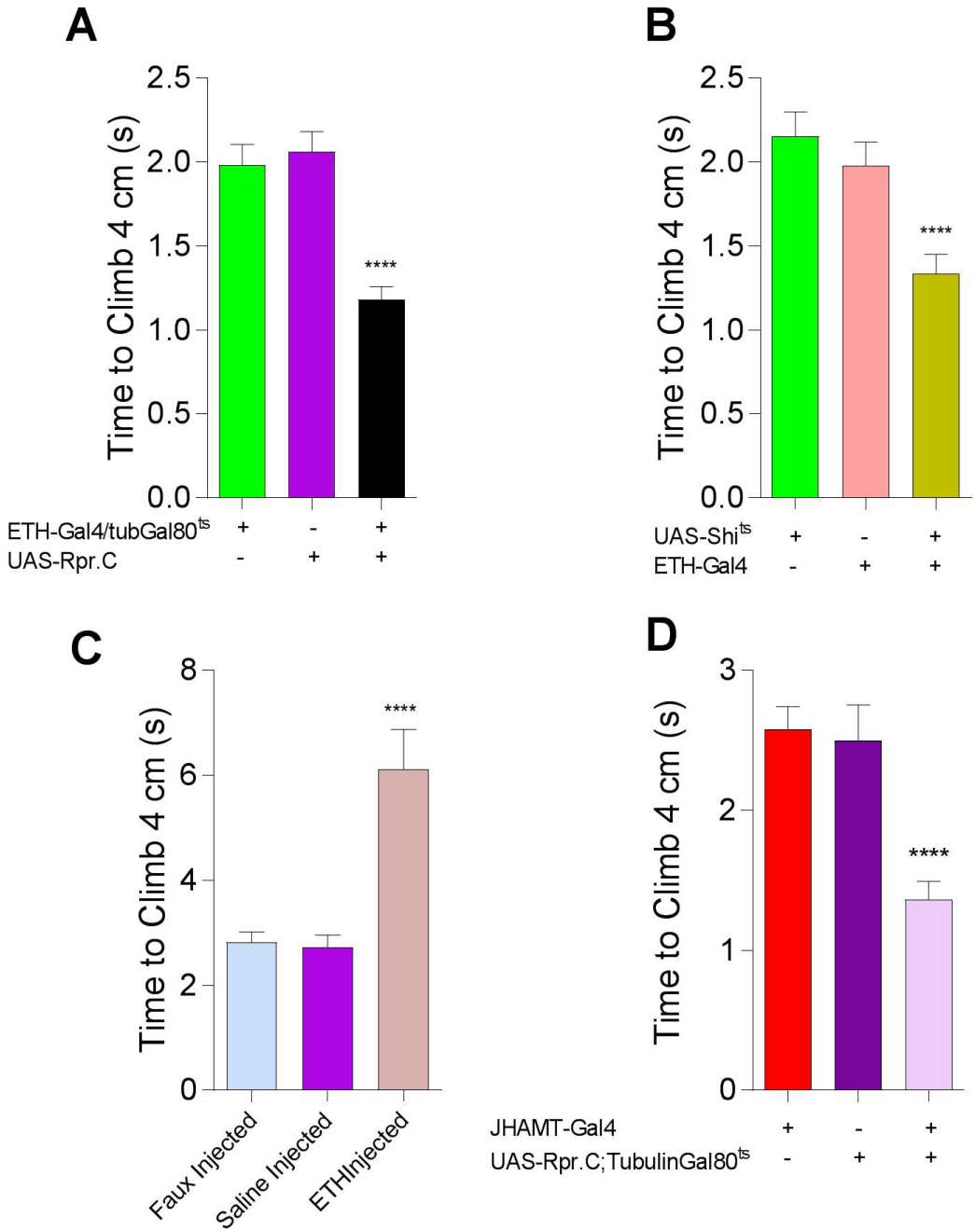
A
Time to Climb 4 cm (s)
2.5
2.0
1.5
1.0
0.5
0.0
****
ETH-Gal4/tubGal80ts + - +
UAS-Rpr.C - + +
B
Time to Climb 4 cm (s)
2.5
2.0
1.5
1.0
0.5
0.0
****
UAS-Shits + - +
ETH-Gal4 - + +
C
Time to Climb 4 cm (s)
8
6
4
2
0
****
Faux Injected
Saline Injected
ETHInjected
D
Time to Climb 4 cm (s)
3
2
1
0
****
JHAMT-Gal4 + - +
UAS-Rpr.C;TubulinGal80ts - + +

Figure 5.1. ETH or JH deficiency cause hyperactivity (A) Time to climb 4cm for Inka cell-ablated males (ETH-Gal4;TubulinGal80^{ts}/UAS-Reaper) and genetic controls (n=50). (B) Time to climb 4cm for Inka cell-blocked males (ETH-Gal4/UAS-*Shibire^{ts}*) and genetic controls (n=50). (C) Climbing speed of Canton-S males faux injected, injected with 50nl of saline, and 50nl of 200μM ETH1 (n=30). (D) Climbing speed of CA-ablated (JHAMT-Gal4;TubulinGal80^{ts}/UAS-Reaper) and genetic controls (n=50). **** $p<0.0001$.

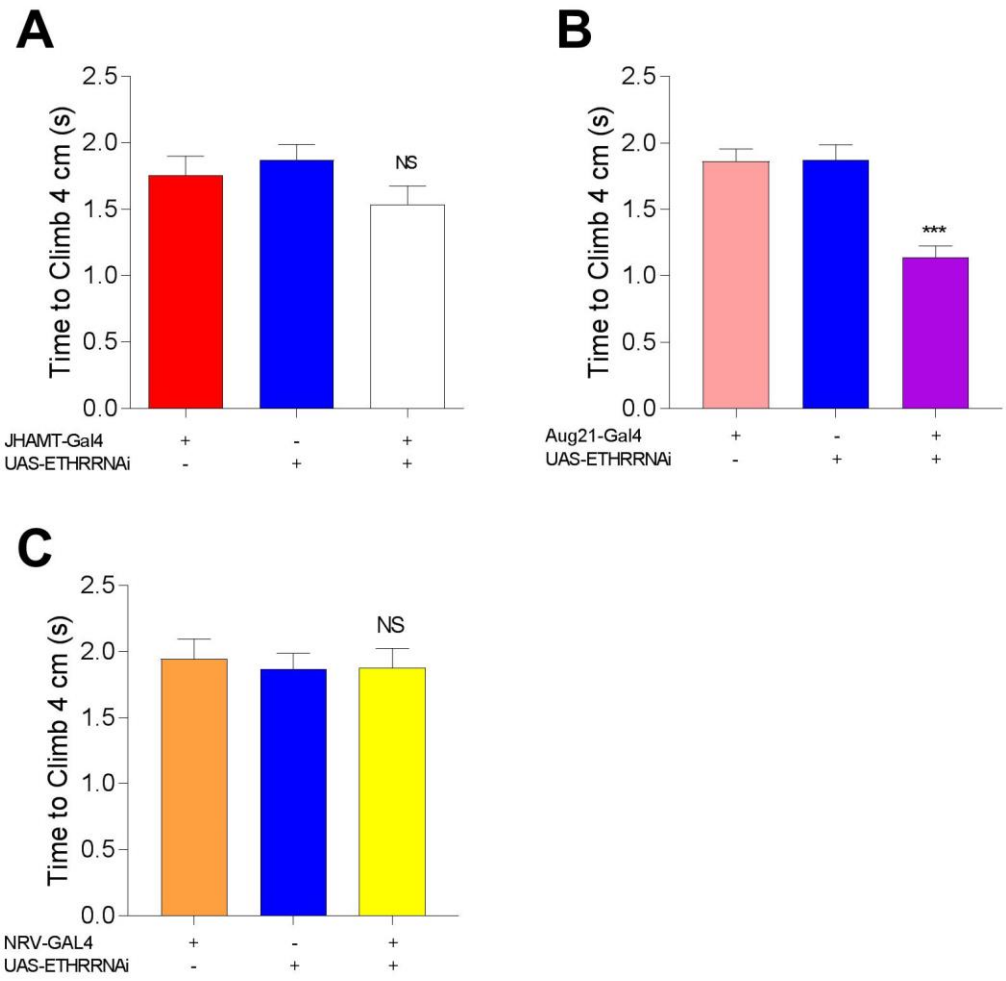

Figure 5.2. Corpus Allatum-driven ETHR silencing causes hyperactivity (A-C) Time to climb 4cm for CA ETHR-silenced specifically (JHAMT-Gal4 /UAS-CM3)(A), using the strong but less-specific Aug21-Gal4 (B), and the pan-neuronal driver Nrv-Gal4 (C) and genetic controls (n=50).

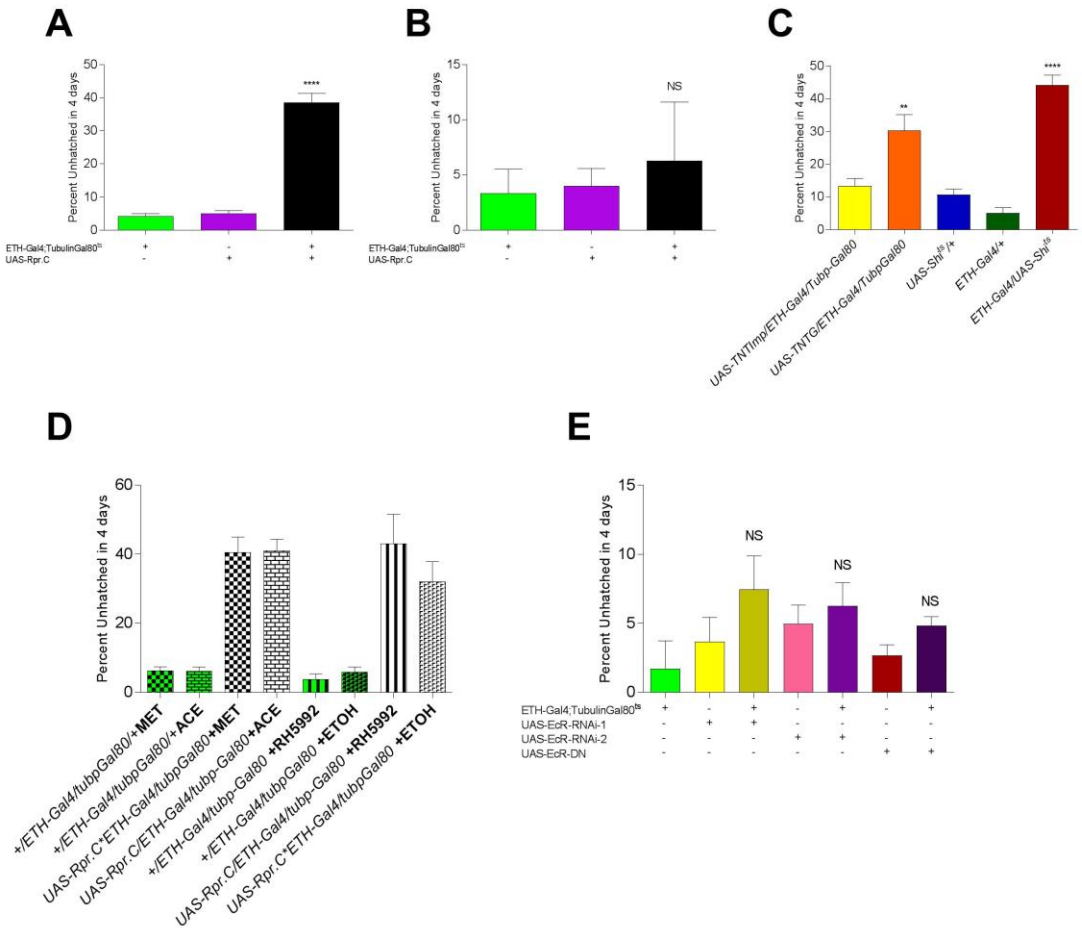

Figure 5.3. Inka cell release is necessary for egg hatching (A-B) Percentage of total eggs laid that hatched for Inka cell ablated (ETH-Gal4;TubulinGal80^{ts}/UAS-Reaper) and genetic controls at a Gal80^{ts} restrictive temperature (25°C)(A) and restrictive temperature control (18°C) (B)(n=30-40). (C) Influence of JH (methoprene) and 20E (RH5992) analog treatments on egg hatchability in Inka cell killed (ETH-Gal4;TubulinGal80^{ts}/UAS-Reaper) and Gal4 control females (n=25-35). (E) Hatch rate in females with ecdysone receptor silenced (ETH-Gal4;TubulinGal80^{ts}/UAS-EcR-RNAi) or a dominant negative isoform (ETH-Gal4;TubulinGal80^{ts}/UAS-EcR-DN) overexpressed in Inka cells (n=30)

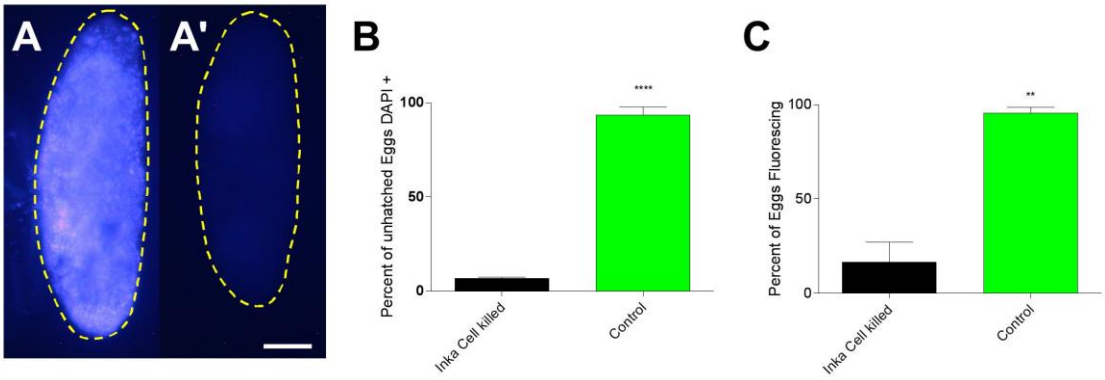

Figure 5.4. Unhatched eggs from Inka cell-ablated females are unfertilized (A) Example of unhatched, DAPI-stained egg taken from control females which generally arrested in late embryogenesis, and one taken from an Inka cell-ablated female (A'), which did not appear to have condensed nuclei (scale bar 50 μm). Yellow dotted line indicates outline of dechorionated, stained oocytes. (B) Quantification of eggs DAPI-positive or DAPI-negative from Inka cell ablated females and controls (n=3x100). (C) Quantification of percentage of unhatched eggs expressing paternally-donated ubiquitin-GFP from Inka cell ablated females and controls (n=3-5x100).

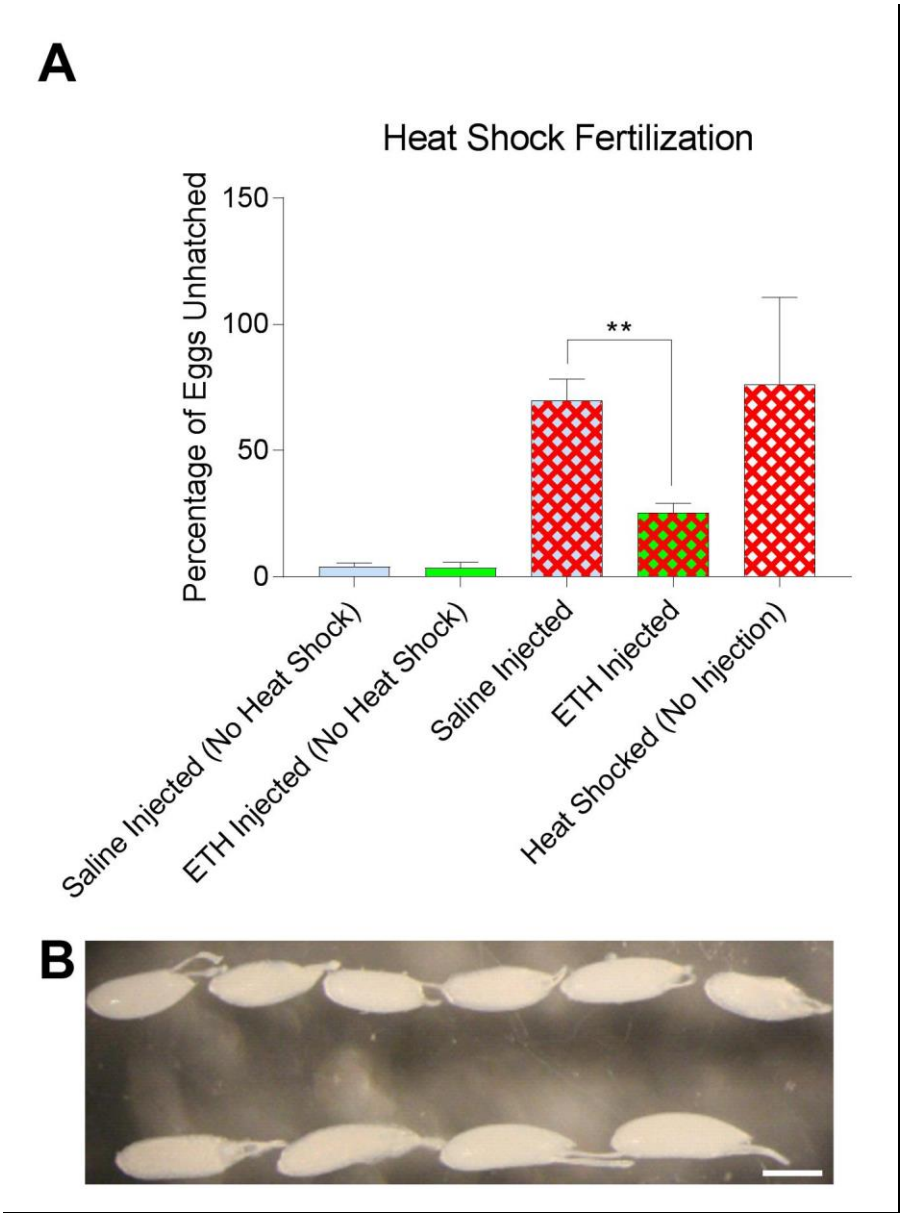

Figure 5.5. Heat shocked females share fertilization deficiency

(A) Heat shocked, Canton-S mated females were injected with ETH or saline prior to a 3 day egg laying period, percentage of eggs failing to hatch were compared (n=7-17). (B) Eggs taken from heat-shocked, methoprene-treated female ovaries (top) and acetone vehicle controls (bottom)(scale bar 100 μm)

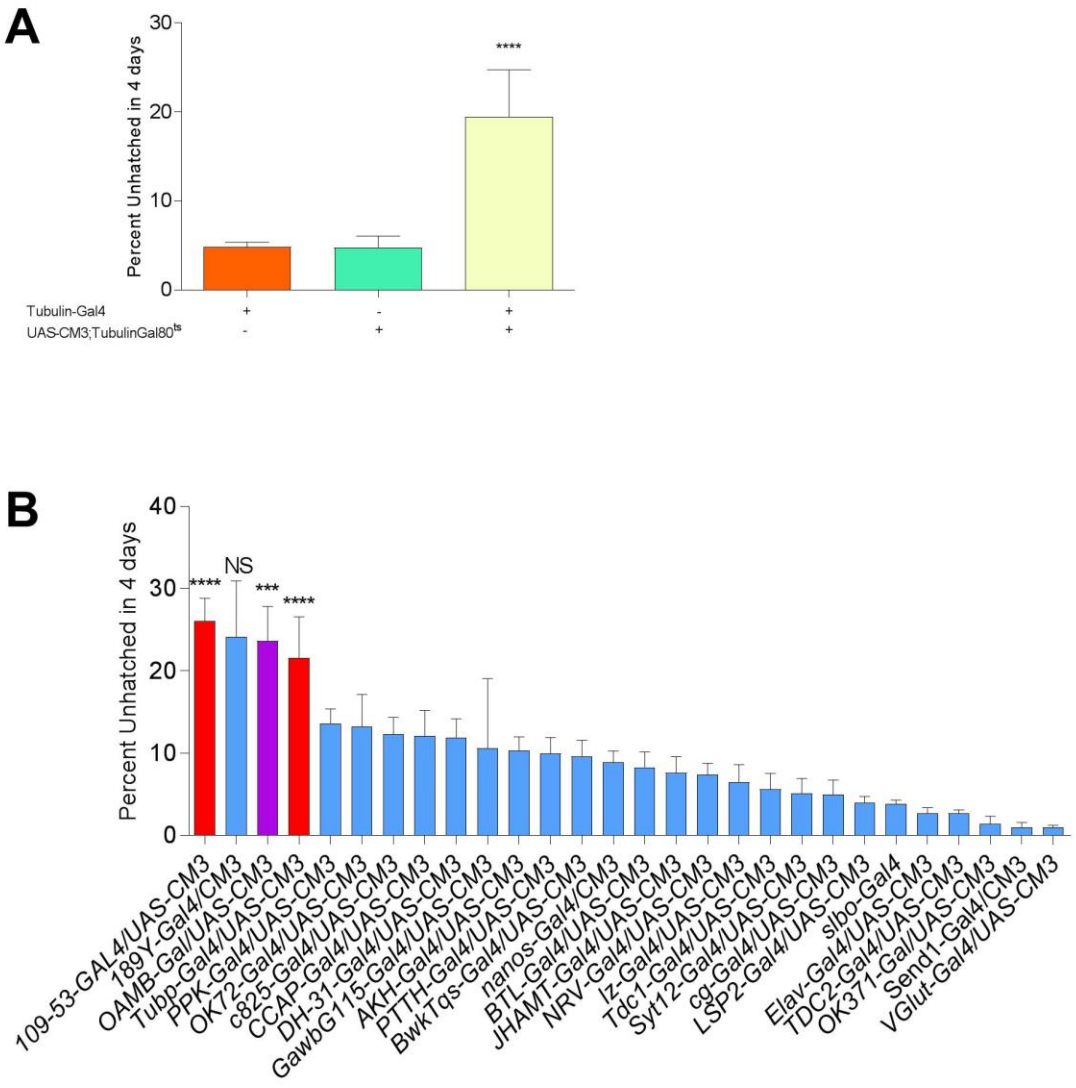

Figure 5.6. Directed Gal4 screen reveals 109-53-Gal4-expressing tissue depends upon ETHR silencing (A-B) ETHR-silencing in regions denoted by an array of Gal4s including the ubiquitous Tubulin-Gal4 (A) and various other Gal4s expressing in neuronal and reproductive tissue (B). Red bars indicate significance from both Gal4 and UAS-control, purple indicates significant but variable results.

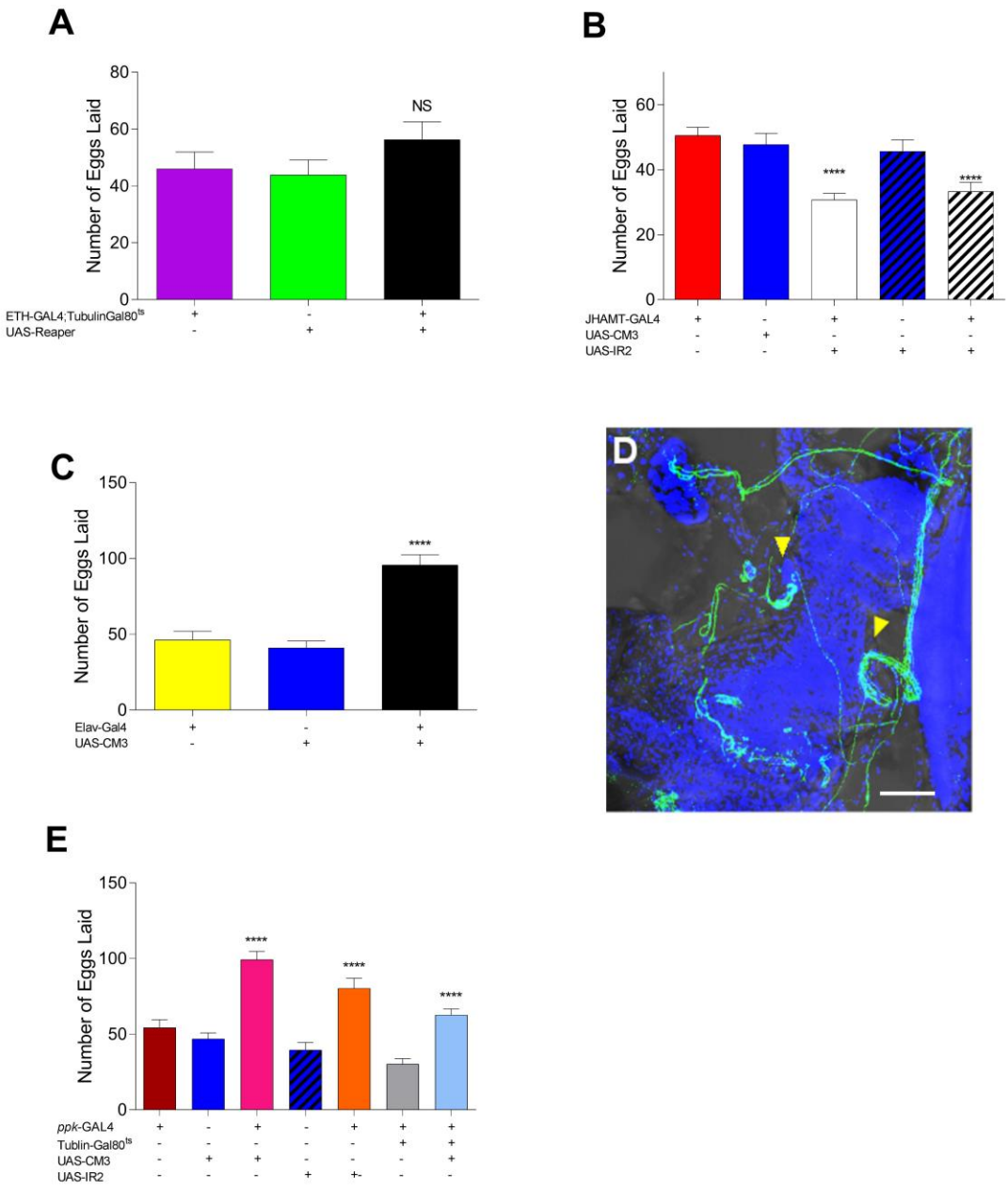
A
Number of Eggs Laid
80
60
40
20
0
NS
ETH-GAL4;TubulinGal80ts
UAS-Reaper
B
Number of Eggs Laid
60
40
20
0
****
****
JHAMT-GAL4
UAS-CM3
UAS-IR2
C
Number of Eggs Laid
150
100
50
0
****
Elav-Gal4
UAS-CM3
D
E
Number of Eggs Laid
150
100
50
0
****
****
****
ppk-GAL4
Tublin-Gal80ts
UAS-CM3
UAS-IR2

Figure 5.7. ETHR in ppk neurons is necessary for virgin-like egg laying (A) Virgin female egg laying in Inka cell-ablated (ETH-Gal4;TubulinGal80^{ts}/UAS-Reaper) females and genetic controls (n=30-40). (B) CA ETHR-silenced virgin female egg laying using two RNAi constructs (CM3 and IR2) (n=30-40). (C) Virgin female egg laying in females with ETHR silenced pan-neuronally (n=30). (D) ETHR-Gal4 expression in the abdominal nerve to the uterus (yellow arrowheads) known to be ppk and fru positive (scale bar 50 μm). (E) Egg laying of females with ETHR silenced in ppk neurons using two RNAi's, whole life, and specifically in the adult stage (n=20-30).

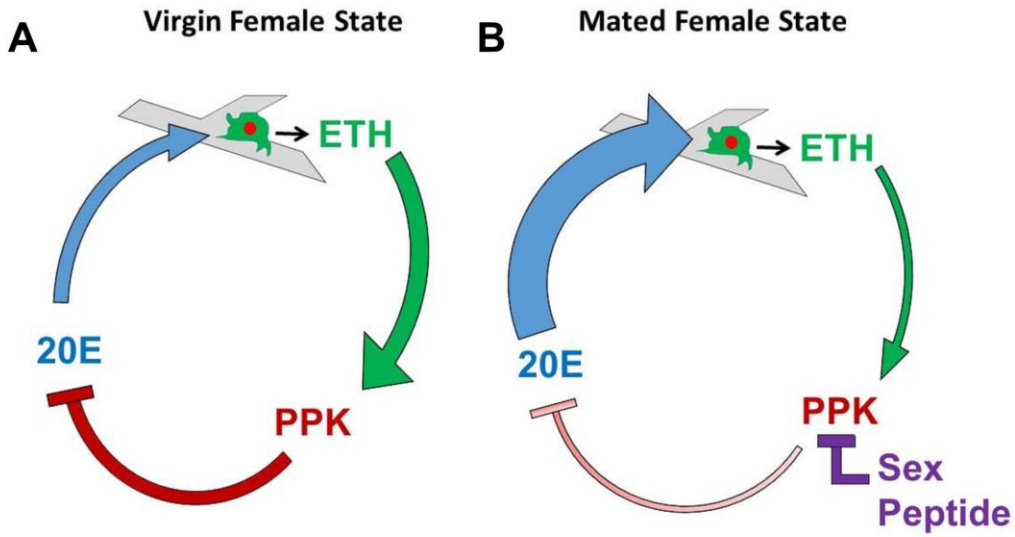

Figure 5.8. Model for maintenance of virgin female endocrine state (A) In the virgin female, 20E produced in the ovaries leads to ETH production, which maintains activity in ppk neurons, inhibiting greater 20E production, and thus maintaining state. (B) When mating occurs, sex peptide injected into female hemolymph inhibits the ppk neurons, reducing inhibition of 20E production. High 20E inhibits ETH release which, in turn, further decreases activity in ppk neurons.

Conclusions

While we rarely think of it as much, reproduction is undoubtedly the acme of an individual's fitness. To reproduce, an individual must not only possess a well-coordinated, successful system, it must also convey a variety of signals to express that success to a discerning member of the opposite sex. Its dependency on more simplistic systems makes the understanding the emergent and required complexity of reproductive endocrinology the pinnacle for the field. The work here not only establishes an endocrine hierarchy and hormonal axes for the first time in invertebrates, it goes on to examine the structural relationship of this hierarchy and the behaviors manipulated by these systems.

20hydroxyecdysone, ecdysis triggering hormone and juvenile hormone are highly conserved across the arthropod phylum, as are their canonical actions which govern molting (Klowden, 2013). The revelation that they work synergistically to optimize reproductive physiology is broadly relevant for management of arthropod friends (honeybees, brine shrimp, lobsters) and foes (anthropophillic mosquitoes, emerald ash borers, locusts) alike. Prior to the work from chapter 2, it was unclear whether ETH had any role whatsoever in the adult stage. We reveal that ETH is an allatotropin and that normal juvenile hormone levels depend upon input from ETH. Chapter 2 also maps the expression pattern of the glandular source of ETH, the Inka cells, for the first time. Their pattern and proximity to their targets in the ovaries and CA may suggest a paracrine regulation, given that the eth receptors depend upon relatively high concentrations of eth for activity (Park, Kim, Dupriez, & Adams, 2003). We also show that the eth genes follow common regulation in males and females, but expression patterns are sexually

dimorphic, an observation that laid the foundation for investigation of Eth and reproduction. We also found that transcript levels of the eth genes change after mating and synchronization dissipates.

While the influence of ETH on the corpus allatum had been suggested(Areiza, Nouzova, Rivera-Perez, & Noriega, 2014; Yamanaka et al., 2008), its influence lacked functional significance. This dissertation suggests that JH depends upon ETH and this dependency regulates the rate of preparation of physiology for reproduction. As ETH is regulated by ecdysterioid levels(Kingan & Adams, 2000), this suggests large-scale coordination for regulation of reproductive behavior, specifically for oogenesis. Micro pulses of 20E facilitate ovariole progression (Morris & Spradling, 2012), we show here that this initiates a hierarchical hormone cascade. While ETHR does not express in ovarioles, ecdysteroid receptor expresses in germaria and in late stage follicle cells, JH receptor expresses in midoogenetic oocytes and octopamine receptor expresses in the follicular terminal during very late oogenesis. JH and 20e levels are inversely related in the female(Dubrovsky, Dubrovskaya, & Berger, 2004; Terashima & Bownes, 2006), and octopamine and JH occur simultaneously(Gruntenko, Karpova, & Alekseev, 2007). *Drosophila melanogaster* is known among insects for its asynchronous egg production (Huynh & St Johnston, 2004), but the hormonal dynamics suggest a model wherein 20E micro pulses causes "semi-synchronous" progression of oogenesis, with the amplitude of either phase determining the rate of reproduction.

The identification of octopamine in the female reproductive tract was also an important revelation for the dynamics of reproductive behavior. The terminal step in

oogenesis is typically ovulation, which involves octopamine activation of both follicle rupture and egg release from ovaries (Middleton et al., 2006; Sun, Spradling, & Banerjee, 2013). We find here that in addition to regulation of JH levels, ETH regulates egg release, again, as a systemic coordinator. When steroid levels surge in response to a variety of stressors, hemolymph ETH levels decline leading to lower JH and OA, and lower oogenesis, ovulation, and emergent fecundity as a result. The discovery of these dynamics establishes an elegant new paradigm for understanding stress and reproduction, and the larger question of how biological systems interact with their environment. The pressing question of 20E's relationship with ETH emerges, as ETH is simulataneously dependent on EcR for normal fecundity in the unstressed context and inhibited by it during stress. More than likely, the Inka cell exhibits a state-specific response to ecdysone signaling, in the unstressed context secretory competence is permitted, where high steroid concentration in the stressed state inhibits it. The obvservation that "unstressed" females have capacity for lower 20E and higher ETH suggests further complexity to the endocrine system, and the inevitable expansion of *Drosophila melanogaster*'s should reveal coregulators which may elucidate the answers to these questions of dynamics.

The establishment of causality in this system led to a simple new assay for stress; egg retention. Exploited this causality with the discovery of stress imposed by carcass-derived scent. Animals can detect species-specific information on decomposing bodies, but whether this is active adaptation or exploitation of natural occurring species specific volatile chemical profiles remains unclear (Kats & Dill, 2016). In nature, this is a highly

beneficial mechanism, because it allows an individual to detect regions where threats exist. In chapter 3, we suggest stress-induced arrest of reproduction can occur as an energy conservation mechanism. The stress response in this case is unlikely to be for the purpose of energy conservation. Instead, the organism arrests ovulation because not only is there a perceived threat to the individual, but also to the offspring. This may be some of the first evidence of invertebrates predicting an outcome without the aid of higher order processing.

Chapter 4 investigates the role of ETH in the male. While we find in the first chapter that ETH in the male is critical for synthesis of fecundity-stimulating accessory gland proteins, the largest abnormalities we observed in the males were in behavior, rather than physiology. Males courted virgin and mated females as well as males indiscriminately; suggesting the neural circuitry necessary for courtship discrimination was severely impaired. Courtship discrimination depends upon pheromone sensation. Examination of the expression pattern of ETHR-Gal4 revealed a variety of sensory neurons necessary for pheromone sensation and processing, suggesting the increased courtship was due to inability to sense inhibitory pheromones. Indeed, when we silenced ETHR specifically in GR32A and OR67D we saw reduced neuronal sensitivity to ligands and disinhibition of courtship behavior.

In context, this suggests ETH plays a somewhat similar role in both males and females. In females, high ETH coincides with resumption of reproductive behaviors and of egg laying. In the male, it stimulates courtship discrimination and synthesis of components of male seminal fluid. When ETH is elevated in a male and female

counterpart, one would expect reproduction would be optimized. However, the elimination of ETH from hemolymph resulted in *elevated* courtship levels, which may suggest its role as a courtship inhibitor is stronger than its potential role as a courtship stimulator through OR47B and NP3056-labeled neuron stimulation. However, injection of ETH into wild-type males in a variety of contexts, including stress, was unable to perturb courtship behavior toward virgin, mated females or males. The implication is that ETH levels are at their maximum in laboratory conditions or that ETH's role as a courtship inhibitor has not been properly addressed with the experimental conditions. An intriguing third possibility is that ETH deficiency and ETHR silencing may be working through different mechanisms. ETH deficiency over time causes JH deficiency, which could delay maturation of critical courtship neurons. ETHR silencing may be eliminating a receptor that is necessary for cell homeostasis, as it has been shown that the mammalian homolog of ETHR, growth hormone secretagogue receptor (GHSR) is highly constituitively active (Pantel, 2006). While these questions remain unanswered, we have presented clear evidence that both ETH and ETHR are critical for proper courtship discrimination in males and have laid a foundation for a landmark study into behavioral endocrinology.

Many questions about the role of ETH and its receptors have yet to be clarified. ETH causes hyperactivity by regulating JH levels and targeting unknown neurons. Further examination is needed, but flies deficient in ETH may be deficient in JH and broadly in catecholamines (Gruntenko et al., 2005). If this is the case, ETH deficient

adults may be a better model for ADHD than the current dopamine based models (De Luca et al., 2002; Lebestky et al., 2009; van Swinderen & Brembs, 2010).

In line with our model, we found female ETH deficiency also suppresses fecundity through decreased fertilization of oocytes. The current body of evidence suggests that ETH receptor in the oviduct epithelium may also be critical for proper fertilization. One mechanism by which this could occur is impaired hydration of the oviductal lumen leading to incomplete egg activation preventing proper sperm penetrance. Again, further testing is needed to examine this hypothesis.

The ability of ETH to activate ppk neurons is an interaction with profound consequence for our established female endocrine network. After mating, female hemolymph becomes laden with sex peptide (Lung & Wolfner, 1999; Pilpel, Nezer, APPLEBAUM, & Heifetz, 2008). This protein acts as a hormone activating both the CA to turn on JH production and to inihibit ppk neurons, leading to increased production of 20E. The CA and ppk neurons are both targets of ETH and it seems ETHR activity is somewhat obviated by sex peptide after mating. Additionally, it suggests a feedback mechanism that allows for elevation of both JH and steroid levels simultaneously after mating (Harshman, Loeb, & Johnson, 1999; Sugime et al., 2017), despite their antagonistic roles. In virgins, ETH stimulates ppk neurons, inhibiting 20E release and elevating JH levels, leading to oocyte synthesis in preparation for mating. However, after mating, 20E levels increase, which should suppress ETH release according to the principles we've established here. Simultaneously, JH levels, which depend upon ETH increase. If sex peptide can turn off ppk and turn on the CA, mating should dampen the

effect of ETH signaling. However, as we know steroid strongly upregulates ETHR in females (Meiselman et al., 2017), depressed levels of ETH relative to the virgin may still have a critical role.

In this dissertation we establish the role of ETH as a critical adaptor in the adult stage. A prolific liberin in the developmental stages, the hormone continues to diversify and proliferate signals from its regulator in 20E to a variety of tissues and glandular targets. Hormones have a probabilistic effect on behavior, manipulating neuroeconomics and tissue in a spatiotemporally isolated manner. One could go so far as to suggest that the endocrine system is essentially a "second brain," changing the machinations of the neural circuitry already in place. Neurological and physiological plasticity depend deeply on the endocrine system, and in order for organismal biology to progress, we must all learn to appreciate the endocrine context.

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