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A Hormonal Signaling Cascade During an Early Adult Critical Period Required for Courtship Memory Retention in Drosophila

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Summary

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

Introduction

The faculty to acquire and preserve information is essential for adapting to environmental changes and species propagation. As in vertebrates, a variety of studies in invertebrates have revealed the importance of hormones in learning and memory. Regarding the fruit fly *Drosophila melanogaster*, biogenic amines contribute to diverse memory forms by influencing neuronal activity in the brain [1-4]. Recent studies have shown that the steroid 20-hydroxyecdysone contributes to memory formation and retention through distinct mechanisms [5, 6]. However, hormonal regulation of circuits mediating learning and memory in *Drosophila* is still poorly understood.

During juvenile development, insects perform periodic, hormonally-driven ecdysis behaviors that are obligatory for shedding of the old cuticle at the end of each molt. Ecdysis triggering hormones (ETH) released from epitracheal gland Inka cells initiate each ecdysis sequence via activation of ETH receptors (ETHRs), G protein-coupled receptors activating Gαq pathways in separate neuronal groups [7-13]. Although Inka cells and transcripts of *ETH*/*ETHR*s are present in adult *Drosophila*, roles of ETH signaling in adult stage have not been described [14-16].

Release of the sesquiterpenoid JH from the *corpora allata* (CA) promotes juvenile body plan and its effects during the pre-adult period have been extensively studied in a broad range of insect species. In *Drosophila*, the *Methoprene-tolerant* (*Met*) gene encodes a bHLH-PAS protein, which functions as a JH receptor. Functions of Met in developmental and reproductive events are complemented by the paralog germ-cell expressed (gce) [17]. During adulthood, JH is re-purposed as a gonadotropic hormone, coordinating vitellogenesis, ovary maturation, pheromone synthesis of females, and mating behaviors of both males and females [18-29]. In previous studies of the honeybee, JH determines social status and regulates olfactory memory of adult animals, possibly by affecting aminergic circuits in brain [30-34]. However, roles of JH in adult *Drosophila* behaviors remain largely undescribed.

Recent reports indicate that ETH functions as an allatotropin in mosquitoes (*Aedes aegypti*) and flies (*Drosophila melanogaster*) [35, 36]. In this study, we found that the ETH-JH signaling cascade is required for memory performance of male *Drosophila* by applying a simple learning paradigm known as courtship conditioning. In this paradigm, male courtship intensity is modified by previous experience with a courtship partner [37]. Virgin females are highly receptive, but mated females are unreceptive because of the presence of sex peptide (SP) in the seminal fluid given by previous partner [38, 39]. We provide evidence that ETH regulates courtship memory maintenance of male *Drosophila* through promotion of JH synthesis and activation of dopaminergic neurons. Together, our study thus reveals a hormonal cascade consisting of ETH-JH-dopamine to regulate a critical period for learning and memory during adulthood.

Results

ETH is an obligatory regulator of JH levels in adult males

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

To confirm presence of ETHR in male CA, we expressed double-stranded RNA constructs targeting the *ETHR* gene in the CA by using the CA-specific driver, *JHAMT-GAL4* (Fig. 1B) [28]. *JHAMT* gene encodes JH acid *O*-methyltransferase, which is an enzyme catalyzing one of the final steps of JH synthesis. JHAMT is predominantly present in the CA [40]. Quantitative PCR measurements showed knockdown of relative transcript number by ~42% in males (Fig. 1C, females in Fig. S1).

To investigate actions of ETH on CA of adult males, we monitored intracellular calcium levels *in vivo* by preparing a transgenic fly expressing the Ca^{2+} indicator GCaMP5 in CA via the *JHAMT-GAL4* driver (Fig. 1D-a). We observed robust increases in Ca^{2+}-associated fluorescence

in the CA following exposure to ETH. In contrast, CA of *ETHR*-silenced males exhibit sharply decreased calcium mobilization in response to ETH exposure (Fig. 1D-b and 1D-c). Analysis of Ca^{2+}-associated fluorescence traces provides evidence that RNAi silencing of *ETHR* in the CA not only suppresses cytoplasmic Ca^{2+} accumulation, but also delays the response to ETH (Fig. E).

Silencing of *ETHR* expression specifically in the CA (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym*) leads to marked reduction (>70%) in JH levels compared to genetic control males (Fig. 1F), demonstrating an essential role for ETH targeting adult CA for regulation of JH synthesis. This reduction has serious consequences for memory retention, as we demonstrate in subsequent sections of this paper.

ETH regulates courtship memory through downstream JH signaling

We investigated whether reduction of JH levels by RNA knockdown of *ETHR* affects social-context-dependent learning and memory of males using the courtship conditioning paradigm. In this paradigm, males experiencing rejection by unreceptive, mated females reduce courtship activity when paired subsequently with virgin females. We found that courtship index (CI) of *ETHR*-silenced males was not significantly suppressed after the training, while both genetic controls showed significant suppression toward the tester females (Fig. 2A). Memory performance is expressed as memory performance index (MPI) (see Materials and Methods section). Impaired courtship memory performance was observed using RNAi lines directed at independent sequences in the *ETHR* gene: *UAS-ETHR RNAi-Sym* and *UAS-ETHR RNAi-IR2*. These results indicate that ETH signaling in the CA is necessary for short-term courtship memory performance (representative courtship activity changes in Fig. S2; statistical analyses of each genotype are listed in Table S1).

We next tested the hypothesis that reduction of JH levels during adulthood affects memory performance by employing rescue experiments with the JH analog methoprene. Methoprene application immediately after eclosion (day 0) rescued memory deficiency of *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* males, whereas vehicle-treated *ETHR*-silenced and methoprene-treated genetic control males showed no significant changes in memory performance (Fig. 2B). This provides direct evidence that JH deficiency is of crucial importance for normal memory performance.

Since memory indices presented here (CI, MPI) are based on male locomotory activity directed toward females, changes in basal locomotory activity may influence apparent courtship activity. Influences of JH on behavioral basal activity and courtship activity have been reported, in particular hyperactivity resulting from JH deficiency [18, 21, 22, 25, 29]. Whether JH levels influence basal activity of males or not, it is notable that the hyperactivity in male locomotion is likely not correlated with courtship avidity [18]. To clarify, we first tested male locomotion using a negative geotaxis (climbing) assay and found that mean velocity of JH-deficient males was statistically similar to that of genetic controls. We also found no statistical differences from controls in successful copulation rates and courtship behavior (courtship singing) toward mature virgin females. Since we used immobilized virgin females as testers in conditioning trials, courtship indices were analyzed toward a decapitated virgin female. As expected, CI measures of JH-reduced males were not significantly different from control males (Table S2).

JH is essential for courtship memory retention

Decreased memory performance (MPI) may be caused either by loss of learning ability during the training session or by defective retention of memory during the post-training assay period. To assay for learning during the training session, we measured learning performance index (LPI). Upon experiencing continuous rejection during a 1-hour training period with a mated female, both genetic control and *ETHR*-knockdown males exhibit decreased courtship index (CI) during the final 10 min of training as compared to the initial 10 min interval (Fig. 2C). Reduced CI during the training period is considered as memory acquisition or learning ability [41]. These results thus indicate that marked reduction of MPI in JH-deficient males during the subsequent test period is not attributable to loss of learning ability.

To test whether reduction of JH levels by *ETHR* silencing negatively affects memory retention, we performed a memory decay assay. Following 1-hour training with mated females, males were tested with immobilized, decapitated virgin females at sequential intervals over a total of 10 min. Whereas control males showed no significant loss of memory during this post-training period, *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* males exhibited a gradual decline in memory performance over the 10 min interval (Fig. 2D). These data demonstrate that JH deficiency leads to loss of memory retention, even during the short-term post-training interval.

Although JH-deficient males exhibit olfactory deficits, courtship memory occurs in absence of aversive, mating-associated chemical cues

Males rejected by mated females are exposed to aversive chemical cues (e.g., cVA) considered to be primary factors contributing to reduction of subsequent courtship intensity, defined as courtship memory [42, 43]. However behavioral rejection cues also may contribute to courtship memory. We therefore asked: 1) whether ETH-JH deficiency leads to loss of chemosensory sensitivity to post-mating chemical cues, and 2) whether courtship memory following training with mated females can be demonstrated in the absence of aversive pheromonal cues from the mated trainer female. To address these questions, we dissociated influences of aversive chemical cues from behavioral rejection cues by pairing males with either pseudovirgin (Ψ_v) or pseudomated (Ψ_m) females [44].

We prepared Ψ_v females by crossing Canton-S virgin females with sex peptide-null mutant males (SP_0). Even after mating, Ψ_v females are still receptive to courting males because they did not receive the gift of sex peptide from the prior partner, but nevertheless smell “bad”, having been “perfumed” with the aversive male pheromone, cVA [45]. As expected, *GAL4* control males showed reduction in accumulated copulation rates when paired with Ψ_v females compared to pairings with virgin females (Fig. 3A-a). Notably, JH deficient males showed relatively less suppression of copulation rate when paired with Ψ_v females under the same conditions. Likewise, although control males displayed lower courtship indices (CI) toward Ψ_v females, JH-deficient males showed no significant suppression of courting activity toward Ψ_v females compared to virgin females (Fig. 3A-b). These data indicate that JH deficiency indeed reduces sensitivity to aversive chemical cues associated with a mated female, which could account for some measure of elevated CI - defined as loss of courtship memory - in courtship-conditioned, JH-deficient males.

To determine relative importance of behavioral cues vs. aversive chemical cues during training with mated females, we tested memory performance of control males using pseudo-mated (Ψ_m) female (*elav-GAL4*/*UAS-SP*) trainers, which are virgins that express sex peptide. Since virgin females expressing sex peptide are refractory to male advances without prior mating, we could assay for behavioral cues in the absence of aversive post-mating pheromonal cues. When trained with Ψ_m females, control males exhibited high MPI for suppression of subsequent courtship activities that were indistinguishable to those shown when they were trained with mated females (F_m), indicating that rejection in the absence of aversive chemical cues is sufficient for induction of short-term courtship memory (Fig. 3B). We found that MPI exhibited by *ETHR*-silenced, JH-deficient males was equally low, whether they were trained by mated or Ψ_m females (Fig. 3B). These data suggest that rejection in the absence of aversive chemical cues (i.e., solely on the basis of behavioral cues) is sufficient to elicit optimum MPI levels in controls and that JH deficiency markedly reduces sexual-deprivation-dependent memory in spite of olfactory deficiencies during training.

Influences of ETH-JH signaling on memory are specified during the adult period

Rescue of memory deficits by methoprene (Fig. 2B) suggests essential roles for JH in memory performance during adulthood. Our findings suggest further that ETH plays a critical role in regulating memory performance through its maintenance of JH levels. We were concerned whether this regulation was a residual effect of ETH released at eclosion or whether continued release from Inka cells persists in mature adults.

We therefore investigated the timing of ETH release during adulthood using several genetic approaches. First, Inka cells were ablated by applying the TARGET (temporal and regional gene expression targeting) system [46]. Temporal expression of pro-apoptotic genes (rpr and hid; [47, 48]) targeting Inka cells for cell killing resulted in significant memory performance deficit (Fig. 4A). This was confirmed by applying the ligand-inducible GAL4-based GeneSwitch/UAS system using an Inka cell-specific GeneSwitch line (*ETH-GeneSwitch*, *EUG8*) [49]. As in the TARGET experiment, conditional Inka cell-ablation significantly impaired memory performance. We next performed conditional block of ETH release by expressing tetanus toxin light chain (TeTxLC) via the same GeneSwitch driver (*EUG8*). TeTxLC catalytically inhibits vesicle release once present in the cytosol by cleaving synaptobrevin [50]. Adult-specific expression of active TeTxLC in Inka cells (*UAS-TNT$_G$*) significantly impaired memory performance compared to vehicle-fed males, whereas the inactive TeTxLC expressing males (*UAS-TNT$_{imp}$*) showed no significant change (Fig. 4A). These data confirm that ETH release from Inka cells, as well as *ETHR* expression in the CA, are essential for normal memory performance through regulation of downstream JH signaling.

We next investigated whether ETH signaling during development is required for proper "wiring" of the CNS through stage-specific *ETHR* silencing in the CA using the TARGET system. *ETHR* silencing in the CA during the pre-adult period led to no deficits in normal memory performance, while post-eclosion (adult period only) *ETHR*-silenced males and positive controls showed significantly impaired memory performance (Fig. 4B). JH reduction driven by *ETHR* knockdown in the CA showed no gross morphological abnormalities in the brain (Fig. S3). These observations, along with our previous methoprene rescue data, show that ETH signaling-dependent JH levels during adulthood are essential for normal memory performance, and that

ETH-induced developmental events do not contribute to the memory deficit phenotypes we describe here.

ETH-JH signaling is functional during an early adult critical period for memory performance

During adulthood, JH may play distinctive functional roles in the CNS during different age periods [18, 30, 33]. Since age-dependent JH levels are different in males and females (female in [51], male in Fig. S4), we hypothesized that a critical period for JH action may influence memory performance. We therefore tested age-dependent efficacy of methoprene rescue of memory deficits in JH-deficient males. Interestingly, impaired memory performance of JH deficient males (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym*) could be rescued by methoprene only during early adulthood. In the first round of experiments, methoprene was applied topically on the following days posteclosion to separate groups of males: day 0, 1, 2, 3, 4, 10 (courtship conditioning and memory assay were performed 4 days after application in each instance). Methoprene treatment on posteclosion males days 0 and 1 rescued memory performance, while treatment on day 2 showed some degree of MPI improvement that did not reach statistical significance (Fig. 5A, B). Later methoprene treatments on days 3, 4, and 10 were clearly ineffective. Day 2-6 methoprene-treated males show significant courtship suppression (Table S1), but no significant difference in MPI compared to vehicle-treated animals. Memory performance of *GAL4* control males was not affected by methoprene (Fig. 5A and B).

GAL4 control males also exhibited gradual loss of memory performance with age; older (day 10) males have low levels of JH (Fig. S4), however age-dependent memory loss is likely JH-independent, since methoprene treatment is ineffective in restoring memory performance after day 6 (Fig. 5B).

To define more precisely the critical period for methoprene-dependent memory recovery, we applied methoprene to progressively older posteclosion *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* males and assayed for memory performance 24 hr later. We treated groups of individuals on posteclosion days 0, 1, 2, 3, 4, and 5 with either 64 pmol (1x) or 322 pmol (5x). While the lower dose of methoprene was ineffective, the higher dose clearly rescued memory performance of males treated on posteclosion days 0, 1, and 2 (Fig. 5C). Although the courtship indices of day 0 and 1 *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* males are lower than *GAL4* control groups, higher dose methoprene treatment likely increases courtship activity of *ETHR*-silenced males, indicating that JH may also affect sexual maturation in early period (Table S3). Taken together, our evidence demonstrates that promotion of courtship memory performance by JH is confined to a critical period during the first three days of adulthood.

JH regulates memory performance by targeting TH-positive neurons

Although *Drosophila* expresses two JH receptor paralogs (*Met* and *gce*) in the brain [15, 52, 53], their functions in adult behavior remain unclear. Since JH likely plays a role in formation and/or function of the memory circuit, we employed RNA knockdown of both receptors in candidate brain regions thought to be important in memory and behavior. It is well established that mushroom body (MB) neurons are involved in both short-term and long-term memories [54, 55], in part through monoamine-based signaling. Glutamate is a key neurotransmitter contributing to

cognitive ability and learning and memory in a variety of species. A recent study revealed that subsets of glutamatergic neurons innervating MB neurons operate in the memory retrieval (recall) following short-term conditioning [56]. *Orco* (Or83b), a co-receptor expressed in broad range of olfactory receptor neurons (ORNs), is essential for ORN functions contributing to associative learning and memory [57].

We assessed memory performance following RNAi knockdown of both *Met* and *gce* in a number of different neuronal types, including mushroom body (MB; *OK107-GAL4*), dopaminergic (DA; *TH-GAL4*), octopaminergic (OA; *Tdc2-GAL4*), serotonergic (5-HT; *Trh-GAL4*), glutamatergic (Glut; *OK371-GAL4*), and olfactory receptor (*Orco-GAL4*) neurons. Silencing of *Met*/*gce* in DA neurons significantly impairs memory performance without affecting basal courtship intensity, whereas *Met*/*gce* knockdown in OA, 5-HT, Glut, and olfactory receptor neurons did not cause memory deficiency (Fig. 6A). Notably, although *Met*/*gce* silencing in ORNs (*Orco-GAL4*/*UAS-gce RNAi;UAS-Met RNAi*) led to significant reduction in courtship of naïve males toward immobilized virgin females (tester) (Fig. S5), they showed normal memory performance following training with a mated female. This is consistent with our prior result (Fig. 3), showing that behavioral cues associated with rejection (sexual deprivation) are equally or more important than exposure to chemical cues for the memory performance. RNA knockdown of either *Met* or *gce* alone using the *TH-GAL4* driver did not impair memory performance, indicating that the two receptor subtypes are effective in compensating for memory deficits (Fig. 6B). Taken together, our results identify DA neurons as targets for ETH-JH signaling in establishment of short-term courtship memories.

Discussion

Key findings reported in this study are that ETH signaling is required for maintenance of normal JH levels in adult *Drosophila* males and that JH deficiency brought about by interruption of ETH signaling leads to rapid memory loss. These basic findings, along with important mechanistic details, can be summarized as follows. First, CA cells respond to ETH by mobilizing calcium, while genetic suppression of *ETHR* expression specifically in the CA reduces calcium mobilization leading to a ~70% drop of JH levels during the first week of adult life (Fig. 1). Second, JH deficiency produced by interruption of ETH signaling results in impairment of STM under the courtship conditioning paradigm; this phenotype is rescuable by treatment with methoprene (Fig. 2A and 2B). Third, JH-dependent memory performance relates to memory retention as opposed to acquisition (Fig. 2C and 2D). Fourth, optimal memory performance of trained males toward subsequent encounters with virgin females is induced by behavioral cues provided by mated (pseudo-mated) trainer females in absence of aversive post-mating chemical cues; ETH-JH deficiency leads to rapid extinction of this memory. Fifth, JH dependence of memory performance occurs during a critical period - the first 2-3 days of adult life (Fig. 2B, Fig. 4, and Fig. 5). Finally, cellular targets of JH that mediate STM are TH-positive dopaminergic neurons (Fig. 6A and 6B). We propose a model summarizing these findings (Fig. 6C). Our results are further discussed below in the context of previous accounts of JH influences on adult behaviors.

ETH functions as an obligatory allatotropin for courtship memory in adult *Drosophila*

Neuropeptide allatotropins (AT) known to stimulate JH production in a wide range of insects [58], but these peptides have not been found in *Drosophila*, although some allatotropic actions of

neurotransmitters have been reported, including glutamate [51, 59]. We recently reported on peptidergic regulation of JH synthesis in *Drosophila* [36]. In both sexes, ETH-JH signaling is essential for attainment of normal reproductive potential, including vitellogenesis and egg production in females. However, the functional significance of ETH as an allatotropin with respect to cognitive behavior is not known. Here we report for the first time evidence that ETH signaling is critical for courtship memory performance. Calcium is critical for JH biosynthesis, since CA cells cannot produce JH in calcium-free medium *in vitro* [60]. A well-known consequence of Gαq-coupled signal transduction is liberation of IP_3 (inositol 1,4,5-triphosphate)-dependent intracellular calcium release from stores. We found that ETH mobilizes calcium in CA cells, but much less so following RNAi knockdown of *ETHR* in the CA. These results provide clear evidence that ETH functions as an obligatory allatotropin crucial for STM in adult male *Drosophila*.

Influences of JH on *Drosophila* courtship behavior and memory performance

Drosophila courtship involves a sophisticated behavioral sequence involving neural circuitry integrating multiple sensory inputs for decision-making [61, 62]. Roles of JH in male courtship behavior are diverse, depending on the insect species. For example, it is well known that JH influences social interactions through pheromone recognition. In the locust *Schistocerca gregaria* and the moth *Agrotis ipsilon*, JH plays a critical role in setting male sensitivity to pheromones, which promotes context-specific behavioral responses toward both genders [63-66]. In male *Drosophila*, JH esterase-binding protein overexpression, which enhances JH esterase function and hence JH degradation, is reported to reduce pheromone production, thus enhancing homosexual tendencies [22]. A recent study showed the importance of the *JHAMT* gene in male courtship, and that reduced courtship index observed in *JHAMT*-silenced males is likely caused by reduction of JH biosynthesis [28]. Another study provides a clue for the neural mechanism underlying JH promotion of male courtship. Expression of *Methoprene-tolerant* (*Met*) in Or47b neurons enhances male sensitivity to female cuticular hydrocarbons, thereby facilitating successful courtship [21]. This account provides compelling evidence supporting a role for JH in regulation of pheromone sensing by male *Drosophila*.

In the present study, we also found that JH may influence pheromone recognition of males. Elevated courtship activity and successful copulation rates of JH-deficient males paired with receptive, pseudo-virgin females (Ψ_v in Fig. 3A) suggest two possible explanations: hypersensitivity to aphrodisiac pheromones (e.g. 9-pentacosene [67] and palmitoleic acid [21]) or insensitivity to anti-aphrodisiac (e.g. cVA) pheromones. Since *Met*-expressing Or47b neurons promote courtship [21], we hypothesize that JH may be also important in recognition of anti-aphrodisiac pheromones (e.g., cVA). This hypothesis is supported by two lines of evidence produced in this study. First, JH-deficient males produced in this study show no significant change in courtship toward virgin females (Table S2), indicating that JH-reduction does not promote hypersensitivity to female pheromones. Second, we found that RNAi knockdown of JH receptors broadly in ORNs suppressed courtship significantly, likely caused by poor detection/recognition of target females (Fig. S5). Variability in courtship activity of naïve JH receptor-silenced males (*Orco-GAL4*/*UAS-gce RNAi;UAS-Met RNAi*) line could be caused by low expression of *Orco* (*Or83b*) in Or47b neurons [68].

Our finding that JH deficient, ETHR-silenced males exhibit no change in courtship index differs from results recently reported by Wijesekera et al., who showed that silencing of the *JHAMT* gene in CA suppresses male courtship activity significantly [28]. Although JH levels

were not assessed in this study, it was presumed that JH deficiency resulted from *JHAMT* knockdown, since the phenotype was rescued with methoprene. The apparent discrepancy between the two studies likely arises from differences in courtship assay protocols used. Wijesekera et al. paired males with immature, pheromone deficient females (day 0 posteclosion), whereas we used mature, day 4 decapitated virgin female testers in this study. To clarify the apparent discrepancy between our study and that of Wijesekera et al., we compared courtship indices of JH-deficient males produced by CA-specific silencing of *JHAMT* paired either with immature females (day 0 posteclosion) or mature 4-day posteclosion females (Fig. S6). As reported by Wijesekera et al., *JHAMT*-silenced males showed significant reduction of courtship activity toward immature females; this reduction is attributable in part to increased latency to courtship initiation (orientation followed by one-wing extension) compared to genetic controls (Fig. S6A). However, when paired with decapitated day-4 females, *JHAMT*-silenced males showed normal courtship indices and courtship latency (Fig. S6B). Silencing of *JHAMT* also caused significant courtship reduction and courtship delay of males when paired with immobilized immature females (Fig. S6C). Indeed, when paired with decapitated immature females, *JHAMT*-silenced males exhibited even more pronounced courtship latency compared to intact, mobile immature females (compare Fig. S6A with S6C). Increased latency may be attributable to loss of visual inputs provided by mobile, behaving females that are detected by JH-deficient males. We therefore hypothesize that, although JH deficiency in males likely causes reduced sensitivity to aphrodisiac pheromones, normal levels of these pheromones in mature females are sufficient for promotion of normal courtship activity of males. A previous study revealed that pheromone synthesis during female maturation strongly influences courtship latency [20]. Although results of this study and those of Lin et al. [21] showed that JH receptor expression in olfactory neurons influences detection of mature female pheromones, our findings suggest that JH deficiency caused by silencing of *ETHR* or *JHAMT* in the CA may reduce, but not abolish pheromone sensitivity.

Although JH deficiency may influence male sensitivity to pheromones and hence alter courtship drive, we find that robust courtship memory occurs in the absence of chemical cues such as cVA. In particular, we found that control males show normal MPI following training with pseudo-mated females (Ψ_m in Fig. 3B). Furthermore, JH-deficient males display normal courtship behavior and learning ability during training (Fig. 2C), but impaired memory performance following pairings with Ψ_m trainer females (Fig. 3B). We therefore conclude that: 1) rejection behavior exhibited by a mated female is the dominant factor driving memory performance under our courtship conditioning protocol, and 2) JH is essential for sexual deprivation-dependent memory retention (Fig. 2D).

Previous studies also suggest that drastic reduction of JH levels increases locomotory activity, which under our paradigm could influence memory performance [18, 22]. However, we found that partial JH deficiency (e.g., 70% reduction) caused by *ETHR* knockdown in the CA alters neither climbing nor courtship activities of mature males (Table S3). In contrast, lower courtship activity of immature animals (day 0-2 post-eclosion), which have minimum JH levels, was partially increased by methoprene (Table S3), suggesting that absence of JH reduces courtship activity of males. Taken together, it seems possible that the degree of JH deficiency is of crucial importance in determining phenotypic outcomes related to locomotion, courtship, and memory maintenance.

JH influences dopaminergic neurons and courtship memory during a critical period

We found that the obligatory role of ETH-JH signaling in courtship memory is limited to early adulthood. In particular, methoprene rescue of courtship memory in JH-deficient males was successful only in day 0-3 post-eclosion males (Fig. 2B, Fig. 4, Fig. 5). This critical period for hormonal action on memory may be attributable to neurogenesis and/or completion of CNS circuit assembly in young adult males [69]. Notably, we did not observe enhancement of STM by methoprene treatment of control males, confirming that rescue did not involve enhancement of MPI over normal levels (Fig. 2B, Fig. 5). We therefore propose that memory circuit maturation is complete under the influence of normal JH levels.

It is well-known that JH promotes brain dopamine levels and learning in male honeybees [30, 33]. Interestingly, methoprene treatment of young males enhances brain DA levels, with likely consequences for sexual and behavioral maturation. The role of JH in aversive learning of young drones therefore can be understood by this hormone-amine signaling cascade [31, 32]. Here we show that JH receptor expression in TH-positive DA neurons is necessary for normal courtship memory performance (Fig. 6A). In the *Drosophila* brain, approximately 130 TH-positive DA neurons occur as clusters, including protocerebral anterior medial (PAM), protocerebral anterior lateral (PAL), protocerebral posterior medial (PPM), posterior lateral (PPL) subgroups. These neurons innervate diverse central brain regions, including distinct zones of the mushroom body neuropil, which are considered as a memory hub. The *TH-GAL4* line labels most TH-positive DA neurons, with the exception of most PAM subgroups [70, 71]. Since JH-deficient males fail to retain memories, further investigation is required to show JH influences DA neuronal morphologies that contribute to memory maintenance. Although previous studies provided strong evidence for involvement of DA neurons in *Drosophila* behaviors, precise functional roles for dopamine circuits in memory processes is complicated. In particular, recent studies of aversive conditioning demonstrated that distinct populations of dopaminergic neurons contribute to either acquisition or extinction of information [1, 72, 73]. In courtship conditioning, it has been reported that dopamine is important role in the consolidation of short-term memory into long-term memory [74].

Although suppression of both JH receptors (*Met*, *gce*) in TH-positive DA neurons resulted in MPI deficiency, RNA silencing of either *Met* or *gce* alone did not produce the phenotype (Fig. 6B). Previous reports revealed that these receptor types are redundant and compensate the loss of function in mutant lines, especially during *Drosophila* development [75, 76].

In summary, JH signaling is conserved across a wide range of insect species. Functional parallels between JH and the mammalian thyroid hormone signaling have been proposed [77]. Beyond metamorphosis and reproductive processes, recent studies suggest involvement of thyroid hormone signaling in cognitive functions, especially learning and memory during a critical period [78, 79]. We propose here yet another potential conservation of hormonal function between JH and thyroid hormone signaling: that of social context-dependent neural and behavioral plasticity. Since thyroid hormone also influences persistent memories, further investigations on ETH-JH regulation of long-term memory are underway.

Experimental Procedures

Fly Strains

Flies were raised on standard-cornmeal-agar medium at room temperature. Crosses were maintained at room temperature on a 12-12 hr light/dark cycle. Wild- type flies were *Canton-S*. To reduce variation arising from genetic background, we backcrossed all flies for at least five generations to the *wCS* strain. Many fly lines used in this study were kindly provided by colleagues and institutions as follows: *JHAMT-GAL4* and *UAS-Dicer;UAS-JHAMT RNAi* flies, Brigitte Dauwalder (University of Houston) [28]; *ETH-GAL4* flies, David Anderson (California Institute of Technology); *UAS-Met RNAi* and *UAS-gce RNAi* lines, Lynn Riddiford (Janelia Research Campus); sex peptide null mutant (SP_0) and *UAS-SP* flies, Barry Dickson (Janelia Research Campus); *UAS-ETHR RNAi-Sym* and *UAS-ETHR RNAi-IR2* were described previously [7]; *UAS-rpr,hid* flies, Paul Taghert (Washington University); *OK107-GAL4*, *TH-GAL4*, *Tdc2-GAL4*, *Trh-GAL4*, *OK371-GAL4*, *Orco-GAL4*, *UAS-GCaMP5*, *UAS-RedStinger*, *UAS-Shi*^{ts1}, *TubPGAL80*^{ts}, tetanus toxin light chain lines (*UAS-TNT*$_G$ and *UAS-TNT*$_{imp}$), Bloomington Stock Center. The ETH GeneSwitch line (*EUG8*) was described previously [49].

Quantitative RT-PCR Analysis

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

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

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

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

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

Immunohistochemistry and *in vivo* Ca^{2+} Imaging of CA

For immunohistochemical detection of ETH in adult males, day 4 to 5 males were dissected in ice-chilled PBS. The ventral side of the thorax and abdomen was opened to remove muscle and intestines prior to fixation in 4% paraformaldehyde overnight at 4°C. After five 10-min washes with PBST (0.5% Triton X-100 in PBS) and 1 hour blocking with 5% NGS (normal goat serum) in PBST at room temperature, samples were incubated with rabbit anti-DmETH1 (1:1,000) for 2 days at 4°C. After six 10 min washes with PBST, samples were incubated with goat anti-rabbit

Alexa Fluor 488 (1:500). After five washes with PBST and one wash with PBS, samples were mounted in the mounting media (Aqua Poly/Mount, Polysciences Inc.).

For CA staining, overall CNS and gut of day 4 *JHAMT-GAL4/UAS-mCD8-GFP* males were dissected in ice-chilled PBS. Tissues were fixed in 4 % paraformaldehyde overnight at 4 °C. After five 10 min washes with PBST and 1 hour blocking with 5 % NGS in PBST at room temperature, samples were incubated with rabbit anti-JHAMT (1:100) [40] and mouse anti-GFP (1:500) for overnight at 4°C. Then, samples were incubated with goat anti-rabbit Alexa Fluor 647, and goat anti-mouse Alexa Fluor 488 (1:500 for each). Images were captured with Zeiss LSM510 confocal microscope.

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

Analysis of JH III Levels

Adult males (4-day posteclosion) were collected on the dry ice and kept at -80°C until extraction. JH III was labelled with a fluorescent tag DBD-COCl (4-(N,N-Dimethylaminosulfonyl)-7-(N-chloroformylmethyl-N-methylamino)-2,1,3-benzoxadiazole), and analyzed by reverse phase High Performance Liquid Chromatography coupled to a Fluorescence Detector (HPLC-FD) using a Dionex Summit System (Dionex, CA) equipped with a 680 HPLC pump, a TCC oven, a UV detector and an fluorescence detector connected in series. Details of the procedures were described previously [80].

Behavioral Assays

Experimental male pupae were individually sorted into 96-well plates, then housed for 4 days post-eclosion in individual clean glass tubes with fly food to prevent pretest social experiences. For preparation of mated female trainers, 3-4 day old *Canton-S* virgins were placed with *Canton-S* males prior to assay the following day. For preparation of immobilized tester females, 4-5 day old Canton-S virgins were anesthetized with CO_2 and decapitated with fine scissors immediately before experimentation. Courtship assays were performed in a 14-multi-mating chamber (10 mm diameter, 5 mm depth) [81].

For the courtship conditioning, overall experimental procedures followed those described previously, with some modification [82]. A single 4-day-old test male was placed in a chamber with a mated female for one hour (training). After a 10-minute post-training isolation period, courtship behavior of the trained male toward a tester (decapitated virgin) female was recorded with a digital camcorder (Sony HDR-XR260). A sham-trained male was kept alone in the

514 courtship chamber for one hour and paired with an immobilized tester female in another chamber
515 for 10 minutes. Training, sham-training, and test sessions were performed under the same
516 conditions. Courtship chambers were washed with 70% ethanol at least 10 min before the
517 experiment to prevent carryover influences from odor artifacts.

518 Pseudomated females (Ψ_m) were *elav-GAL4*/*UAS-SP* virgins. Although these females have
519 not mated, they reject males due to transgenic expression of sex peptide. Pseudovirgin females
520 (Ψ_v) were *Canton S* females that had been mated with sex peptide null (SP_0) homozygous males
521 one day before the courtship conditioning. Although they are receptive to males, aversive
522 pheromone signaling brought about by mating causes male avoidance [44].

523 Inka cells were selectively ablated using the TARGET system. *ETH-*
524 *GAL4;TubPGAL80^{ts}*/*UAS-rpr,hid* males were transferred from 19°C to 31°C within two hours
525 after eclosion, and kept in 31°C until courtship conditioning. For drug-dependent conditional
526 gene expression, flies were raised on standard fly food to the pupal stage. 200 μM RU486
527 (mifepristone, Sigma)- containing or 1.6 % ethanol containing fly food was poured into 96-well
528 plates and stored in 4°C. Individually eclosed males in plates transferred into glass tubes with
529 RU486 or ethanol containing fly food. Courtship conditioning was performed 4 days after
530 individual housing. Stage-specific *ETHR* knockdown using the TARGET system was achieved
531 by transferring flies from 19°C to 31°C (after eclosion) or from 31°C to 19°C (before eclosion).
532 Control flies were raised at 19°C (negative) or 31°C (positive) during their entire life until
533 immediately before the courtship assay. Detailed procedures for TARGET and GeneSwitch
534 experiments were previously described [46].

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

543

544 **Statistical analysis**

545 Courtship index (CI) is defined as the proportion of time devoted to courtship behavior
546 during a 10-min assay period (e.g., total seconds devoted to courtship behavior over a total of
547 600 sec). Courtship memory performance index (MPI) is expressed as ratio of the difference
548 between CI of trained males (CI_T) and mean of sham-trained males (CI_{Sm}) to CI_{Sm}; MPI = (CI_{Sm}-
549 CI_T)/CI_{Sm}. No memory is indicated by 0 MPI, since courtship level of the trained male is
550 equivalent to that of the sham-trained males. Test males that copulated during the training period
551 were excluded from the test session. At least 20 males were tested under equivalent training and
552 test conditions. All indices were scored manually in a blind fashion by two investigators. The
553 Mann-Whitney *U* test was used to test statistical significance between CIs of trained and those of
554 sham-trained males. Permutation tests were used to compare MPIs, with 100,000 permutations of
555 the raw data. Learning performance index (LPI) was determined by comparing mean CI from the
556 first 10-min interval of the 1-h training period (CI_{Im}) to the CI of the last 10-min interval (CI_F);
557 LPI = (CI_{Im}-CI_F)/CI_{Im}. The Mann-Whitney *U* test was applied to test statistical difference

between initial and final CIs. Student's *t* test was used to compare courtship activities of males toward virgin females and toward Ψ_v females.

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Figure Legends

Fig. 1. ETH signaling is essential for maintenance of JH levels in adult male *Drosophila*.

(A) Presence of Inka cells and ETH peptides in adult male (Day 4 or 5 after eclosion) shown by ETH1 immunohistochemistry (green) and a nuclear marker RedStinger expression (red) in the *ETH-GAL4* transgenic line. Two pairs of Inka cells are located on the thoracic trachea (Tr1 and Tr2) and seven pairs are detected on the abdominal trachea (Ab1 to Ab7). Scale bar: 10 μm.

(B) *JHAMT-GAL4* labels CA specifically. CA of *JHAMT-GAL4*/*UAS-mCD8-GFP* males were stained with anti-JHAMT (left, red) and anti-GFP (middle, green) antibodies; superimposed images are shown in the right panel. Scale bar: 50 μm.

(C) Relative *ETHR* transcript abundance in CA of control (*JHAMT-GAL4*/*UAS-mCD8-GFP;* white bar) and *ETHR*-silenced (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym;UAS-mCD8-GFP;* purple bar) males measured by qPCR. Error bar represents s.e.m (*t*-test, **P*<0.001).

(D) *In vivo* Ca^{2+}-induced fluorescence in CA of a day 4 male CA expressing *GCaMP5* after application of ETH1 (1 μM). (a) Diagram experimental setup for *in vivo* CA Ca^{2+} imaging. (b) Representitive Ca^{2+}-mediated fluorescence in CA of vehicle- or ETH1-treated *JHAMT-GAL4*/*UAS-GCaMP5* male. (c) Representative fluorescence ($\Delta F/F_0$) of the CA following ETH1 (1 μM) application. Upper trace (red) represents Ca^{2+} elevation in CA of a *JHAMT-GAL4*/*UAS-GCaMP5* male, while the trace below (blue) shows Ca^{2+} elevation in CA of an ETHR-silenced male (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym;UAS-GCaMP5*) in response to 1 μM ETH1 application. (E) Analysis of Ca^{2+} dynamics at the CA responding to ETH application. (a) Mean maximum fluorescence responses of male CA exposed to fly saline (-, white bar) or 1 μM ETH1(+, red bar for *JHAMT-GAL4*/*UAS-GCaMP5*; blue bar for *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym;UAS-GCaMP5*). (b) Cumulative fluorescence changes (area under the curve) over a 10 min interval starting from onset of the response. (c) Latency to maximum fluorescence amplitude following ETH application. Error bar represents s.e.m. (n = 6-9, *t*-test, ****P*<0.001, ***P*<0.01, **P*<0.05).

(F) Silencing of *ETHR* in CA reduces JH levels in day 4 adult males. JH III levels are represented as mean ± s.e.m. of 4 independent replicate groups (total numbers of animals tested: 264 *JHAMT-GAL4*/+; 268 *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* (*t*-test, ***P*<0.01)).

Fig. 2. JH deficiency creates deficit in short-term memory retention, not acquisition.

(A) Short-term memory performances of JH-deficient males (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* or /*UAS-ETHR RNAi-IR2*) subjected to courtship conditioning; males were tested 10-min after completion of 1-hour training with a mated female). Upper plot compares courtship indices (CI) of sham-trained (left) and trained (right) males following each treatment. Genetic controls are shown with white bars; test males using two independent RNAi constructs are shown in either red (*JHAMT-GAL4*/*ETHR RNAi-Sym*) or purple (*JHAMT-GAL4*/*ETHR RNAi-IR2*). Bottom plot shows memory performance indices (MPI) of genetic control and test males. "*" represents significant difference between MPI of *GAL4* control and test males (****P* < 0.001), and "#" indicates the significant difference between MPI of *UAS* control and test males ($^{\#\#\#}P$ < 0.001) (n = 40-57).

(B) CI distributions and MPI of methoprene-treated *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* males. Acetone was applied as a vehicle; "*" denotes the significant difference between MPI of vehicle-treated and that of methoprene-treated males (^{a}P < 0.01) (n = 40-46).

(C) During the hour-long training period, both control and JH-deficient males exhibit learning through reduction of CI and LPI. (JH-deficient fly genotype: *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* and *JHAMT*/*UAS-ETHR RNAi-IR2*). Asterisks indicate significant differences between CI during the initial 10

min interval (I) of the 1 hr pairing period and CI during the final (F) 10 min interval of the pairing period (Mann-Whitney *U* test, $^{**}P < 0.01$, $^{*}P < 0.05$) (n = 40-52).

(D) Memory decay assay following 1-hour exposure to mated females (n = 48-56).

Fig. 3. JH-deficient males exhibit olfactory deficits, but robust courtship memory occurs in absence of aversive, mating-associated chemical cues.

(A) (a) Accumulated time to copulation of JH-deficient and *GAL4* control males paired with mature virgin (F_v) or pseudovirgin (Ψ_v) females (n = 20-23). (open circle: *JHAMT-GAL4*/+ paired with F_v; filled circle (red): *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* paired with F_v; filled circle (gray): *JHAMT-GAL4*/+ paired with Ψ_v; filled circle (brown): *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* paired with Ψ_v). (b) CI of those males toward F_v or Ψ_v females until copulation (Student's *t* test, $^{*}P < 0.05$).

(B) CI and MPI of JH-deficient and *GAL4* control males trained with either mated (F_m) or pseudomated (Ψ_m) females. "a" represents significant difference between MPI of *GAL4* control and test males trained with equivalent trainer type (*JHAMT-GAL4*/+ vs. *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* trained with F_m, or trained with Ψ_m ($^{a}P < 0.01$) (n = 44-57).

Fig. 4. ETH-driven JH functions in memory performance during the adult period.

(A) CI and MPI following temporal ablation of Inka cells and suppression of ETH release by conditional expression of pro-apoptotic genes and tetanus toxin light chain in Inka cells. In *GeneSwitch* experiments, "a" denotes significant difference between MPI of vehicle-treated and RU486-treated animals ($^{a}P < 0.01$) (n = 48-64).

(B) Upper schematic diagram shows conditional *ETHR* knockdown in the CA. *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym;TubPGAL80^{ts}* males were kept for entire life at 19℃ (X), at 31℃ (pre/post), pre-adult stage at 31℃ (pre) or adult stage at 31℃ (post). CI distributions and MPI of conditional knockdown males. Significant differences: "*"- *GAL4* control and test males ($^{***}P < 0.001$); "#"- UAS control and test males ($^{\#\#}P < 0.01$); "a"- negative control (X) and that of positive control (pre/post) males ($^{a}P < 0.01$); "b"- negative control (X) and that of test (post) males ($^{b}P < 0.001$) (n = 40-56).

Fig. 5. JH action on memory performance operates during a critical period during the first week of adulthood.

Memory deficits following *ETHR*-silencing were rescued by topical application of methoprene to different aged flies. Acetone was used as a vehicle.

(A) CI distributions and MPI of GAL4 control (left) and JH-deficient (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym*) males. Empty bars indicate MPI of vehicle-treated, and purple bars represent MPI of methoprene-treated males (n = 40-58).

(B) Dynamics of MPI of aged control and test males. "$" denotes significant difference between MPI of day 0-4 and day 10-14 vehicle or methoprene-treated males ($^{\$}P < 0.01$). "a" indicates the significant difference between MPI of vehicle-treated and that of methoprene-treated animals ($^{aa}P < 0.01$, $^{a}P < 0.05$).

(C) Precise methoprene sensitive period of *JHAMT-GAL4*/*UAS-ETHR RNAi-Sym* males. Two dosages of methoprene (1x, 64.4 pmol; 5x, 322 pmol) were treated to each age and courtship conditioning was performed at 24-hour after the methoprene application ($^{**}P < 0.01$, $^{*}P < 0.05$)

Fig. 6. Dopaminergic (DA) neurons are functional targets of JH by recruiting JH receptors.

(A) Knockdown of *met* and *gce* was accomplished through use of diverse *GAL4* drivers for introduction of dsRNAs directed against *Met* and *gce* sequences. CI distribution and MPI of *GAL4* controls (left), and those of *UAS* control and test males (right) (n = 44-52). Drivers: *OK107-GAL4*, whole mushroom body; *TH-GAL4*, tyrosine hydroxylase (DA); *Tdc2-GAL4*, tyrosine decarboxylase 2 (neuronal OA); *Trh-GAL4*, tryptophan hydroxylase (5-HT); *OK371-GAL4*, glutamatergic neurons; *Orco-GAL4*, broad odorant receptor neurons (co-receptor *Or83b*).

(B) Suppression of *Met* or *gce* expression in TH-positive DA neurons was performed by preparing *TH-GAL4*/*UAS-Met RNAi* and *TH-GAL4*/*UAS-gce RNAi* lines (n = 49-54).

(C) A model for the hormonal cascade regulating *Drosophila* short-term courtship memory. Proposed model as described in the text describing the function of ETH-JH signaling in regulating male's courtship memory maintenance.

Figure

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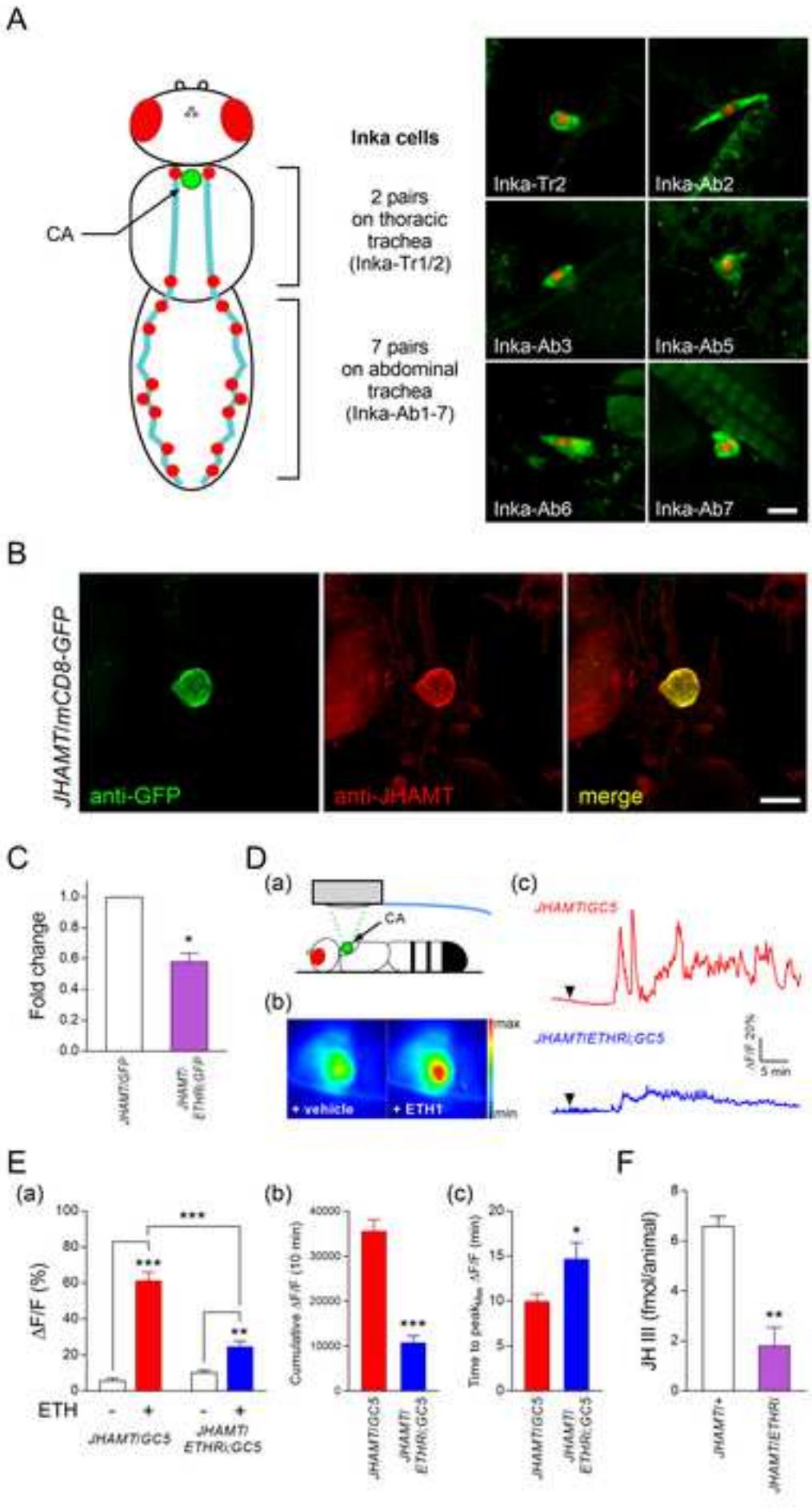

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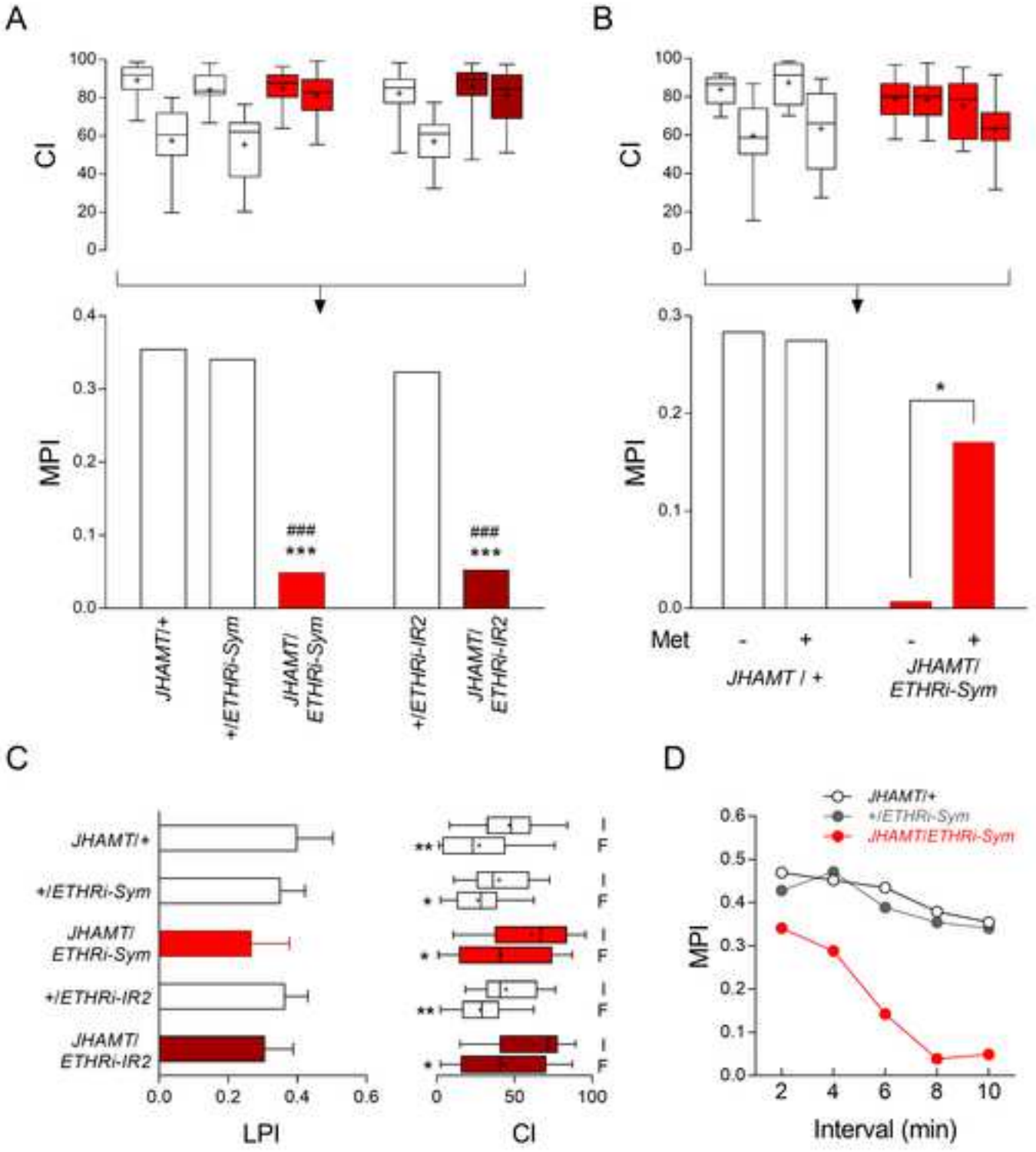

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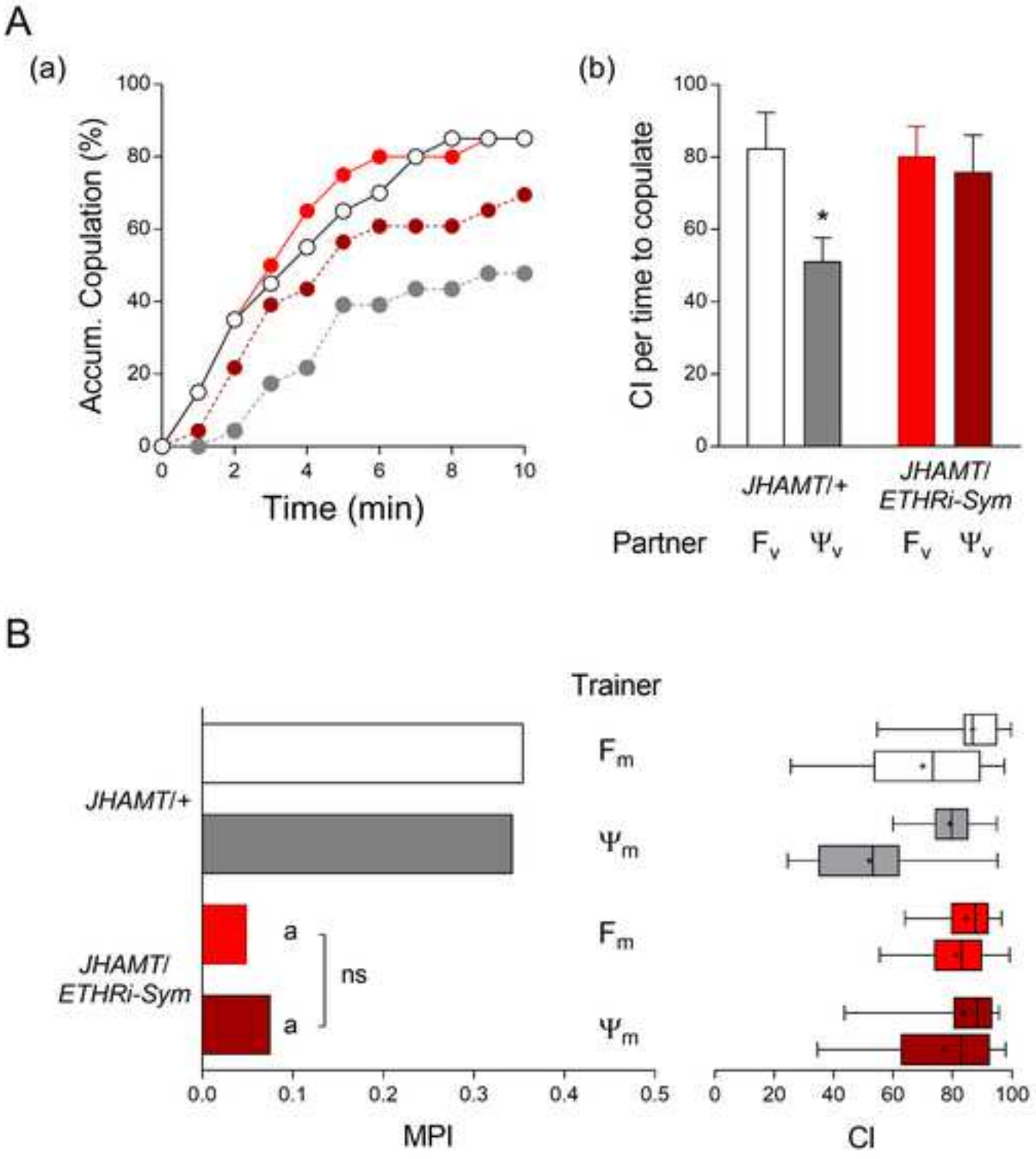

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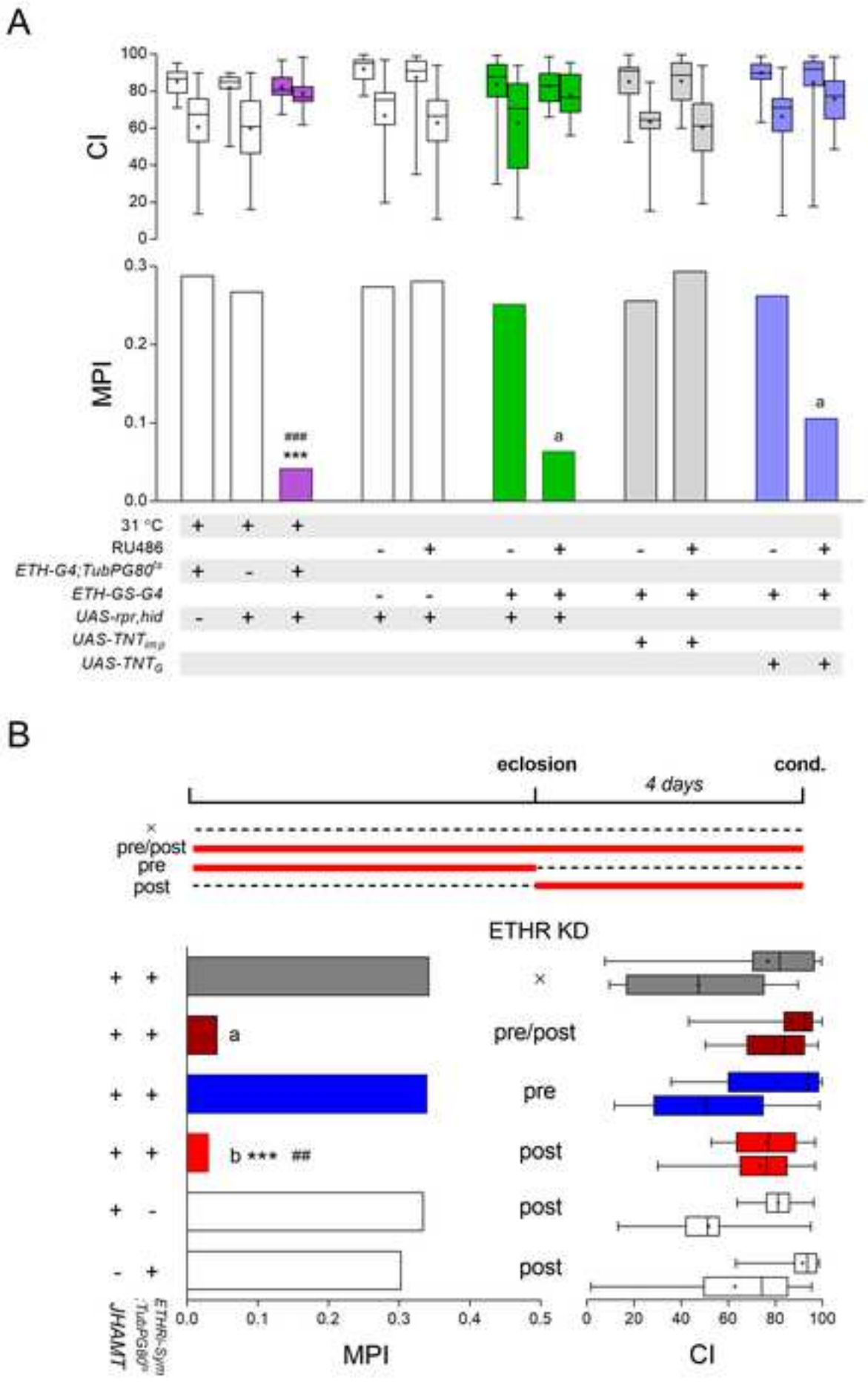

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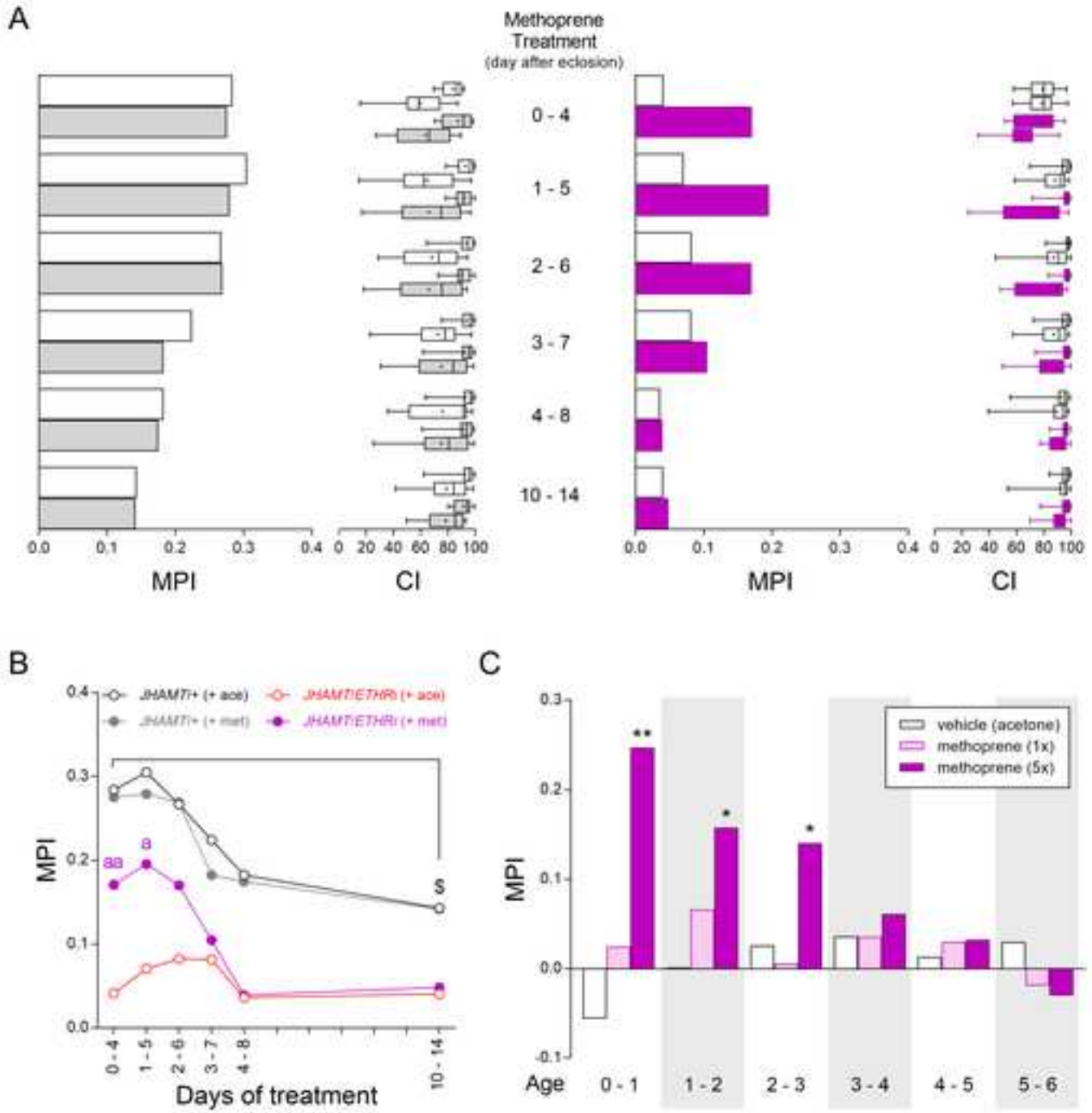

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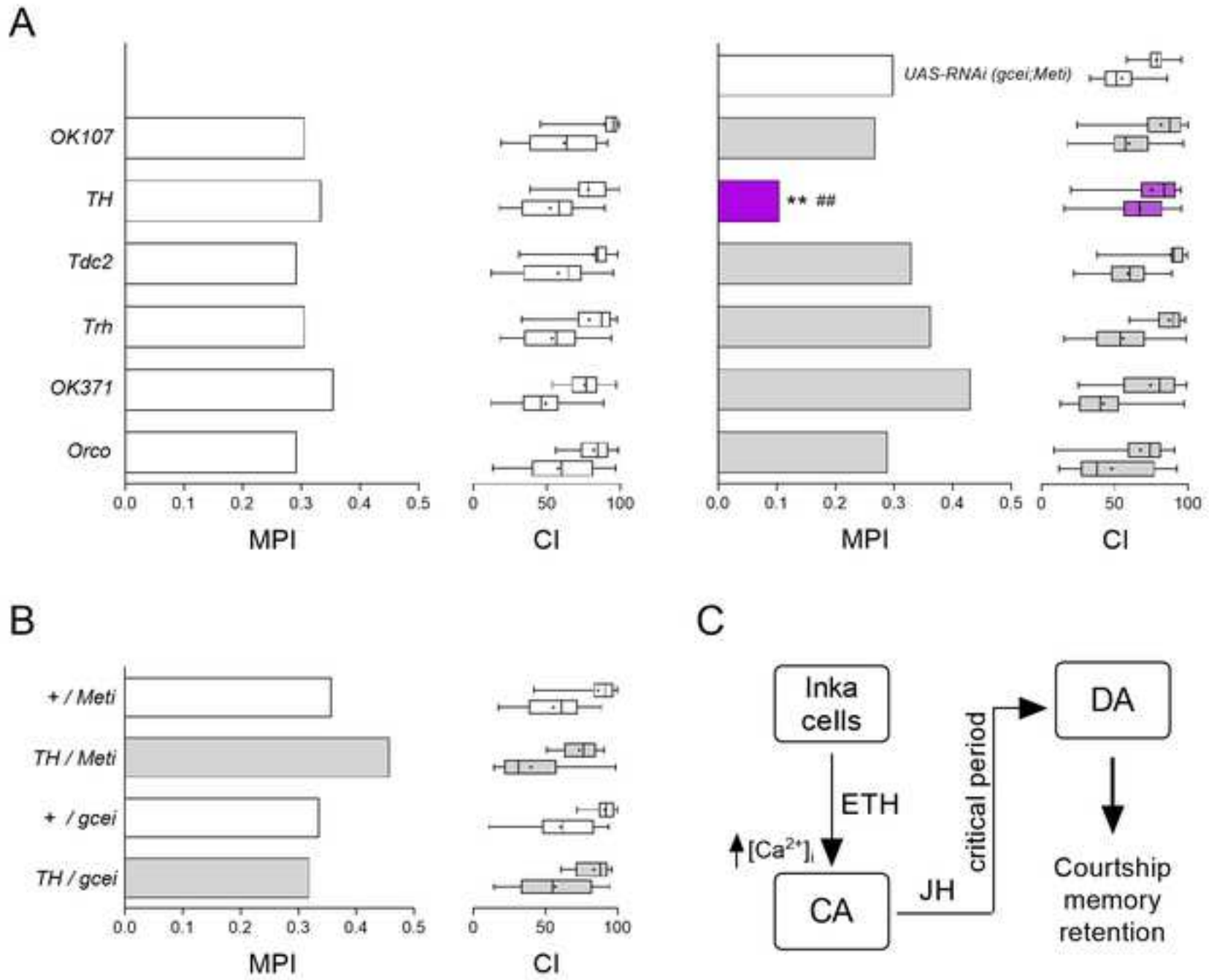

Supplemental Information

Table S1. Statistical analysis summary for courtship memory tests (Mann-Whitney U Test).

Fig.	Experiment	Genotype / Condition	*P*-Value
2A	Short-term courtship conditioning (compare CIs b/w trained & sham-trained)	*JHAMT-GAL4/+*	*< 0.001*
		+/UAS-ETHR^{RNAi}-Sym	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym	*0.155* (ns)
		+/UAS-ETHR^{RNAi}-IR2	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-IR2	*0.245* (ns)
2B	Methoprene rescue (compare CIs b/w trained & sham-trained)	*JHAMT-GAL4/+* (acetone day 0-4)	*< 0.001*
		JHAMT-GAL4/+ (met day 0-4)	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 0-4)	*0.495* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (met day 0-4)	*0.013*
2D	Memory decay assay (compare CIs b/w trained & sham-trained)	*JHAMT-GAL4/+* (2-min interval)	*< 0.0001*
		JHAMT-GAL4/+ (4-min interval)	*< 0.0001*
		JHAMT-GAL4/+ (6-min interval)	*< 0.001*
		JHAMT-GAL4/+ (8-min interval)	*< 0.001*
		+/UAS-ETHR^{RNAi}-Sym (2-min interval)	*< 0.001*
		+/UAS-ETHR^{RNAi}-Sym (4-min interval)	*< 0.0001*
		+/UAS-ETHR^{RNAi}-Sym (6-min interval)	*< 0.001*
		+/UAS-ETHR^{RNAi}-Sym (8-min interval)	*0.003*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (2-min interval)	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (4-min interval)	*0.022*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (6-min interval)	*0.096* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (8-min interval)	*0.399* (ns)
3C	Dissociation experiment (compare CIs b/w trained & sham-trained by Ψ_m)	*JHAMT-GAL4/+*	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym	*0.345* (ns)
4A	Inka cell ablation: TARGET (compare CIs b/w trained & sham-trained)	*ETH-GAL4;TubPGAL80^{ts}/+* (31℃)	*< 0.001*
		+/UAS-rpr,hid (31℃)	*< 0.001*
		ETH-GAL4;TubPGAL80^{ts}/UAS- rpr,hid (31℃)	*0.092* (ns)
	Inka cell ablation: GeneSwitch (compare CIs b/w trained & sham-trained)	*+/UAS- rpr,hid* (-RU486)	*< 0.001*
		+/UAS- rpr,hid (+RU486)	*< 0.001*
		EUG8/UAS- rpr,hid (-RU486)	*< 0.001*
		EUG8/UAS- rpr,hid (+RU486)	*0.084* (ns)
	Blocking vesicle release: TeTxLC (compare CIs b/w trained & sham-trained)	*EUG8/UAS-TNT$_{imp}$* (-RU486)	*< 0.001*
		EUG8/UAS-TNT$_{imp}$ (+RU486)	*< 0.001*
		EUG8/UAS-TNT$_G$ (-RU486)	*< 0.001*
		EUG8/UAS-TNT$_G$ (+RU486)	*0.016*
4B	Conditional ETH KD: TARGET (compare CIs b/w trained & sham-trained)	*JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym;TubPGAL80^{ts}* (X)	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym;TubPGAL80^{ts} (pre/post)	*0.103* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym;TubPGAL80^{ts} (pre)	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym;TubPGAL80^{ts} (post)	*0.386* (ns)
		JHAMT-GAL4/+ (post)	*< 0.001*
		+/UAS-ETHR^{RNAi}-Sym;TubPGAL80^{ts} (post)	*0.001*
5A	Periodic methoprene rescue (chronic application) (compare CIs b/w trained & sham-trained)	*JHAMT-GAL4/+* (acetone day 1-5)	*< 0.001*
		JHAMT-GAL4/+ (met day 1-5)	*< 0.001*
		JHAMT-GAL4/+ (acetone day 2-6)	*< 0.001*
		JHAMT-GAL4/+ (met day 2-6)	*0.001*
		JHAMT-GAL4/+ (acetone day 3-7)	*< 0.001*
		JHAMT-GAL4/+ (met day 3-7)	*0.001*
		JHAMT-GAL4/+ (acetone day 4-8)	*0.001*
		JHAMT-GAL4/+ (met day 4-8)	*0.016*
		JHAMT-GAL4/+ (acetone day 10-14)	*0.009*
		JHAMT-GAL4/+ (met day 10-14)	*0.004*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 1-5)	*0.118* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (met day 1-5)	*0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 2-6)	*0.197* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (met day 2-6)	*0.004*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 3-7)	*0.111* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (met day 3-7)	*0.083* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 4-8)	*0.187* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (met day 4-8)	*0.151* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 10-14)	*0.156* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (met day 10-14)	*0.205* (ns)
5C	Periodic methoprene rescue (24-hour application) (compare Cis b/w trained & sham-trained)	*JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym* (acetone day 0-1)	*0.221* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (1x met day 0-1)	*0.071* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (5x met day 0-1)	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 1-2)	*0.449* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (1x met day 1-2)	*0.098* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (5x met day 1-2)	*< 0.001*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 2-3)	*0.272* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (1x met day 2-3)	*0.164* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (5x met day 2-3)	*0.021*
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 3-4)	*0.212* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (1x met day 3-4)	*0.136* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (5x met day 3-4)	*0.071* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 4-5)	*0.196* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (1x met day 4-5)	*0.225* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (5x met day 4-5)	*0.099* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (acetone day 5-6)	*0.123* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (1x met day 5-6)	*0.361* (ns)
		JHAMT-GAL4/UAS-ETHR^{RNAi}-Sym (5x met day 5-6)	*0.195* (ns)
6A	JH receptor knockdown (*Met* & *gce*) (compare CIs b/w trained & sham-trained)	*+/UAS-gce^{RNAi};UAS-Met^{RNAi}*	*< 0.001*
		OK107-GAL4/+	*< 0.001*
		OK107-GAL4/UAS-gce^{RNAi};UAS-Met^{RNAi}	*< 0.001*
		TH-GAL4/+	*< 0.001*
		TH-GAL4/UAS-gce^{RNAi};UAS-Met^{RNAi}	*0.041*
		Tdc2-GAL4/+	*< 0.001*
		Tdc2-GAL4/UAS-gce^{RNAi};UAS-Met^{RNAi}	*< 0.001*
		Trh-GAL4/+	*< 0.001*
		Trh-GAL4/UAS-gce^{RNAi};UAS-Met^{RNAi}	*< 0.001*
		OK371-GAL4/+	*< 0.001*
		OK371-GAL4/UAS-gce^{RNAi};UAS-Met^{RNAi}	*< 0.001*
		Orco-GAL4/+	*< 0.001*

		Orco-GAL4/UAS-gce^{RNAi};UAS-Met^{RNAi}	*0.005*
6B	JH receptor knockdown (*Met* or *gce*) in *TH-GAL4* (compare CIs b/w trained & sham-trained)	*+/UAS-Met^{RNAi}*	*< 0.001*
		TH-GAL4/UAS-Met^{RNAi}	*< 0.001*
		+/UAS-gce^{RNAi}	*< 0.001*
		TH-GAL4/UAS-gce^{RNAi}	*< 0.001*

Table S2. Locomotion test and heterosexual activity assay

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

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

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

b. An indicator of courtship activity, one-wing extension (courtship singing) was analyzed by counting the proportion of the length of wing extension of a male to the time to copulation with a virgin Canton-S female (day 4-5). The total copulation rates were tested by counting the number of males copulated with virgin females in 10 minutes.

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

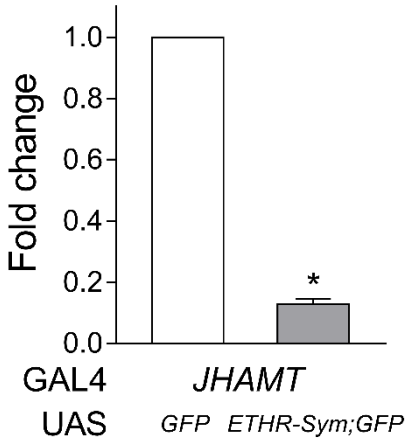

Figure S1. Silencing *ETHR* using *JHAMT-GAL4* reduces gene expression in female CA.

Relative expression ratio of *ETHR* genes in the CA of control (*JHAMT-GAL4*/*UAS-mCD8-GFP*) and *ETHR*-silenced (*JHAMT-GAL4>UAS-ETHR RNAi-Sym*/*UAS-mCD8-GFP*) females showed significant reduction (87.3%) in gene expression. Error bars represent s.e.m (t-test, **P*<0.0001).

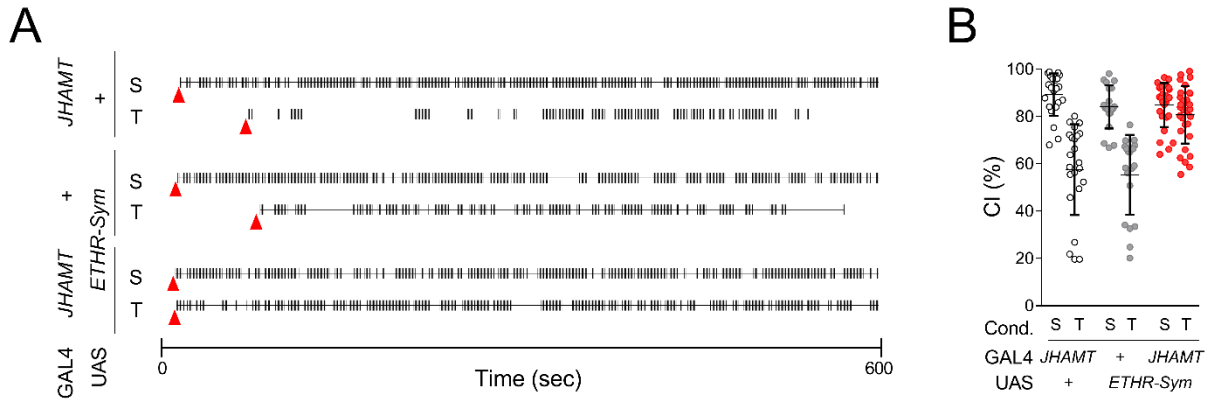

Figure S2. Silencing *ETHR* in the CA impairs courtship suppression after the training.

(A) Bouts of male courtship behavior toward a decapitated virgin female following training or sham-training. Down-regulation of *ETHR* expression in the CA (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym*) impairs subsequent courtship suppression toward an immobilized mature virgin female even after the training with a mated female, whereas two genetic control show dramatic courtship suppression by previous experience. "S" indicates sham-trained and "T" represents "trained" male. Arrowheads show the starting points of courting behavior toward a tester decapitated virgin female.

(B) Individual courtship distribution of sham-trained and trained males. Lines indicate mean ± STD.

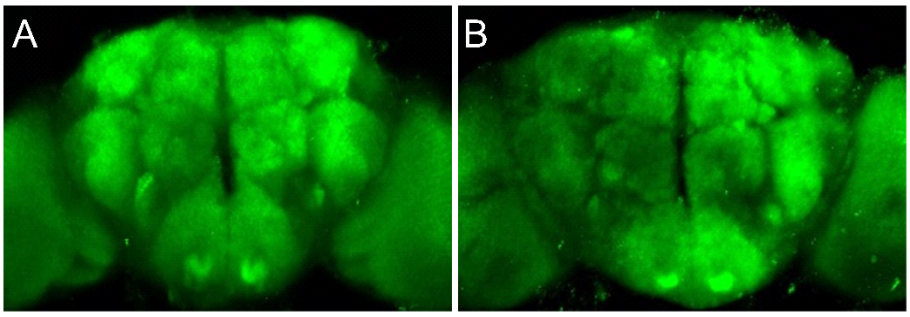

Figure S3. Reduction of JH does not have morphological defects in brain.

Brains dissected were day 4 adult males.

(A) nc82 staining in a representative GAL4 genetic control (*JHAMT-GAL4*/+) center brain.

(B) nc82 staining in a JH-reduced male (*JHAMT-GAL4*/*UAS-ETHR RNAi-Sym*) center brain. No gross morphological differences were observed in neuropil structure between genetic control and JH-suppressed animals.

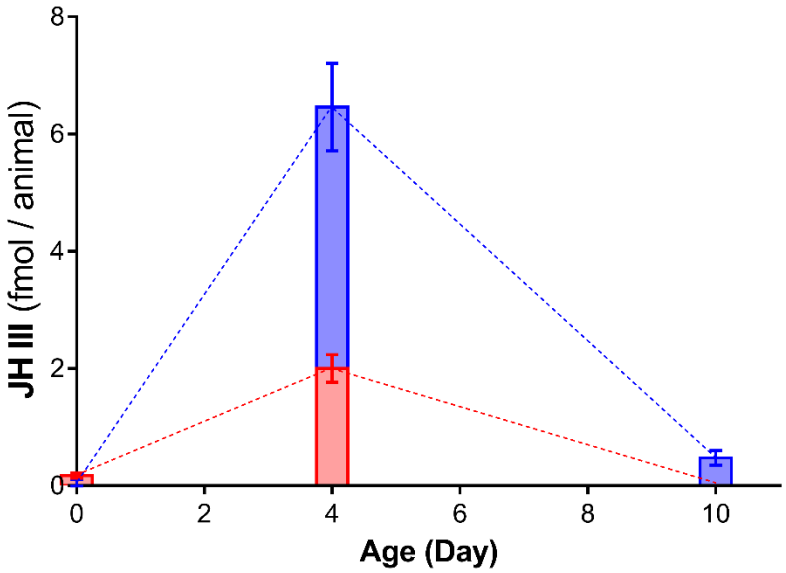

Figure S4. JH level is changed by aging of adult males.

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

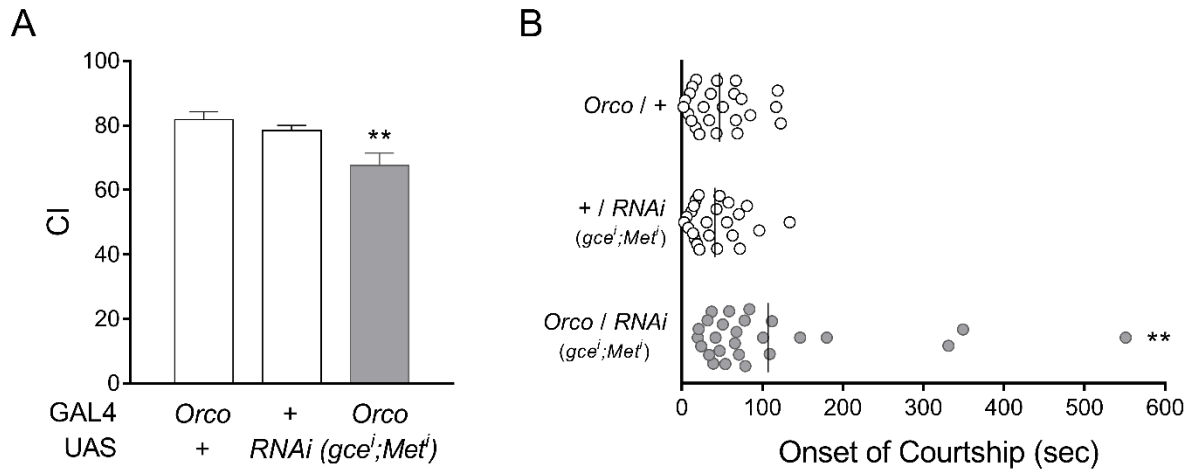

Figure S5. JH receptors in ORNs play a role in courtship behavior of naïve males.

In the courtship conditioning, courtship activities of sham-trained animals toward decapitated virgin females were analyzed.

(A) Overall courtship behaviors of test and genetic control males (mean ± s.e.m, One-way ANOVA, $^{**}P<0.01$, n = 24-26).

(B) Onset of courtship behavior of individual animals in plot (A). Lines indicate mean time of courtship onset (Kruskal-Wallis nonparametric test, $^{**}P<0.01$).

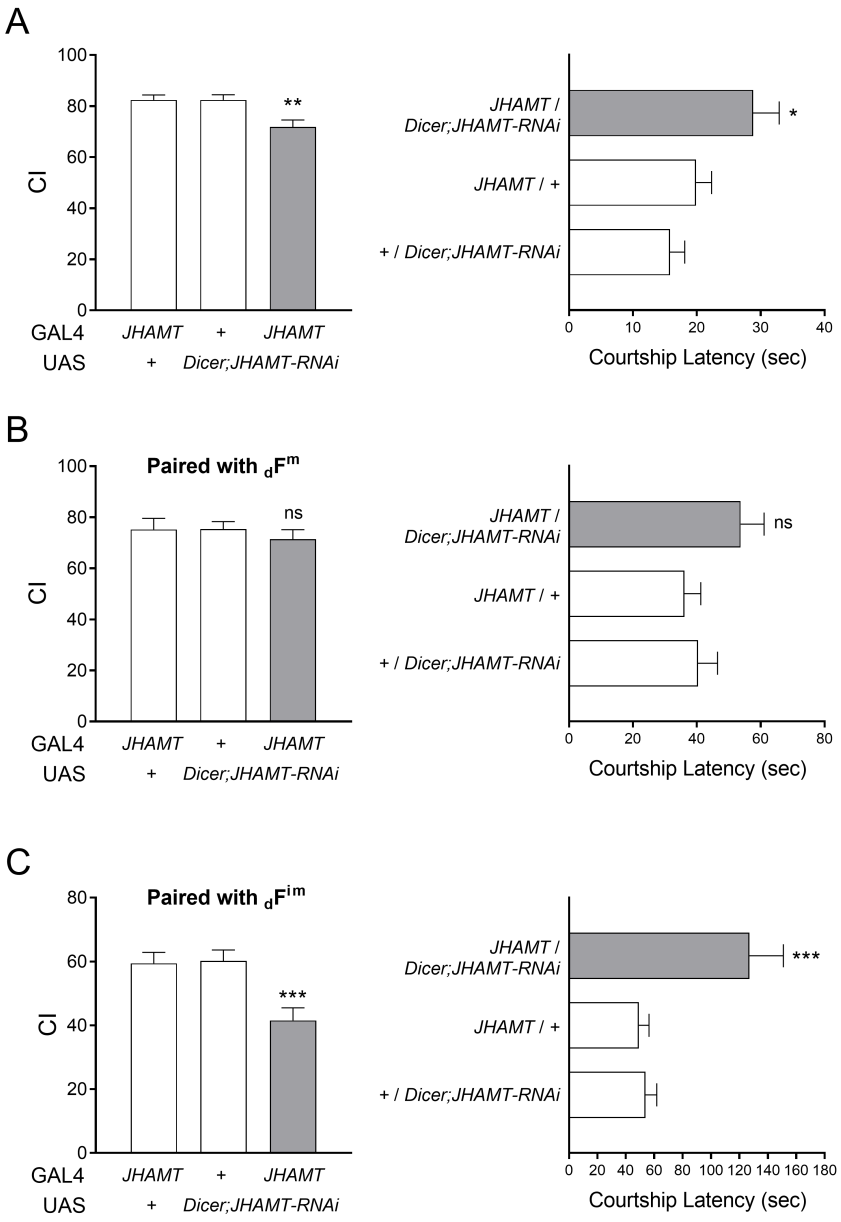

Figure S6. *JHAMT* knockdown in the CA reduces male courtship specifically toward immature tester females, but not mature tester females..

(A) Overall courtship indices (CI, left) and courtship latencies (right) of *JHAMT* knockdown and genetic control males toward immature (2-hour post-eclosion) tester females (n = 25-27).

(B) Overall CI (left) and courtship latencies (right) of JHAMT knockdown and genetic control males toward immobilized (decapitated) mature (day-4 post-eclosion) tester females (n = 23).

(B) Overall CI (left) and courtship latencies (right) of JHAMT knockdown and genetic control males toward immobilized (decapitated) immature (2-hour post-eclosion) tester females (n = 22). mean ± s.e.m, One-way ANOVA for CIs and , Kruskal-Wallis nonparametric test for courtship latencies. **P*<0.05; ***P*<0.01; ****P*<0.001; ns, no significance.