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Ecdysis triggering hormone receptors regulate male courtship behavior via antennal lobe interneurons in *Drosophila*

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ABSTRACT

Ecdysis triggering hormone receptors (ETHR) regulate the behavioral sequence necessary for cuticle shedding. Recent reports have documented functions for ETHR signaling in adult *Drosophila melanogaster*. In this study, we report that ETHR silencing in local interneurons of the antennal lobes and *fruitless* neurons leads to sharply increased rates of male-male courtship. RNAseq analysis of ETHR knockdown flies reveals differential expression of genes involved in axon guidance, courtship behavior and chemosensory functions. Our findings indicate an important role for ETHR in regulation of *Drosophila* courtship behavior through chemosensory processing in the antennal lobe.

1. Introduction

Ecdysis triggering hormone receptors (ETHRs) in coordination with its primary ligand, ecdysis triggering hormone (ETH), orchestrate critical behaviors involved in cuticle shedding at the conclusion of each molt in insects. These G protein-coupled receptors are expressed in distinct ensembles of peptidergic neurons including myoinhibitory peptides, FMRFamides, eclosion hormone, kinin, bursicon and crustacean cardioactive peptide. These receptors and ETH are conserved in a wide variety of insect species (Žitňan et al., 1996, Park et al., 1999, Žitňan et al., 2002, Žitňan et al., 2003, Clynen et al., 2006, Hummon et al., 2006, Amare and Sweedler, 2007, Dai and Adams, 2009). In *Drosophila melanogaster*, endocrine Inka cells, the sole source of ETH, persist into adulthood, during which ecdysis behavior is absent. Likewise, *ETHR* transcripts are present in the adult stage (Graveley et al., 2011, Lee et al., 2017, Meiselman et al., 2017), raising questions regarding functional roles of ETHRs during adulthood.

During larval development, ETH targets central neurons expressing ETHRs to regulate sequential ecdysis-associated behaviors (Kim et al., 2006, Žitňan and Adams, 2012, White and Ewer, 2014, Kim et al., 2015). A possible role for ETH in regulation of juvenile hormone levels was first recognized upon detection of ETHR transcripts in *corpora allata* (CA) in larvae of the silkworm, *Bombyx mori* (Yamanaka et al., 2008). Subsequently, ETH was demonstrated to function as an obligatory allatotropin essential for fecundity and courtship memory in adult *Drosophila* (Deshpande 2012, Lee et al., 2017, Meiselman et al., 2017). Because expression of genes encoding ETH and ETHR are regulated by ecdysone (Žitňan et al., 1999, Kim et al., 2006, Meiselman et al., 2017), we reasoned that reproductive phenotypes associated with ecdysone deficiency may result from aberrant ETH signaling. We

Abbreviations: ETHRs, ecdysis-triggering hormone receptors; JH, juvenile hormone; CI, courtship index; WEI, wing-extension index; CA, corpora allata

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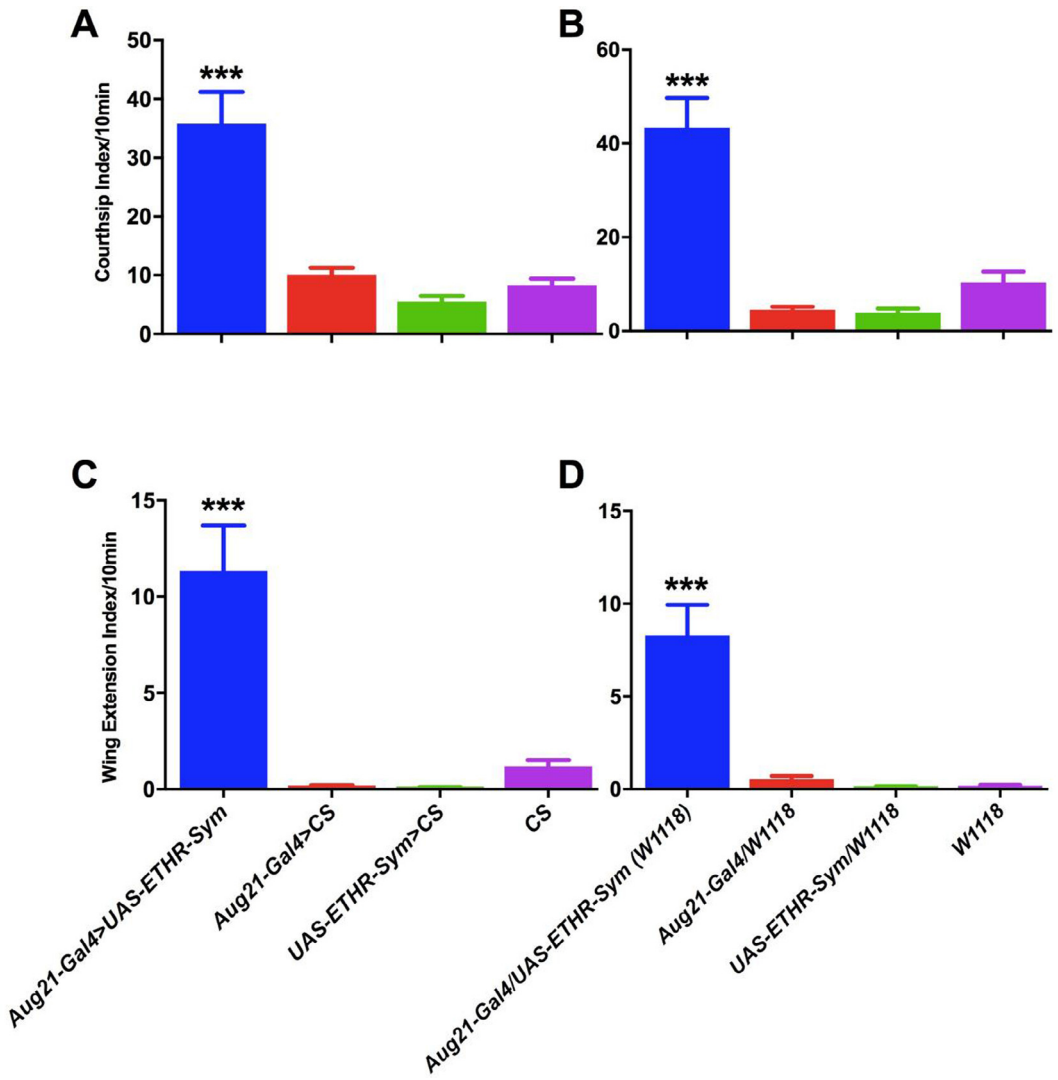

Fig. 1. ETHRs regulate male courtship behavior. Courtship indices of A) cantonized or B) w^{1118} background males towards Canton-S or w^{1118} males, respectively. Wing extension indices of C) cantonized and D) w^{1118} background males towards Canton-S or w^{1118} males, respectively. n = 20 for each genotype. Error bars indicate SEM. ***P < .001 (Kruskal-Wallis One-way ANOVA).

therefore examined whether ETHR deficiencies might explain elevated male-male courtship reported in ecdysone receptor mutants (Ganter et al., 2007, Schwedes and Carney, 2012).

In this study, we investigated functional roles for ETHR in adult *Drosophila* male behavior by RNAi silencing using the *Aug21-Gal4* driver line, which is reported to be specific for CA labeling during juvenile stages (Mirth et al., 2005). We found that ETHR knockdown with this driver causes a sharp increase in male-male courtship correlated with a male-specific reduction in juvenile hormone (JH) levels. However, we observe an expansion of *Aug21-Gal4* labeling to the CNS during adulthood and find that the male-male courtship phenotype involves ETHR-expressing interneurons of the antennal lobe rather than JH deficiency. Our findings reveal a novel role for ETHR signaling in central olfactory processing.

2. Materials and methods

2.1. Fly lines

Drosophila melanogaster were reared on regular cornmeal medium at 25 °C under 12:12 light: dark cycle. *ETH-Gal4*, *UAS-ETHR-Sym*, and *UAS-ETHR-IR2* flies were described previously (Kim et al., 2006, Kim et al., 2015). We obtained wCS (cantonized w^{1118}) flies from Anupama Dahanukar, *Aug21-Gal4/CyO* flies from Günter Korge, *elav-Gal4/tub-Gal80^{ts}* from Anandasankar Ray, *fru-Gal4* flies from Bruce Baker, and NP3056, Krasavietz-Gal4, LCCH-Gal4, NP6277-Gal4, HB4-94-Gal4 from Liqun Luo. All other fly lines were obtained from the Bloomington Stock Center. Canton-S (CS) flies were used as wild type for cantonized flies and w^{1118} flies were used as controls for non-cantonized flies. Flies with w^{1118} background were labeled as (w) and cantonized flies were labeled as (cs). *Aug21-Gal4/+*, *UAS-ETHR-Sym/+* flies were obtained by crossing *Aug21-Gal4/CyO* and *UAS-ETHR-Sym* respectively with wild type flies. *Aug21-Gal4/+*, *UAS-ETHR-Sym/+*, *Aug21-Gal4/ UAS-ETHR-Sym* or wild type flies were used as test flies and wild type flies were used as subject. RNAi was induced under *elav-Gal4;tub-Gal80^{ts}* by crossing *UAS-ETHR-Sym* flies with *elav-Gal4;tub-Gal80^{ts}*. Flies were raised at 18 °C until pupal ecdysis and 24–72 hr later the flies were raised at 30 °C until they were tested. Control *elav-Gal4/+;tub-Gal80^{ts}* flies were also treated similarly by incubating adults at 30 °C until tested for male-male courtship. Flies were collected within 12 hr after eclosion under CO_2 anesthesia. Males were individually aged after collection in 12 × 75 mm Pyrex glass culture tubes (Corning, NY, United States) with about 1.5 cm food at the bottom, whereas virgin females were aged in groups of 5–7 per vial.

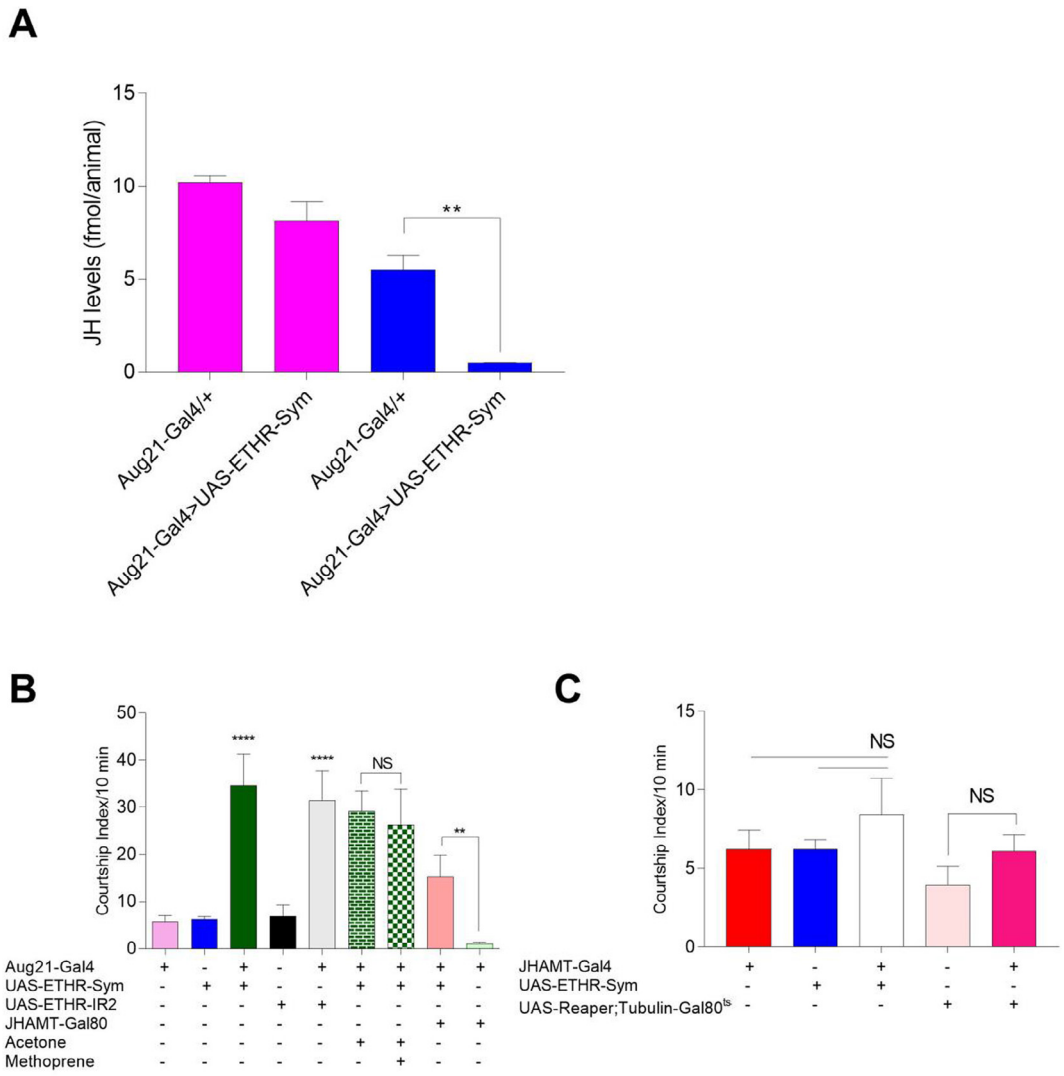

Fig. 2. JH deficiency in ETHR-silenced males does not cause male-male courtship phenotype. A) JH-III levels in 3–4-day old adult females (pink bars-left) and males (blue bars-right). B) Methoprene treatment does not rescue increased male-male courtship in ETHR-RNAi males, demonstrated using two independent RNAi constructs (UAS-Sym, UAS-IR2). C) Male-male courtship indices do not increase after CA specific silencing of ETHRs or ablation using the *JHAMT-Gal4* driver. n = 10–25. Error bars indicate SEM. **P < .01; ****P < .0001 (One-way ANOVA).

2.2. Immunohistochemical staining

The expression pattern of the *Aug21-Gal4* driver was determined by crossing it with *UAS-mCD8EGFP* flies, dissecting CNS, CA and gut, and staining the tissue against anti-GFP. All tissue samples were dissected in phosphate saline buffer and were stained using standard protocols. Samples were immediately fixed by transferring into 4% paraformaldehyde and stored at 4 °C overnight. After 3 × 5 min washes with PBST (0.2% PBST: 50 ml PBS + 100 µl Triton X 100), 5% normal goat serum in PBST was used for blocking at 4 °C overnight. Tissue samples were washed 3 times with PBST and were incubated with primary antibody, Rabbit anti-GFP (Abcam) a dilution of 1:2000 and stored at 4 °C overnight. After 3 washes with PBST, tissue samples were incubated with the secondary antibody, Alexa 488 Goat anti-Rabbit (Jackson ImmunoResearch) at 4 °C overnight. After 3 washes with PBST and 1 wash with PBS, samples were exposed through a series of gradually increasing concentrations of glycerol solutions. Tissue samples were mounted on a glass slide in 100% glycerol. Confocal images were taken at the Institute for Integrative Genome Biology (IIGB), University of California, Riverside (UCR) using a Leica SP2 confocal microscope.

2.3. Video recording

Videos were recorded using a Sony HDR-XR150 camera for 10 min and behavior analysis was done manually. Video analysis was done on a Toshiba DVD recorder (model RD-XS35) by slowing down the video by 16x. All data analysis was done blind. All data were recorded and analyzed completely randomly across the genotype.

2.4. Courtship assays

Courtship assays were performed using 48-well polystyrene plate with chamber dimensions of 15 mm height and 10 mm diameter. One test and one subject fly were carefully aspirated into the arena and a maximum of six pairs were recorded at a time. Courtship behavior included following, orientation, tapping, singing and attempted copulation (bending of abdomen). Courtship index (CI) for male-male courtship was calculated as percentage of time spent courting other males during a 10 min test session. In the case of male-female courtship, courtship index was calculated as percentage of time spent by a male courting a female either until either copulation occurred or 10 min in the absence of copulation. Wing-extension index (WEI) was calculated as the percentage of time a male extends its wing perpendicular to its

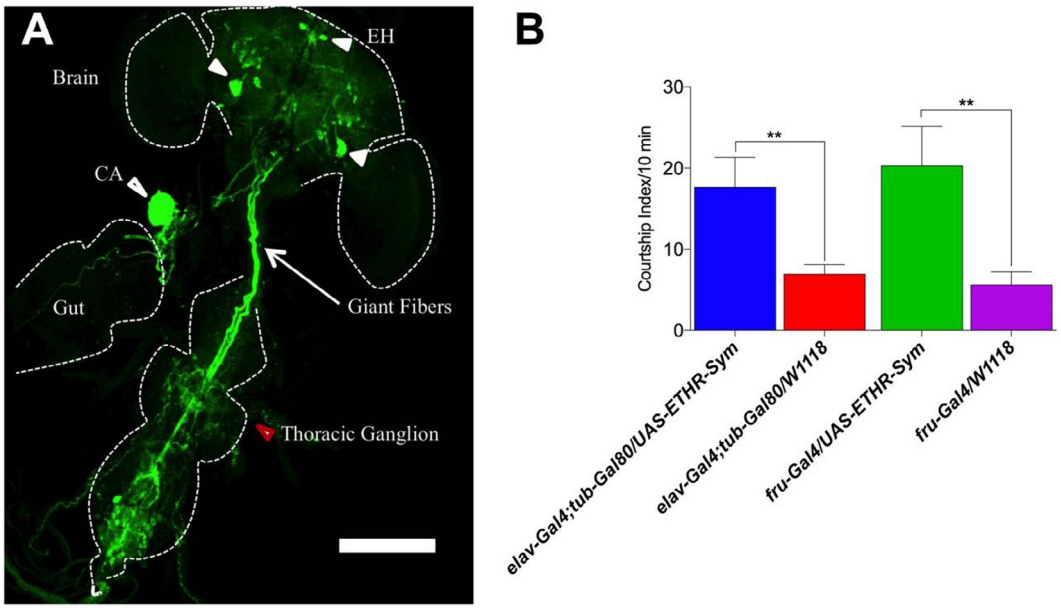

Fig. 3. ETHRs in fru-positive neurons regulate male courtship behavior. A) Immunostaining of 3-day old adult male *Aug21-Gal4/UAS-mCD8-GFP* indicates expression in the CNS along with CA. Red arrow - thoracic ganglion, open white arrowhead – CA and closed white arrowheads - neurons. White scale bar indicates 150 µm. B) Increased courtship indices of males towards WT males using pan-neuronal (*elav-Gal4*) or *fruitless* neuron specific (*fru-Gal4*) drivers. n = 5–20. Error bars indicate SEM. **P < .01 (Mann-Whitney Rank Sum Test).

body during 10 min of interaction time. A total of 10–20 pairs were tested for each genotype and an average CI and WEI was calculated and standard error mean (SEM) was determined for each genotype. All the courtship assays were performed between Zeitgeber time 7 to 9 at 23 °C (Goldman and Arbeitman, 2007).

2.5. 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 et al., 2012), with ~150 flies for each genotype divided into three groups for statistical analysis.

2.6. Methoprene treatment

Adult flies were topically treated with 0.01% methoprene dissolved in acetone as described previously (Meiselman et al., 2017).

2.7. RNAseq analysis

Adult fly samples from control flies (*Aug21-Gal4/+*) and ETHR-RNAi flies (*Aug21-Gal4 > UAS-ETHR-Sym*) were collected in 2 groups. For each set, 50 adult naïve male heads (3–5 day posteclosion) were collected by snap freezing flies into liquid nitrogen at ZT 7. All head samples were processed similar to whole fly samples with size selection of 300 bps. Samples were submitted to the IIGB, UCR and were subjected to single-end sequencing on the HiSeq2000 (Illumina). Libraries were run at a concentration of 1.375 pM using 100 cycles. A total of 4 samples were multiplexed together in one lane.

Data obtained from IIGB, UCR were de-multiplexed based on barcode sequences using custom written Practical Extraction and Reporting Language (PERL) scripts (Wall, 2000). Reads matching ribosomal RNA sequences (using the BLAT alignment program (Kent, 2002)) were removed from further analysis, since they likely were degradation products that had contaminated the library. The remaining reads obtained from Illumina were aligned against the *D. melanogaster* genome, Berkeley *Drosophila* Genome Project (BDGP) assembly release 5 (Trapnell et al., 2009), using default parameters. Expression levels of known *D. melanogaster* transcripts were estimated using Reads Per Kilobase of transcript per Million mapped reads (RPKM values) (Mortazavi et al., 2008). Differential gene expression analysis was performed using the DEseq R-package (Anders and Huber, 2010) comparing control versus RNAi libraries. A p-value cut-off of < 0.1 was used, due to lack of replicates; p-values instead of p-adjusted were used for analysis. Only transcripts with reads ≥10 in at least one of the libraries were used for analysis (Illumina, 2011).

Gene identifiers for differentially expressed genes were uploaded to www.flymine.org and chromosomal distribution, tissue distribution and gene names were converted into database identifiers (eg. FBgn0028738). A database identifier list was used for gene ontology enrichment analysis. Gene ontology analysis for both up-regulated and down-regulated genes, based on biological process, cellular function and molecular function, was performed on www.flymine.org using the Holm-Bonferroni multiple hypothesis test correction at p < 0.05. Data was uploaded on www.flymine.org (Lyne et al., 2007) and GO terminologies were generated for each dataset. Genes falling into each category were transferred to an Excel file and pie charts were generated using MATLAB.

2.8. Statistics

Statistical analyses were performed on all data sets using http://faculty.vassar.edu/lowry/VassarStats.html (last accessed in March 2012). The Kruskal-Wallis test with Mann-Whitney *post hoc* test was performed on non-parametric data sets for statistical comparison of behaviors exhibited by ETHR silenced flies and control flies.

3. Results

3.1. Aug21-Gal4 driven ETHR-RNAi elevates male-male courtship

Drosophila courtship is a complex innate behavior coordinated by chemosensory, auditory, mechanosensory and visual senses (Krstic et al., 2009). Since ETHRs are expressed in adult male CA and are necessary for maintenance of normal JH levels (Meiselman et al., 2017),

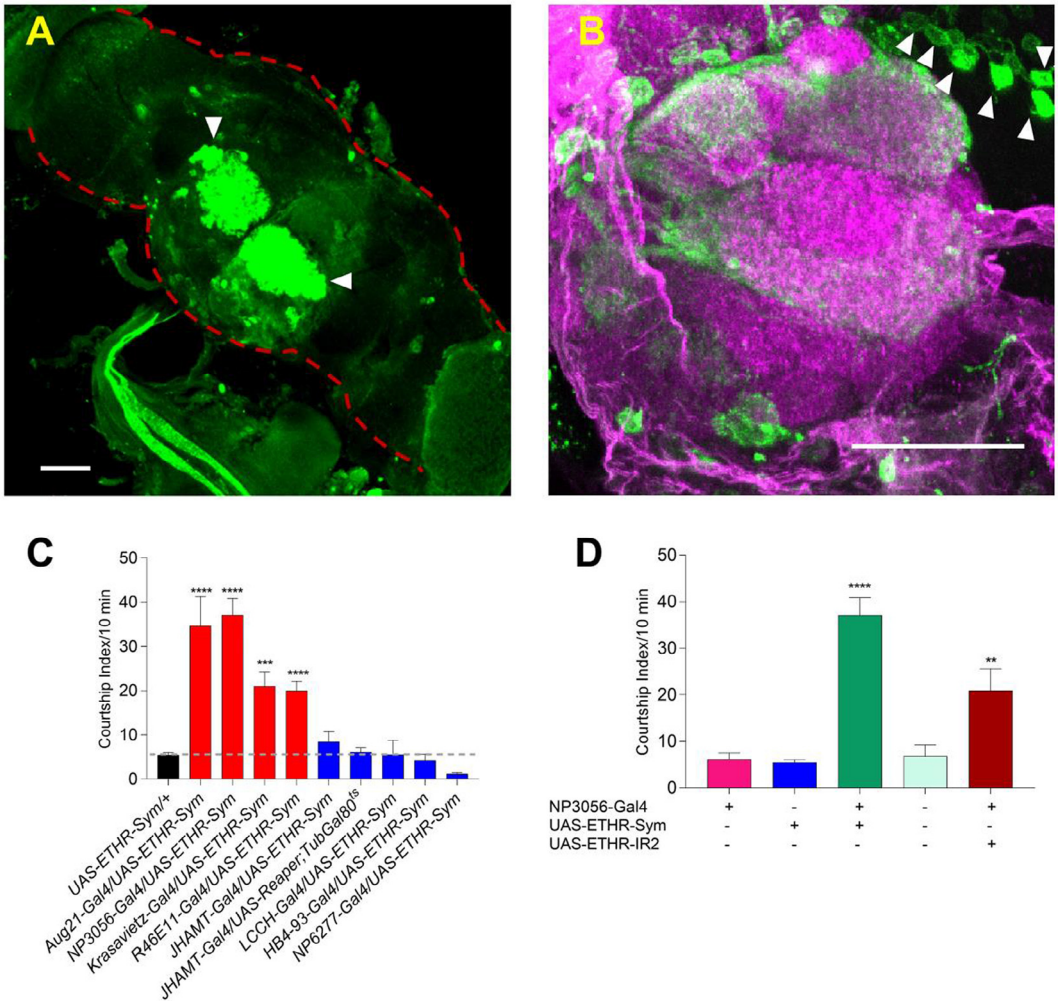

Fig. 4. ETHRs are expressed in antennal lobe interneurons and recapitulate increased male courtship behavior towards WT males. A) Expression of *Aug21-Gal4/UAS-mCD8-GFP* in antennal lobe. Closed white arrowheads indicate antennal lobes. Scale bar indicates 50 µm. B) *ETHR-Gal4* expression in AL interneurons using the Trojan-ETHR-Gal4 driver expressing *UAS-mCD8GFP*. Scale bars indicate 20 µm. Arrowheads indicate cell bodies of antennal lobe interneurons. C) Comparison of increased courtship CI following ETHR silencing using an assortment of *Aug21-Gal4* and AL interneuron Gal4 drivers. n = 10–15 D) AL Gal4 driver, *NP3056-Gal4* recapitulates elevated male-male courtship index similar to that obtained using the *Aug21-Gal4* driver. n = 10–20. Error bars indicate SEM. **P < .01; ****P < .0001 (One-way ANOVA).

we hypothesized that ETHR and consequent JH deficiency may affect male courtship behavior. To test this hypothesis, we used the *Aug21-Gal4* driver known to target the CA (Colombani et al., 2005, Mirth et al., 2005) to silence ETHR using two independent RNAi constructs: (*UAS-ETHR-Sym* and *UAS-ETHR-IR2*). Five- to seven-day old males were monitored for 10 min intervals for interactions with wild-type (WT) males having two genetic backgrounds (Canton S or w^{1118}). Courtship index (CI) was computed as total time spent engaged in courtship behavior toward WT males, including tapping, wing extension, licking, and copulation attempts. Wing extension index (WEI) was calculated as amount of time spent extending a single wing towards the WT subject male, a behavior unique to the courtship display, which is used to "sing," or convey an auditory cue to the subject.

We observed sharp elevation of male-male CI after ETHR silencing (Fig. 1A, B, 2B, 3B, 4C, D), with ETHR-knockdown males displaying CI values up to 35 compared to genetic controls, where CI values were < 10. Similarly, mean WEI of ETHR silenced males of both Canton-S and w^{1118} backgrounds is greatly increased (Fig. 1C, D).

3.2. JH deficiency does not cause male-male courtship

Since *Aug21-Gal4* drives expression in the CA (Colombani et al., 2005, Mirth et al., 2005), we examined whether male JH levels are reduced upon ETHR-silencing. Indeed, JH levels decrease by 90% in *Aug21-Gal4 > UAS-ETHR-Sym* males compared to Gal4 controls (Fig. 2A). Of particular note, *Aug21-Gal4 > UAS-ETHR-Sym* females showed no significant drop in JH levels, indicating a sex-specific action of the *Aug21-Gal4* driver.

JH plays a critical role in male courtship behavior (Wijesekera et al., 2016, Lee et al., 2017). We therefore asked whether JH deficiency contributes to the elevated male-male courtship observed in *Aug21-Gal4 > UAS-ETHR-Sym* and *Aug21-Gal4 > UAS-ETHR-IR2* flies. We first treated males with the juvenile hormone analog methoprene, but found no significant rescue in the elevated male-male courtship phenotype when compared to untreated males (Fig. 2B). We then examined whether the *Aug21-Gal4* driver could cause elevated male-male courtship in the absence of JH deficiency through co-expression of *JHAMT-Gal80*, so as to block Gal4 expression in the CA. *JHAMT-Gal4* is known to be specific for the CA during adulthood (Wijesekera et al., 2016, Meiselman et al., 2017). We nevertheless observed significant elevation of male-male courtship in *Aug21-Gal4; JHAMT-Gal80 > UAS-ETHR-Sym* flies (Fig. 2B), providing further evidence that JH deficiency does not contribute to the male-male courtship phenotype.

Additional experiments were devoted to silencing ETHR using the

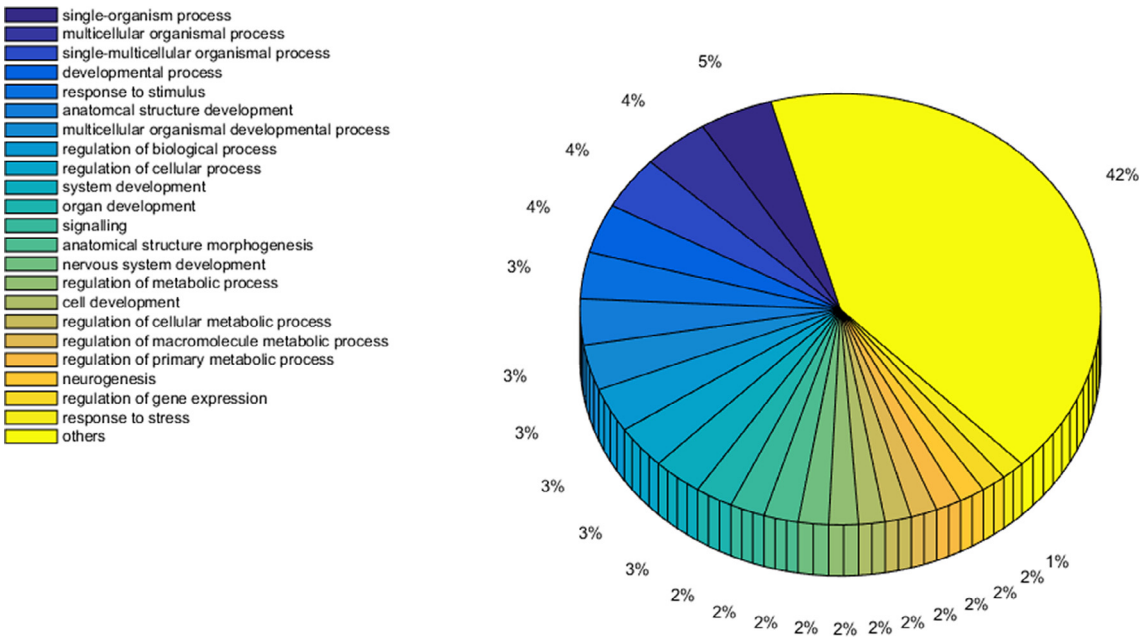

Fig. 5. Functional characterization of genes up-regulated in heads after ETHR silencing. GO terms enriched indicates role of ETHRs in various biological processes, including neurogenesis.

JHAMT-Gal4 driver alone. While silencing ETHR expression in *JHAMT-Gal4 > UAS-ETHR-Sym* males creates JH deficiency (Lee et al., 2017, Meiselman et al., 2017), we observed no difference in male-male CI compared to controls (Fig. 2C). As Aug21-ETHR silencing produces a more severe reduction of JH levels than did JHAMT-driven ETHR silencing reported previously (Meiselman et al., 2017), we asked whether more severe JH depletion may be driving male-male courtship by ablating the CA specifically in the adult stage using *JHAMT-Gal4 > UAS-Reaper;tubulin-Gal80^{ts}*. Again, we found no difference from controls (Fig. 2C), confirming that JH deficiency is not responsible for elevated male-male courtship observed in *Aug21-Gal4 > UAS-ETHR-Sym* flies.

3.3. Aug21-Gal4 expression extends to the adult central nervous system

Although *Aug21-Gal4* is reported to be a CA-specific driver during larval stages (Colombani et al., 2005, Mirth et al., 2005), we found that its pattern of expression in male adults extends to the central nervous system (CNS). Central neurons labeled in *Aug21-Gal4 > UAS-mCD8GFP* flies include giant descending interneurons, eclosion hormone neurons, and local interneurons of the antennal lobe (AL) (Fig. 3A, Fig. 4A). We therefore hypothesized that elevated male-male courtship behavior resulting from the *Aug21-Gal4 > UAS-ETHR-Sym* genotype is a consequence of ETHR silencing in the CNS.

3.4. ETHRs expressed in fru-positive and antennal interneurons are critical for normal male courtship behavior

To test this hypothesis, we first utilized the pan-neuronal driver *elav-Gal4* for ETHR knockdown. To avoid lethal ecdysis deficiencies, we employed the conditional, temperature sensitive driver *tub-Gal80^{ts}* to delay ETH silencing until after eclosion. We found that *elav-Gal4; tub-Gal80^{ts} > UAS-ETHR-Sym* males show elevated male-male CI ($P < 0.01$) (Fig. 3B).

Since *fru*itless neurons regulate male-male courtship, we hypothesized that ETHRs are expressed in *fruitless* neurons and that receptor silencing in those neurons would promote male-male courtship. Hence, a *fru-Gal4* driver (Robinett et al., 2010) was used for ETHR silencing. Courtship index of *fru-Gal4 > UAS-ETHR-Sym* males is elevated significantly ($P < 0.01$) (Fig. 3B).

Aug21-Gal4 expresses prominently in antennal lobe (AL) interneurons (Fig. 4A), which are sexually dimorphic. We therefore examined whether the Trojan *ETHR-Gal4* line (Diao et al., 2016) expresses in local interneurons of the male AL and found *ETHR-Gal4* is indeed prominently expressed (Fig. 4B). We screened several AL Gal4 drivers, including R46E11-Gal4 (Jenett et al., 2012), HB4-94-Gal4, NP6277-Gal4, LCCH-Gal4, NP3056-Gal4 (Chou et al., 2010), and Krasavietz-Gal4 (Dubnau et al., 2003) to drive ETHR RNAi and assessed male-male courtship. ETHR knockdown using three of the aforementioned drivers resulted in significant elevation of male-male CI. In particular, NP3056-Gal4, which labels local interneurons in the antennal lobe specifically (Chou et al., 2010), essentially recapitulates the male-male courtship phenotype observed in *Aug21-Gal4 > UAS-ETHR-Sym* males (Fig. 4C, D). These data suggest that ETHR expression in AL interneurons is critical for regulation of male courtship behavior.

3.5. Differential gene expression in response to ETHR silencing

We assessed differential expression of transcripts in male heads after ETHR silencing (ETHR-RNAi), using Illumina RNAseq. Raw expression data is provided in Dataset S1. Cuffdiff analysis using a p-value cut-off of 0.05 yielded a total of 2045 differentially expressed transcripts in RNAi library as compared to the control library, which includes 456 up-regulated transcripts corresponding to 400 genes and 1589 down-regulated transcripts corresponding to 1474 genes. Out of 1589 down-regulated transcripts, 852 transcripts were only expressed in the control library and no expression was detected in the RNAi library. Up-regulated genes, which were only expressed in RNAi library and not detected in control library, included 64 transcripts. Differential expression of a large number of genes in male heads as a consequence of ETHR silencing indicates significant functional roles for ETHRs in adult male flies (Fig. S1).

We attempted to determine the efficiency of RNAi silencing through analysis of RNAseq data. However, ETHR transcripts read counts were very low (< 10), thus preventing conclusions to be made from this data. Alternatively, we utilized qPCR and determined that, based on $\log_2$-fold changes in transcript number in fly heads, ETHR-A is down-regulated by ~87% and ETHR-B by ~70%, indicating high efficiency of RNAi (Fig. S2). We next determined differential gene expression by

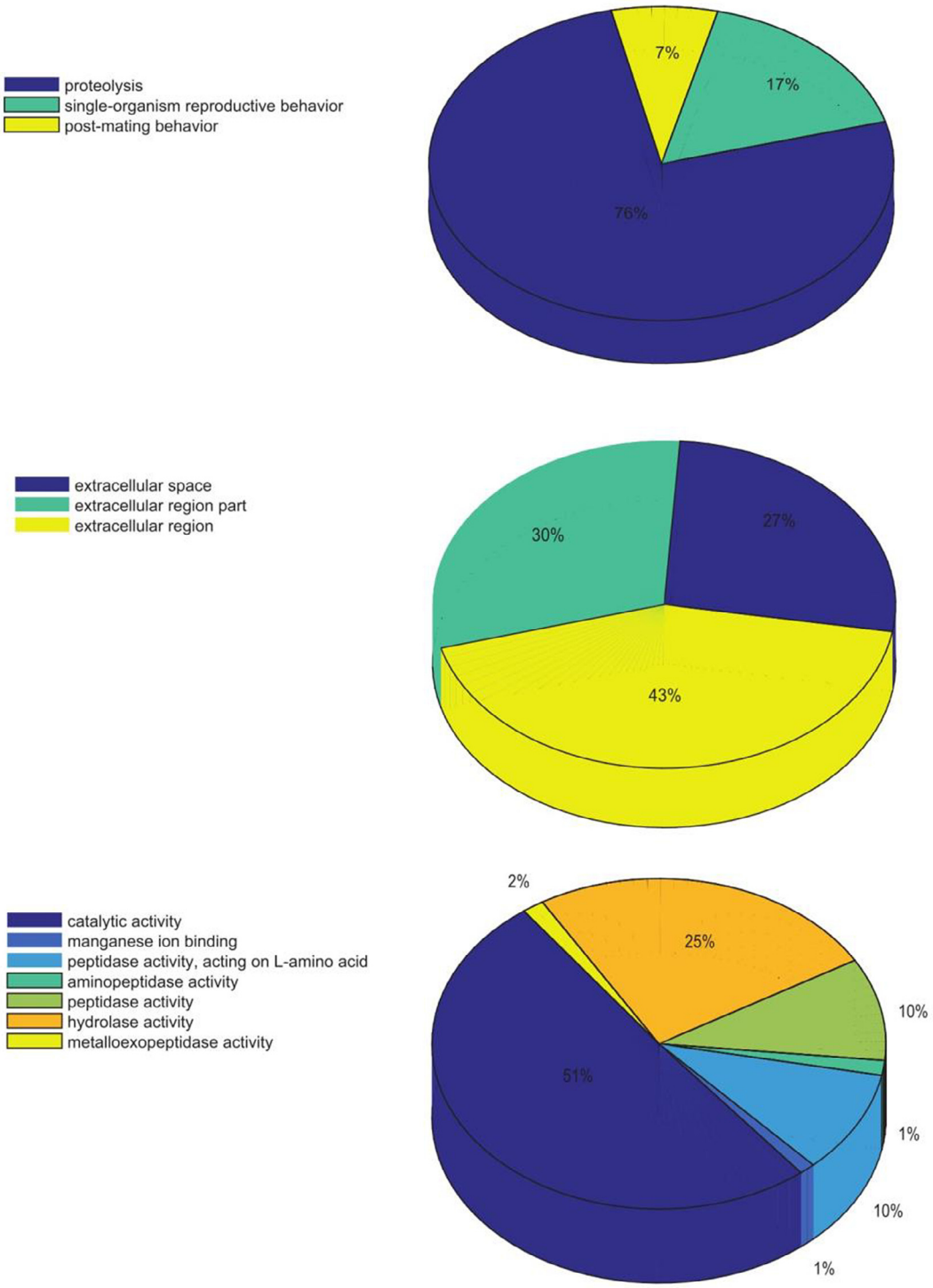

Fig. 6. Functional characterization of genes down-regulated in heads after ETHR silencing. GO terms enriched as biological process (upper) cellular components (middle) and molecular function (lower) with the percentage of genes included in each term indicates role of ETHRs in various adult functions, including reproduction and post-mating behavior.

examining gene ontology categories of up- and down-regulated genes after ETHR silencing. Gene ontology (GO) analysis of up-regulated genes revealed terms enriched under the biological process category, including defense response and developmental processes, metamorphosis and signaling (Fig. 5). This suggests that ETHR is an important signaling molecule in *Drosophila* male heads. Cellular component-based GO enriched two terms, plasma membrane (38) and cell periphery (40). None of the terms were enriched under the GO category molecular function.

Genes implicated in male courtship behavior, including *paralytic (para), pale (ple)* and *shibire (shi)* are up-regulated in male heads after ETHR-RNAi. Two transcripts of the sodium channel gene *para* are up-regulated, whereas one is down-regulated. *para* is a voltage-gated sodium channel known to play a role in courtship song (Peixoto and Hall, 1998). *ple* has a tyrosine 3-monooxygenase activity and is known to be influenced by JH (Neckameyer and White, 1993, Gruntenko et al., 2009). The gene product of *shi* has GTPase activity and functions in vesicle recycling (Ramaswami et al., 1994).

GO analysis of down-regulated genes revealed terms enriched under the biological process category, including proteolysis (1 1 3), single-organism reproduction behavior (25) and post-mating behavior (11) (Fig. 6A). Terms enriched under the cellular component category included extracellular space (69), extracellular region part (79) and extracellular region (1 1 2), indicating presence of structural components downstream of ETHR silencing (Fig. 6B). Terms enriched under molecular function include ~50% of the genes with catalytic activity (4 8 9) and hydrolase activity (2 4 3) (Fig. 6C).

In order to identify genes involved in male courtship behavior, genes associated with the term "male courtship behavior" were searched. Two transcripts of *dlg1* and one transcript each of *para* and *shi* are down-regulated. Interestingly, transcripts of *para* and *shi* were differentially regulated, some were up-regulated and some down-regulated, indicating transcript-specific roles for these genes. 195 genes involved in axon guidance were individually searched. Overall, 39 transcripts related to axon guidance changed after ETHR-RNAi, out of which 22 are down-regulated and 17 are up-regulated (Table S1). Genes interacting with *dsx* and *fru* were individually searched; 14 transcripts including *tra2, Mst84Da, Mst84Dd, hale* and *RSP15Aa* were down-regulated after ETHR silencing.

Some transcripts (64) were expressed only in the RNAi library, but not detected in the control library, indicating their expression may depend upon innate levels of ETHR. Further analysis is required to determine whether these transcripts are regulated by ETHRs. About 852 transcripts were expressed in control libraries, but not in the RNAi head library, after ETHR-RNAi. These include 4 gustatory receptor transcripts (*Gr36d, Gr64e, Gr98a* and *Gr47b),* and axon guidance genes including *Mical, stan, Nedd4, mp, sim, ena, ago, mew, sli, NijA, sbb, exba* and *tok*. Three transcripts (*Acp98AB-RA, BG642163-RA* and *lectin-29Ca-RA)* are accessory gland related proteins, of which *BG642163-RA* and *lectin-29Ca-RA* are thought to be male accessory gland-specific genes (Ranz et al., 2003, Rodgers-Melnick and Naz, 2010). *polo-RA and polo-RB* transcripts are derived from the gene *polo,* possess serine/threonine kinase activity, and are known to be expressed in ovary and testis (Carmena et al., 1998). Six transcripts are found specifically in testis: *ms (2)35Ci* and *ms(2)34Fe* with unknown function*, klhl10-RA*-involved in oxidoreductase activity, *ACXA* with adenylate cyclase activity and casein kinase II β2 subunit, (*CKIIbeta2*) with protein kinase activity and tetraspanin 42A (*Tsp42A*). (Dataset S1).

4. Discussion

We have demonstrated an essential role for ETH signaling in courtship inhibition in adult male *Drosophila.* Reduction of ETHR signaling through RNAi silencing in local interneurons of the male antennal lobe leads to marked elevation of male-male courtship. Furthermore, we show that ETHR expression in *fruitless* neurons is critical for regulation of male courtship behavior. Our findings reveal a crucial function for ETHR in the ability of males to distinguish male from female.

We describe here for the first time that *Aug21-Gal4* is much more broadly expressed during adulthood as compared to larval stages. Although *Aug21-Gal4* is reputed to be a CA specific driver in larval stages (Mirth et al., 2005, Riddiford et al., 2010), we report here that after metamorphosis its expression is not restricted to CA and it expands to CNS, including AL interneurons in adult stage. ETHRs are expressed in CA of the silkworm *Bombyx mori* and *Drosophila melanogaster* (Yamanaka et al., 2008, Lee et al., 2017, Meiselman et al., 2017), the sole source of JH, which influences social interactions such as courtship behavior in various insects, including *Drosophila* (Anton and Gadenne, 1999, Gadenne and Anton, 2000, Wijesekera et al., 2016). Since *Aug21-Gal4* drives expression in the CA, we hypothesized that knockdown of ETHR in CA might create JH deficiency in adult males, thereby affecting male courtship behavior. Indeed, we found that JH levels were reduced in *ETHR-RNAi* males, but unchanged in females. Although previous studies have shown that JH levels are affected by ETHR silencing in female CA using the JHAMT-Gal4 driver (Meiselman et al., 2017), we did not observe this in our study using the *Aug21-Gal4* driver. This discrepancy can be attributed to sexual dimorphism in expression of the *Aug21-Gal4* driver, where it shows significantly lower expression in females as compared to males.

Significantly, we observed no rescue of the male-male courtship phenotype in methoprene-treated males. Furthermore, we created JH deficiency using two additional approaches: 1) using the CA specific driver *JHAMT-Gal4* to silence ETHR expression and 2) using *JHAMT-Gal4* to ablate the CA. In both cases, no elevation of male-male courtship was observed. These findings confirm that JH deficiency does not lead to the male-male courtship phenotype observed using the *Aug21-Gal4* driver. We thus conclude that, although ETHRs regulate JH levels in males, elevation of male-male courtship must be attributed to a different mechanism.

Use of neuronal Gal4 drivers, including *elav-Gal4*, *fru-Gal4* and antennal lobe Gal4 drivers (*R46E11-Gal4*, *NP3056-Gal4*, and *krasavietz-Gal4*) to silence ETHR expression led to markedly elevated levels of male-male courtship. Although some of these Gal4 drivers yielded lower levels of male-male CI as compared to the *Aug21-Gal4* driver, variability in cell-specific strength or combinatorial labeling of heterologous neurons by Gal4 drivers can account for some of these discrepancies (Pramatarova et al., 2006). Of particular note, the AL-specific driver *NP3056-Gal4* recapitulates high male-male courtship indices observed with the *Aug21-Gal4* driver, indicating a crucial role for AL local interneurons in courtship regulation.

Drosophila males discriminate males from females according to gender-specific pheromone profiles. For example, 11-*cis*-vaccenyl acetate (cVA) functioning as an olfactory cue activates olfactory receptors (ORs) OR67d and OR65a (Kurtovic et al., 2007, van der Goes van Naters and Carlson, 2007, Ronderos and Smith, 2010, Lebreton et al., 2014). This information is processed in sexually dimorphic antennal lobe glomeruli (VA1lm and DA1), which play a prominent role in male courtship behavior (Kondoh et al., 2003, Fishilevich and Vosshall, 2005, Manoli et al., 2005, Stockinger et al., 2005). Our observations that ETHR-silencing in local interneurons of the antennal lobe elevates male-male courtship suggests defective processing of male-specific pheromone sensing. Future experiments will be aimed at assessing how ETHR silencing affects pheromone-induced activity in these interneurons. Although ETHRs have higher sensitivity to ETH than to other peptides (Kim et al., 2006), it is possible that peptides such as small cardioactive peptide B, if released in large amounts, could potentially play a role in male courtship regulation. In future experiments, the role of ETH signaling in pheromone processing can be further tested by ablating Inka cells, the sole source of ETH, specifically in adult males.

Numerous sensory and neuronal genes have been implicated in regulation of male courtship behavior. In order to investigate genes influenced by changes in ETHR levels that regulate courtship behavior, RNAseq analysis was conducted to determine differential expression of transcripts in ETHR-RNAi male heads. Pheromones play a crucial role in male courtship behavior, whereby males distinguish a male from female based on divergent pheromone profiles (Dweck et al., 2015). Genes associated with chemosensory functions are differentially expressed as a result of ETHR silencing, indicating their role in pheromone processing. Similarly, axon guidance genes define wiring of the nervous system (Clowney et al., 2015). A large number of axon guidance genes are differentially expressed as a result of ETHR silencing, indicating their role in nervous system wiring. Differential expression of a large number of genes as a consequence of ETHR silencing in male heads indicates significant functional roles for ETHRs in adult male flies. Specifically, genes associated with axon guidance and chemosensory functions are affected due to ETHR silencing, suggesting their role in neuronal regulation of the courtship behavior.

5. Conclusions

Our findings indicate that ETHR signaling in local interneurons of the antennal lobe is essential for male-male courtship inhibition. Although ETHRs were shown originally to play a major role in orchestration of ecdysis behaviors, our results indicate a their novel function in *Drosophila* adult males. Although we have not demonstrated a necessary role for ETH in regulation of antennal lobe interneurons, hormones known to change function after metamorphosis include juvenile hormone and ecdysone, both of which have morphogenetic roles in immature stages, but are re-purposed for regulation of reproduction after eclosion to adulthood. Our findings indicate the possibility of a similar metamorphosis for ETH signaling in the adult stage, where it may play an important role in male courtship behavior.

6. Authors' contributions

Conceived the idea: SAD. Performed wet lab experiments: SAD, MM, RHH, RC, CR-P, DHK. Design of RNA-seq: SAD, RHH. Bioinformatics analysis: SAD, PA. Data analysis: SAD, MM, RHH, CR-P, PA. Interpretation of results: SAD, MM, RHH, CR-P, PA, FGN, MEA. Wrote the manuscript: SAD, MM, MEA. Helped writing the manuscript: PA, RHH, FGN. All authors read and approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.003.

References

Amare, A., Sweedler, J.V., 2007. Neuropeptide precursors in Tribolium castaneum. Peptides 28 (6), 1282–1291.

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11 (10), R106.

Anton, S., Gadenne, C., 1999. Effect of juvenile hormone on the central nervous processing of sex pheromone in an insect. Proc. Natl. Acad. Sci. 96 (10), 5764–5767.

Carmena, M., Riparbelli, M.G., Minestrini, G., Tavares, A.M., Adams, R., Callaini, G., Glover, D.M., 1998. Drosophila polo kinase is required for cytokinesis. J. Cell Biol. 143 (3), 659–671.

Chou, Y.H., Spletter, M.L., Yaksi, E., Leong, J.C., Wilson, R.I., Luo, L., 2010. Diversity and wiring variability of olfactory local interneurons in the Drosophila antennal lobe. Nat. Neurosci. 13 (4), 439–449.

Clowney, E.J., Iguchi, S., Bussell, Jennifer J., Scheer, E., Ruta, V., 2015. Multimodal Chemosensory Circuits Controlling Male Courtship in Drosophila. Neuron 87 (5), 1036–1049.

Clynen, E., Huybrechts, J., Verleyen, P., De Loof, A., Schoofs, L., 2006. Annotation of novel neuropeptide precursors in the migratory locust based on transcript screening of a public EST database and mass spectrometry. BMC Genomics 7 (1), 201.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carré, C., Noselli, S., Léopold, P., 2005. Antagonistic Actions of Ecdysone and Insulins Determine Final Size in Drosophila. Science 310 (5748), 667–670.

Dai, L., Adams, M.E., 2009. Ecdysis triggering hormone signaling in the yellow fever mosquito Aedes aegypti. Gen. Comp. Endocrinol. 162 (1), 43–51.

Deshpande, S.A., 2012. Ecdysis Triggering Hormone Signaling in Adult Drosophila melanogaster. Ph.D Dissertation, University of California, Riverside.

Diao, F., Mena, W., Shi, J., Park, D., Diao, F., Taghert, P., Ewer, J., White, B.H., 2016. The Splice Isoforms of the Drosophila Ecdysis Triggering Hormone Receptor Have Developmentally Distinct Roles. Genetics 202 (1), 175–189.

Dubnau, J., Chiang, A.S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc, F., Scott, R., Certa, U., Broger, C., Tully, T., 2003. The staufen/pumilio pathway is involved in Drosophila long-term memory. Curr. Biol. 13 (4), 286–296.

Dweck, H.K.M., Ebrahim, S.A.M., Thoma, M., Mohamed, A.A.M., Keesey, I.W., Trona, F., Lavista-Llanos, S., Svatoš, A., Sachse, S., Knaden, M., Hansson, B.S., 2015. Pheromones mediating copulation and attraction in < em > Drosophila < /em > Proc. Natl. Acad. Sci. 112 (21), E2829–E2835.

Fishilevich, E., Vosshall, L.B., 2005. Genetic and Functional Subdivision of the Drosophila Antennal Lobe. Curr. Biol. 15 (17), 1548–1553.

Gadenne, C., Anton, S., 2000. Central processing of sex pheromone stimuli is differentially regulated by juvenile hormone in a male moth. J. Insect Physiol. 46 (8), 1195–1206.

Ganter, G.K., Walton, K.L., Merriman, J.O., Salmon, M.V., Brooks, K.M., Maddula, S., Kravitz, E.A., 2007. Increased male-male courtship in ecdysone receptor deficient adult flies. Behav. Genet. 37 (3), 507–512.

Goldman, T.D., Arbeitman, M.N., 2007. Genomic and Functional Studies of *Drosophila* Sex Hierarchy Regulated Gene Expression in Adult Head and Nervous System Tissues. PLoS Genet. 3 (11), e216.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., Brown, J.B., Cherbas, L., Davis, C.A., Dobin, A., Li, R., Lin, W., Malone, J.H., Mattiuzzo, N.R., Miller, D., Sturgill, D., Tuch, B.B., Zaleski, C., Zhang, D., Blanchette, M., Dudoit, S., Eads, B., Green, R.E., Hammonds, A., Jiang, L., Kapranov, P., Langton, L., Perrimon, N., Sandler, J.E., Wan, K.H., Willingham, A., Zhang, Y., Zou, Y., Andrews, J., Bickel, P.J., Brenner, S.E., Brent, M.R., Cherbas, P., Gingeras, T.R., Hoskins, R.A., Kaufman, T.C., Oliver, B., Celniker, S.E., 2011. The developmental transcriptome of Drosophila melanogaster. Nature 471 (7339), 473–479.

Gruntenko, N.E., Karpova, E.K., Chentsova, N.A., Adonyeva, N.V., Rauschenbach, I.Y., 2009. 20-hydroxyecdysone and juvenile hormone influence tyrosine hydroxylase activity in Drosophila females under normal and heat stress conditions. Arch. Insect Biochem. Physiol. 72 (4), 263–272.

Hummon, A.B., Richmond, T.A., Verleyen, P., Baggerman, G., Huybrechts, J., Ewing, M.A., Vierstraete, E., Rodriguez-Zas, S.L., Schoofs, L., Robinson, G.E., Sweedler, J.V., 2006. From the Genome to the Proteome: Uncovering Peptides in the Apis Brain. Science 314 (5799), 647.

Illumina, 2011. RNA-Seq data comparison with gene expression microarrays. Ilumina White paper: Sequencing.

Jenett, A., Rubin, G.M., Ngo, T.T., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J., Iyer, N., Fetter, D., Hausenfluck, J.H., Peng, H., Trautman, E.T., Svirskas, R.R., Myers, E.W., Iwinski, Z.R., Aso, Y., DePasquale, G.M., Enos, A., Hulamm, P., Lam, S.C., Li, H.H., Laverty, T.R., Long, F., Qu, L., Murphy, S.D., Rokicki, K., Safford, T., Shaw, K., Simpson, J.H., Sowell, A., Tae, S., Yu, Y., Zugates, C.T., 2012. A GAL4-driver line resource for Drosophila neurobiology. Cell Reports 2 (4), 991–1001.

Kent, W.J., 2002. BLAT—the BLAST-like alignment tool. Genome Res. 12 (4), 656–664.

Kim, D.H., Han, M.R., Lee, G., Lee, S.S., Kim, Y.J., Adams, M.E., 2015. Rescheduling Behavioral Subunits of a Fixed Action Pattern by Genetic Manipulation of Peptidergic Signaling. PLoS Genet. 11 (9), e1005513.

Kim, Y.-J., Žitňan, D., Galizia, C.G., Cho, K.-H., Adams, M.E., 2006a. A Command Chemical Triggers an Innate Behavior by Sequential Activation of Multiple Peptidergic Ensembles. Curr. Biol. 16 (14), 1395–1407.

Kim, Y.J., Žitňan, D., Cho, K.H., Schooley, D.A., Mizoguchi, A., Adams, M.E., 2006b. Central peptidergic ensembles associated with organization of an innate behavior. PNAS 103 (38), 14211–14216.

Kondoh, Y., Kaneshiro, K.Y., Kimura, K., Yamamoto, D., 2003. Evolution of sexual dimorphism in the olfactory brain of Hawaiian < em > Drosophila < /em > Proc. R. Soc. Lond. B Biol. Sci. 270 (1519), 1005–1013.

Krstic, D., Boll, W., Noll, M., 2009. Sensory integration regulating male courtship behavior in Drosophila. PLoS ONE 4 (2), e4457.

Kurtovic, A., Widmer, A., Dickson, B.J., 2007. A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature 446 (7135), 542–546.

Lebreton, S., Grabe, V., Omondi, A.B., Ignell, R., Becher, P.G., Hansson, B.S., Sachse, S., Witzgall, P., 2014. Love makes smell blind: mating suppresses pheromone attraction in Drosophila females via Or65a olfactory neurons. Sci. Rep. 4, 7119.

Lee, S.S., Ding, Y., Karapetians, N., Rivera-Perez, C., Noriega, F.G., Adams M.E., 2017. Hormonal Signaling Cascade during an Early-Adult Critical Period Required for Courtship Memory Retention in Drosophila. Curr. Biol. 27(18) 2798–2809 e2793.

Lyne, R., Smith, R., Rutherford, K., Wakeling, M., Varley, A., Guillier, F., Janssens, H., Ji, W., McLaren, P., North, P., Rana, D., Riley, T., Sullivan, J., Watkins, X., Woodbridge, M., Lilley, K., Russell, S., Ashburner, M., Mizuguchi, K., Micklem, G., 2007. FlyMine: an integrated database for Drosophila and Anopheles genomics. Genome Biol. 8 (7) R129 R129.

Manoli, D.S., Foss, M., Villella, A., Taylor, B.J., Hall, J.C., Baker, B.S., 2005. Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. Nature 436, 395.

Meiselman, M., Lee, S.S., Tran, R.-T., Dai, H., Ding, Y., Rivera-Perez, C., Wijesekera, T.P., Dauwalder, B., Noriega, F.G., Adams, M.E., 2017. Endocrine network essential for reproductive success in Drosophila melanogaster. Proc. Natl. Acad. Sci. 114 (19), E3849–E3858.

Mirth, C., Truman, J.W., Riddiford, L.M., 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster. Curr. Biol. 15 (20), 1796–1807.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5 (7), 621–628.

Neckameyer, W.S., White, K., 1993. Drosophila tyrosine hydroxylase is encoded by the pale locus. J. Neurogenet. 8 (4), 189–199.

Park, Y., Žitňan, D., Gill, S.S., Adams, M.E., 1999. Molecular cloning and biological activity of ecdysis-triggering hormones in Drosophila melanogaster. FEBS Lett. 463 (1), 133–138.

Peixoto, A.A., Hall, J.C., 1998. Analysis of temperature-sensitive mutants reveals new genes involved in the courtship song of Drosophila. Genetics 148 (2), 827–838.

Pramatarova, A., Ochalski, P.G., Lee, C.H., Howell, B.W., 2006. Mouse disabled 1 regulates the nuclear position of neurons in a Drosophila eye model. Mol. Cell. Biol. 26 (4), 1510–1517.

Ramaswami, M., Krishnan, K.S., Kelly, R.B., 1994. Intermediates in synaptic vesicle recycling revealed by optical imaging of Drosophila neuromuscular junctions. Neuron 13 (2), 363–375.

Ranz, J.M., Castillo-Davis, C.I., Meiklejohn, C.D., Hartl, D.L., 2003. Sex-dependent gene expression and evolution of the Drosophila transcriptome. Science 300 (5626), 1742–1745.

Riddiford, L.M., Truman, J.W., Mirth, C.K., Shen, Y.-C., 2010. A role for juvenile hormone in the prepupal development of Drosophila melanogaster. Development (Cambridge, England) 137 (7), 1117–1126.

Rivera-Perez, C., Nouzova, M., Noriega, F.G., 2012. A quantitative assay for the juvenile hormones and their precursors using fluorescent tags. PLoS ONE 7 (8), e43784.
Robinett, C.C., Vaughan, A.G., Knapp, J.-M., Baker, B.S., 2010. Sex and the Single Cell. II. There Is a Time and Place for Sex. PLoS Biol. 8 (5), e1000365.
Rodgers-Melnick, E.B., Naz, R.K., 2010. Male-biased genes of Drosophila melanogaster that are conserved in mammalian testis. Front Biosci (Elite Ed) 2, 841–848.
Ronderos, D.S., Smith, D.P., 2010. Activation of the T1 neuronal circuit is necessary and sufficient to induce sexually dimorphic mating behavior in Drosophila melanogaster. J. Neurosci. 30 (7), 2595–2599.
Schwedes, C.C., Carney, G.E., 2012. Ecdysone signaling in adult Drosophila melanogaster. J. Insect Physiol. 58 (3), 293–302.
Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirián, L., Dickson, B.J., 2005. Neural Circuitry that Governs Drosophila Male Courtship Behavior. Cell 121 (5), 795–807.
Trapnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25 (9), 1105–1111.
van der Goes van Naters, W., Carlson, J.R., 2007. Receptors and neurons for fly odors in Drosophila. Curr. Biol. 17 (7), 606–612.
Wall, L., 2000. Programming Perl. O'Reilly Associates Inc.
White, B.H., Ewer, J., 2014. Neural and hormonal control of postecdysial behaviors in insects. Annu. Rev. Entomol. 59, 363–381.
Wijesekera, T.P., Saurabh, S., Dauwalder, B., 2016. Juvenile Hormone Is Required in Adult Males for Drosophila Courtship. PLoS ONE 11 (3), e0151912.
Yamanaka, N., Yamamoto, S., Žitňan, D., Watanabe, K., Kawada, T., Satake, H., Kaneko, Y., Hiruma, K., Tanaka, Y., Shinoda, T., Kataoka, H., 2008. Neuropeptide Receptor Transcriptome Reveals Unidentified Neuroendocrine Pathways. PLoS ONE 3 (8), e3048.
Žitňan, D., Adams, M.E., 2012. Neuroendocrine regulation of ecdysis. Insect Endocrinology. L. I. Gilbert. Academic Press, Amsterdam, Boston, pp. 253–309.
Žitňan, D., Hollar, L., Spalovská, I., Takáč, P., Žitňanová, I., Gill, S.S., Adams, M.E., 2002. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. J. Exp. Biol. 205 (22), 3459.
Žitňan, D., Kingan, T.G., Hermesman, J.L., Adams, M.E., 1996. Identification of Ecdysis-Triggering Hormone from an Epitracheal Endocrine System. Science 271 (5245), 88.
Žitňan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., Adams, M.E., 1999. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. Neuron 23 (3), 523–535.
Žitňan, D., Žitňanová, I., Spalovská, I., Takáč, P., Park, Y., Adams, M.E., 2003. Conservation of ecdysis-triggering hormone signalling in insects. J. Exp. Biol. 206 (8), 1275.