REPORT

Hormonal control of *fruitless* expression and male sexual orientation in *Drosophila*

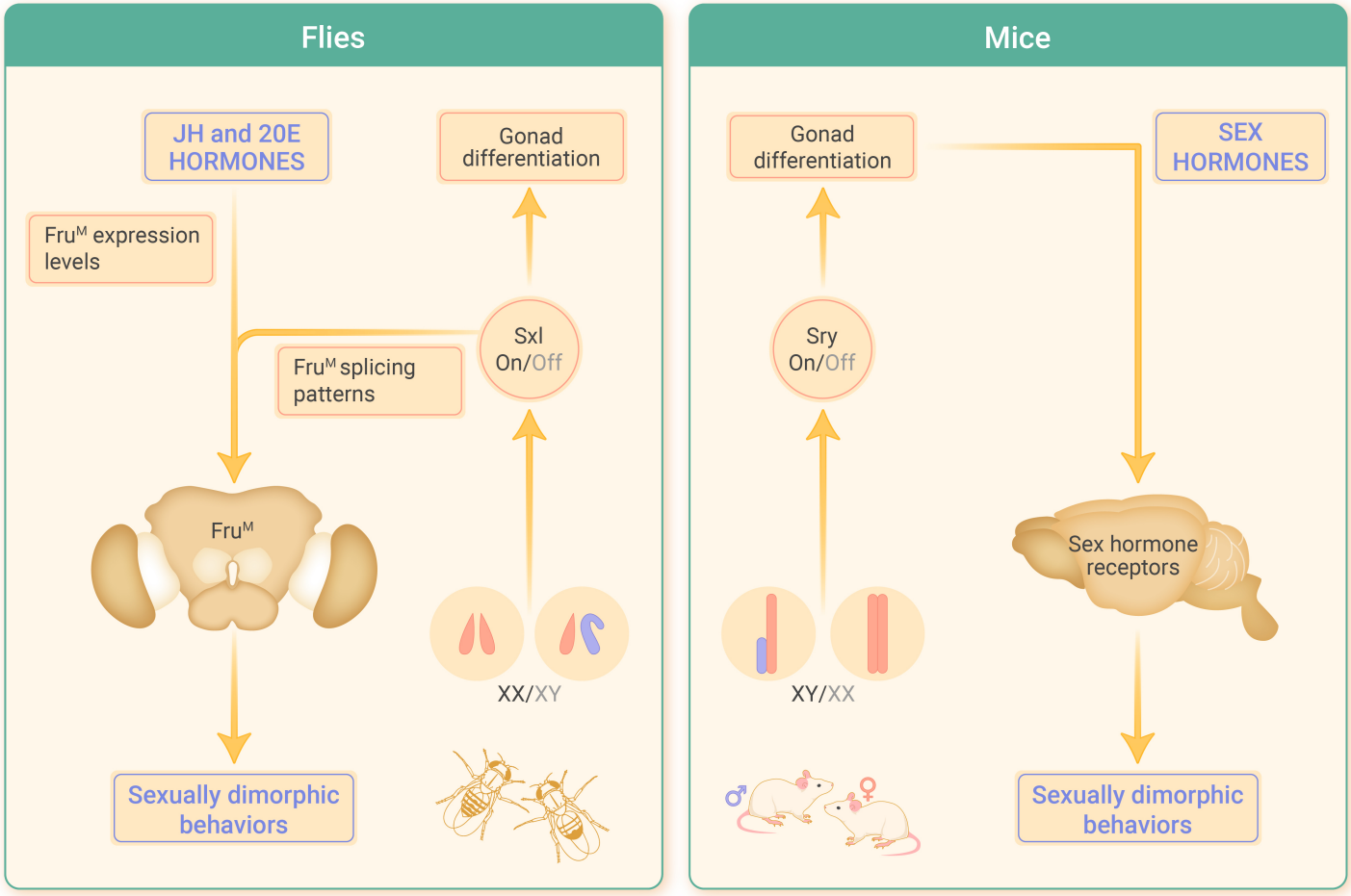
Xiaoxiao Ji,[1] Lin Wang,[1] Xiaolong Li,[1] Sheng Li,[2,3] Suning Liu,[2,3,*] and Yufeng Pan[1,4,*]

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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Ecdysone promotes larval and pupal expression of *fruitless* (*fru*), a master gene for male courtship in flies.
- Juvenile hormone suppresses larval *fru* expression by inhibiting ecdysone signaling.
- Ecdysone regulates the morphology and activity of courtship command-like neurons to alter male sexual orientation.
- Fly hormones and vertebrate sex hormones play conserved and diversified roles in sexual development and behaviors.

REPORT

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Hormonal control of *fruitless* expression and male sexual orientation in *Drosophila*

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Sex determination genes control the differentiation of gonads that synthesize and secret sex hormones to remotely control sexual development and behaviors in mammals. In contrast, sex determination genes in insects directly control sexual development and behaviors in a cell-autonomous way, which is largely independent of hormones. In this study, we find that two important hormones, juvenile hormone (JH) and 20-hydroxyecdysone (20E), oppositely regulate the dynamic expression of Fru^M, the male-specific product of the sex determination gene *fruitless* in *Drosophila*. 20E signaling is essential to the high-level expression of Fru^M proteins in the pupal stage, while JH suppresses larval Fru^M expression through inhibiting 20E signaling. We further show that 20E signaling regulates both the development and physiological function of the male-specific P1 neurons to mediate sexual orientation in male flies. Our results demonstrate both conserved and diversified functions of hormones in sexual development and behaviors in flies and mice.

INTRODUCTION

Sex hormones and sex determination genes control sexual differentiation across animal kingdom. In mammals, the *sex-determining region of the Y chromosome* (*Sry*) gene controls the differentiation of gonad (testis or ovary) synthesizing and secreting sex hormones that remotely act on hormone receptors to control sexual development and behaviors.[1] In *Drosophila* and many other insects, sexual differentiation was controlled directly by sex determination genes in a cell-autonomous way.[2] A sex determination pathway from *sex lethal* (*sxl*) to the sex-specific spliced *fruitless* (*fru*) and *doublesex* (*dsx*) controls almost all aspects of sexual development and behaviors in *Drosophila*.[3] In particular, the male-specific splicing product of *fru* gene (fru^M) is required during a critical developmental period to build the most efficient sex circuitry.[4,5] Moreover, the sex-specific splicing regulation of *dsx* and *fru* genes is highly conserved in other insects, although the upstream of the sex determination pathway may be altered.[6-8] Thus, unlike in mammals, sex differentiation in insects is largely hormone-independent. However, it has been revealed that hormonal signaling is required for the differentiation and maturation of certain neurons in male sexual circuitry in *Drosophila*[9-11] and participates in the regulation of sexual development and behavior in insects, which may be due to the interaction with the sex determination genes.[12-18] Hence there is an urgent need to understand the relationship between hormones and sex determination genes in regulating sexual development and behavior in insects.

Ecdysone and juvenile hormone (JH) are the most important hormones during the development in insects.[19,20] 20-hydroxyecdysone (20E), the main active form of steroid hormone ecdysone, coordinating with the sesquiterpenoid JH determines the major developmental transitions to control the growth and development of insects.[19,21,22] Ecdysone is synthesized in the prothoracic glands (PG) and secreted into the hemolymph, then converted into 20E in the peripheral tissues.[23] 20E combining with its nuclear receptor complex consisting of ecdysone receptor (EcR) and Ultraspiracle (USP) proteins induces a transcriptional cascade, thus initiating the molt and metamorphosis.[19,24] JH is synthesized in the corpus allatum (CA) in *Drosophila*[20,25] and acts through two types of intracellular receptors, Methoprene-tolerant (Met) and its paralog Germ-cell expressed (Gce).[26-28] When JH exists, Met/Gce binds to the JH response region (JHRR) and regulates the expression of different JH response genes.[29] *Krüppel homolog 1* (*Kr-h1*) is identified as a main JH primary-response gene.[29,30] JH acts as a "status quo" factor antagonizing 20E to prevent metamorphosis.[31,32]

Here, we investigate hormonal control of the sex determination gene *fru* and report that *Drosophila* hormones regulate fru^M expression and male sexual behaviors. 20E and JH signaling pathways antagonistically regulate Fru^M expression during development. Furthermore, 20E signaling regulates both the development and physiological function of the male-specific P1 neurons to alter male sexual behaviors. Thus, hormone signaling in fruit flies performs a conserved function comparable to steroid sex hormones in vertebrates on sexual development and behaviors.

MATERIALS AND METHODS

Fly stocks

Flies were normally raised at 22℃ or 25℃ with a 12 h:12 h light:dark cycle at ~60% humidity. Heat-shock flies were raised at the permissive temperature (18℃) and shifted to the restrictive temperature (30℃) during a specific period according to the experimental needs. *Canton-S (WTCS)* and *w1118* were used as wild-type strains. fru^{GAL4},[33] fru^{V5},[4] *P1*^{a}*-splitGAL4 (R15A01-AD; R71G01-DBD)*,[34] and *GMR-GAL4*[35] were used as described previously. *R57C10-GAL4 attP2 (BDSC#39171), tub-GAL80*^{ts} *(BDSC#7019), UAS-myrGFP attP2 (BDSC#32197), UAS-myrGFP su(Hw)attP5 (BDSC#32199)* and *UAS-GCaMP7s VK00005 (BDSC#79032)* were obtained from Bloomington *Drosophila* Stock Center. $Met^{27}Gce^{2.5k}$, $jhamt^2$, *Kr-h1-LacZ-1/Cyo, Kr-h1-LacZ-2/Cyo, UAS-Met::Gce-RNAi, Phm-GAL4, JHRR-LacZ,* $UAS\text{-}EcR.A^{DN}$, $UAS\text{-}EcR.B1^{DN}$, and $UAS\text{-}EcR.B2^{DN}$ were from Dr. Sheng Li (South China Normal University, China).

Tissue dissection, staining, imaging and analysis

Brains and ventral nerve cords of males were dissected in Schneider's insect medium (Thermo Fisher Scientific, 21720024) at different developmental stages including 96 hr after egg laying (AEL), 108 hr AEL, 0 hr after pupal formation (APF), 3 hr APF, 6 hr APF, 24 hr APF, 48 hr APF, 96 hr APF, 1-d-old adult and 5-d-old adult. Larval gender was recognized by the absence or presence of gonads. Tissues were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 to 2 hr on ice, then washed 4 × 15-min in PAT3 (0.5% Triton X-100, 0.5% bovine serum albumin in PBS), followed by incubation with 3% normal goat serum (NGS) in PAT3 for 1 hr at room temperature. Next, Tissues were incubated with primary antibodies diluted in 3% NGS for 1d at 4°C, then washed 4 × 15-min in PAT3, and incubated in secondary antibodies diluted in 3% NGS for 1d at 4°C. After being rinsed in PAT3 for 4 × 15-min, tissues were mounted in Vectorshield (Vector Laboratories, H-1000) for imaging. Primary antibodies: rabbit anti-Fru^M (1:200) previously used,[4] mouse anti-V5-Tag:DyLight 550 (Bio-Rad, MCA1360D550GA; 1:500), mouse anti-GFP (Invitrogen, A11120; 1:1,000), rabbit anti-GFP (Invitrogen, A11122; 1:1,000), mouse anti-EcR (DSHB, Ag10.2; 1:200) and mouse anti-LacZ (Promega, Z3783; 1:200). Secondary antibodies: donkey anti-rabbit IgG conjugated to Alexa 488 (Invitrogen, A21206; 1:500), donkey anti-mouse IgG conjugated to Alexa 555 (Invitrogen, A31570; 1:500), donkey anti-mouse IgG conjugated to Alexa 488 (Invitrogen, A21202; 1:500), and donkey anti-rabbit IgG conjugated to Alexa 555 (Invitrogen, A31572; 1:500). Samples were

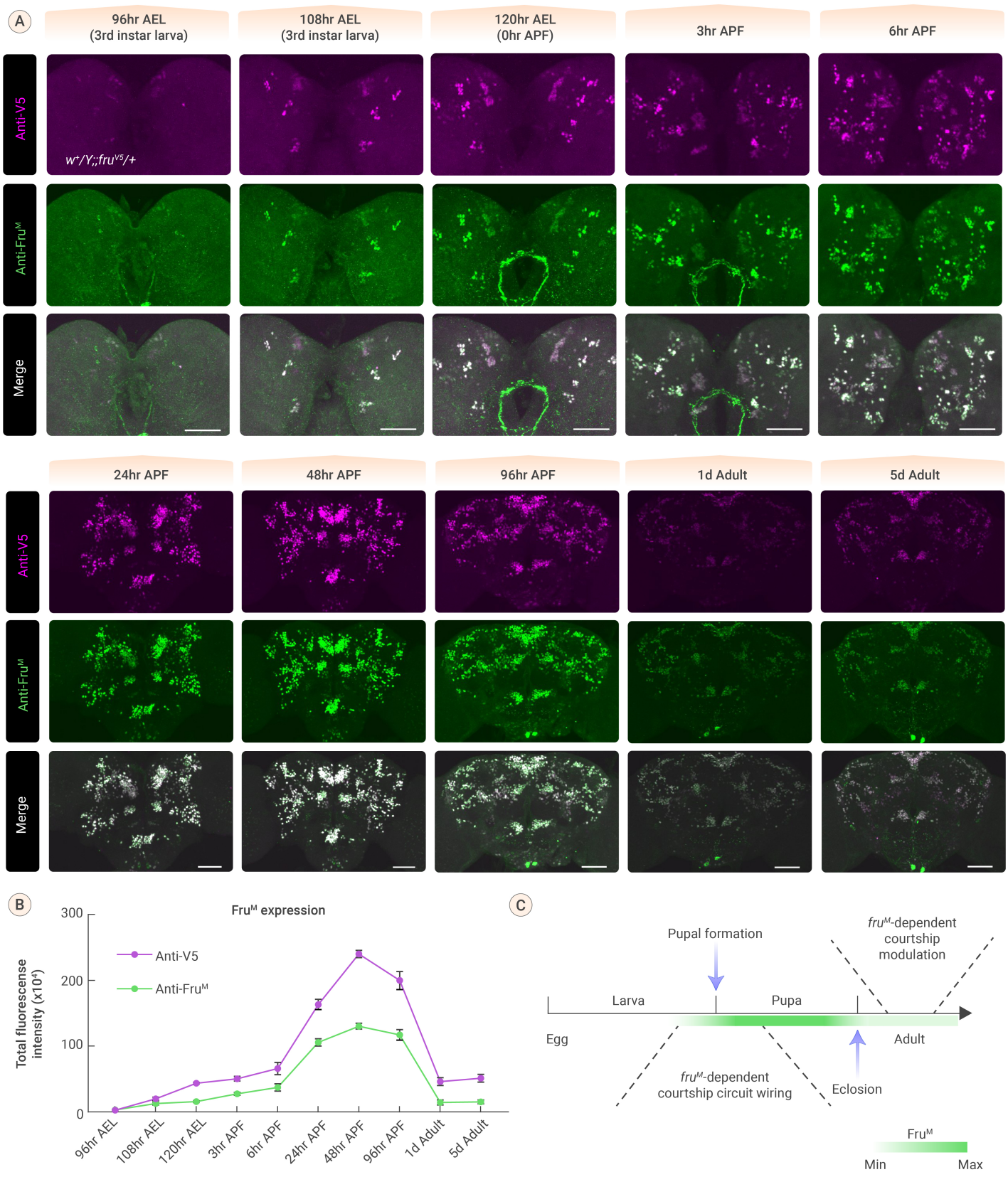


Figure 1. Dynamic expression of Fru^M during development (A) Fru^M expression in *fru^{V5}*/+ male brains from third instar larva to adult. Anti-V5 (magenta) and anti-Fru^M (green) signals were overlapped perfectly. AEL, after egg laying. APF, after pupa formation. 96 hr AEL is the early third instar larval stage and 108 hr AEL is the late third instar larval stage. From 0 hr APF to 96 hr APF are pupal stages. Scale bars, 50 μm. (B) Quantification of Fru^M expression in *fru^{V5}*/+ male brains at different stages. n = 5-7. Error bars indicate SEM. (C) Correlation between dynamic Fru^M expression and temporal regulation of *fru^M*-dependent male courtship. The green gradient indicates the intensity of Fru^M expression from the third instar larva to 5 days old adult.

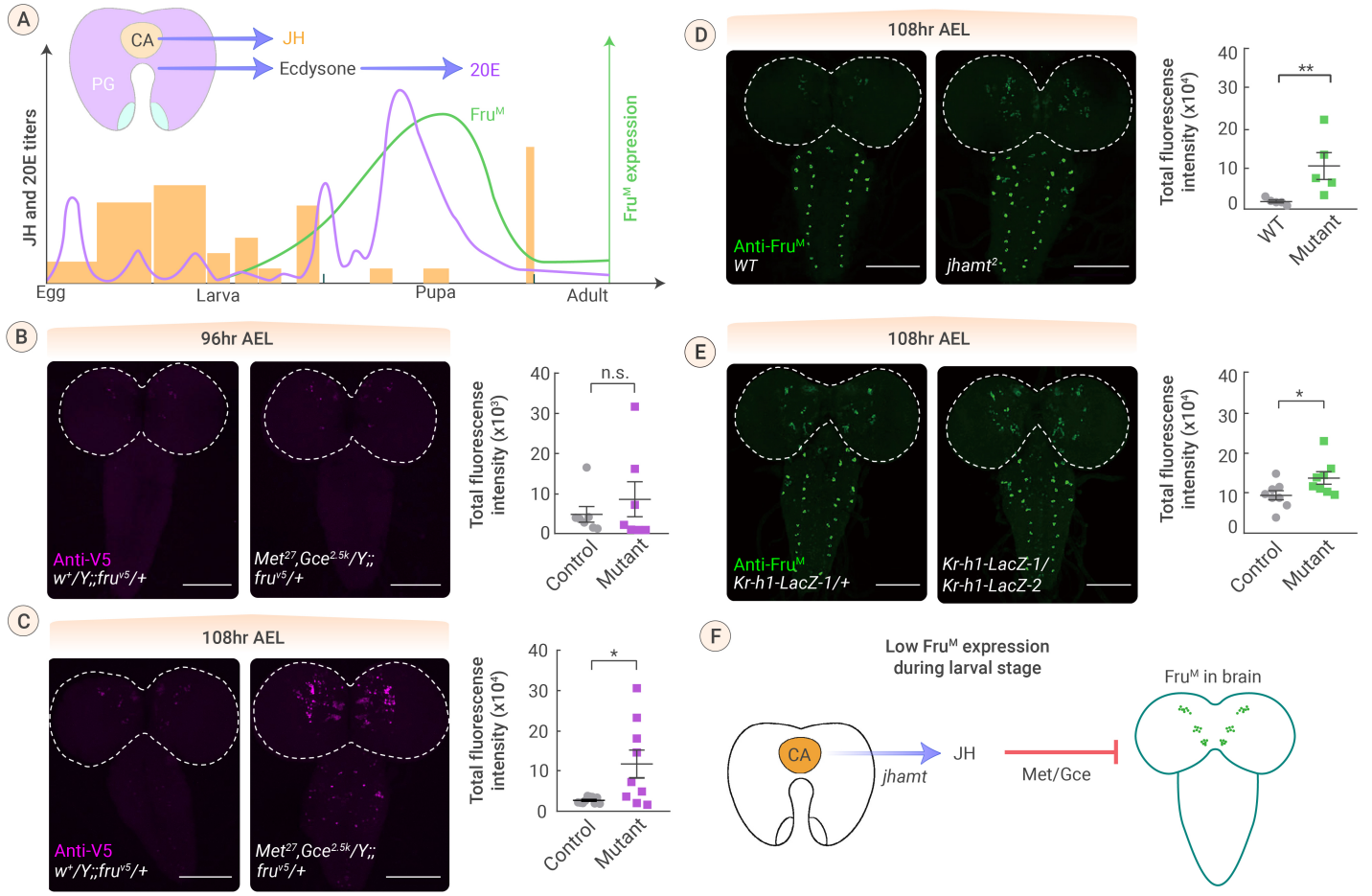


Figure 2. JH signaling inhibits larval Fru^M expression through Met/Gce (A) Changes of JH, 20E titers, and Fru^M expression during development. JH (denoted by the orange rectangle) is produced and secreted by corpus allatum (CA). Ecdysone is first synthesized and released by the prothoracic gland (PG), then converted to its active form 20E (denoted by the purple solid line) in the peripheral tissues. The green solid line indicates Fru^M expression in the male brain. (B) Anti-V5 staining (magenta) and its quantification in *w$^+$/Y;;fru^{V5}/+* and *Met^{27},Gce$^{2.5k}$/Y;;fru^{V5}/+* male brains at 96 hr AEL. Anti-V5 signals represent Fru^M expression. n = 7 for each. n.s., not significant, Mann-Whitney *U*-test. The brains are outlined by the white dotted line. (C) Anti-V5 staining (magenta) and its quantification in *w$^+$/Y;;fru^{V5}/+* and *Met^{27},Gce$^{2.5k}$/Y;;fru^{V5}/+* male brains at 108 hr AEL. n = 10 and 9, respectively. ⋆p<0.05, unpaired *t*-test. (D) Anti-Fru^M staining (green) and its quantification in *jhamt^2* mutant and *WTCS* male brains at 108 hr AEL. n = 5 for each. ⋆⋆p<0.01, Mann-Whitney *U*-test. (E) Anti-Fru^M staining (green) and its quantification in *Kr-h1* mutant (*Kr-h1-LacZ-1/Kr-h1-LacZ-2*) and heterozygous *Kr-h1* control (*Kr-h1-LacZ-1/+*) male brains at 108 hr AEL. n = 8 for each. ⋆p<0.05, Mann-Whitney *U*-test. (F) A schematic for the inhibition of larval Fru^M expression by JH signaling. Error bars indicate SEM. Scale bars, 100 μm.

imaged at 10× or 20× magnification on Zeiss 900 confocal microscope and processed with ImageJ. For quantification of anti-V5, anti-Fru^M, and anti-GFP fluorescence intensity, experimental and control samples in one experiment were stained in the same condition and imaged using the same parameters. We got the summed slice by max intensity Z-projection of the stacks to measure the average fluorescence intensity and fluorescence area within the region of interest (ROI) using the threshold method in ImageJ. The total fluorescence intensity was the product of average fluorescence intensity and fluorescence area.

Calcium imaging

Fly brains expressing GCaMP7s were dissected from 4-d-old males in *Drosophila* Adult Hemolymph-Like Saline (AHLS) which contains 2mM $CaCl_2$, 5mM KCl, 5mM HEPES, 8.2mM $MgCl_2$, 108mM NaCl, 4mM $NaHCO_3$, 1mM NaH_2PO_4, 10mM Sucrose and 5mM Trehalose (pH 7.5). Brains in AHLS were scanned using Zeiss AxoExaminer Z1 upright microscope with W Plan Apochromat 20× water immersion objective. The total fluorescence intensity in ROI was measured as described above.

20E feeding

The 20E powder was dissolved in DMSO to make a 250 μg/uL 20E stock solution. The final concentration of 20E in food was 2.5 μg/uL diluted from the stock solution and DMSO was used as a control. 96 hr AEL larvae were collected and transferred to 20E or DMSO food for 10~13 hr or 24~27 hr at 25℃. Each vial housed ~20 larvae.

Quantitative real-time PCR

Total RNA was extracted from ~20 males with TRIzol (Invitrogen, 15596026) and purified with the DNA-free kit (Ambion, AM1906) according to the manufacturer's protocol. The cDNA was synthesized using the Prime Script reagent kit (Invitrogen, 18091050). qRT-PCR was performed on LightCycler 96 Real-Time PCR System (Roche) using High Fidelity PCR SuperMix (Transgen, AS131-21) and EvaGreen Dye (Biotium, 31000). *actin* was used as the control for normalization. The following primers were used:

actin-forward: 5'- CAGGCGGTGCTTTCTCTCTA -3';
actin-reverse: 5'-AGCTGTAACCGCGCTCAGTA-3';
fru^M-forward: 5'-GTGTGCGTACGTTTGAGTGT-3';
fru^M-reverse: 5'-TAATCCTGTGACGTCGCCAT -3'.
Met-forward: 5'-GCCAGAACCCTATCAGTTGG-3';
Met-reverse: 5'-AGCAGACGGTAGCAGCTCTC-3';
Gce-forward: 5'-ACGGATCCATCCAGGAACTA-3';
Gce-reverse: 5'-CATGGCAGGTGAGTGTGAGA-3'.

Male courtship assay

Virgin males were collected after eclosion and housed individually for 4-7 days until the tests. Virgin *WTCS* females were collected and group-housed for 4-7 days as male-female courtship targets. For the single-pair male-female courtship assay, the tester males and target females were loaded into small round two-layer chambers (diameter: 1 cm; height: 3 mm per layer) under cold anesthesia and separated by a plastic transparent barrier that would be removed after 30~60 min adaption to allow 30 min courtship test at

25°C. For paired male-male courtship assay, two tester males were loaded into one two-layer chamber separated by the transparent barrier and adapted 30~60 min at 25°C. A 30 min recording was taken after removing the barrier. Courtship index (CI), which is the percentage of observation time a fly performs any courtship step in the first 10 min after removing the barrier, was used to measure courtship toward *WTCS* females or between two males and scored manually using the LifeSongX software (lifesong.bio.brandeis.edu/).

Statistics

Experimental and control flies were tested at the same condition in all experiments and data were verified in at least two independent experiments. Sample size for fly behavior is normally around 20 for each group, for tissue staining and quantification ≥ 5. Detailed sample sizes are indicated in each figure legend. All imaging data and behavioral videos were analyzed and checked by at least two investigators. Data analysis was performed using GraphPad Prism 8 and details were indicated in each figure legend. Normal distribution of the data was first determined by D'Agostino–Pearson normality test. If the data were normally distributed, Student's *t*-test was used for pairwise comparisons. If not normally distributed, the Mann-Whitney *U*-test was used for pairwise comparisons. Fisher's exact test was performed to analyze the percentages of different kinds of defects in P1[a] neurons.

RESULTS

Dynamic expression of Fru^M during development

To illustrate the dynamic expression of *fru^M* across development, we utilized the recently generated *fru^{V5}* knock-in flies[4] and examined Fru^M expression from the 3rd instar larva to adulthood using both V5 and Fru^M antibodies (Figures 1A & S1A). Fluorescent signals of the two antibodies in male brains were mostly overlapped and calculated separately (Figure 1B). We found that Fru^M proteins in the male brain were weakly observed at 96 hr after egg laying (AEL) and expressed obviously at about 108 hr AEL, also known as wandering 3rd instar larval stage. Then the expression of Fru^M proteins increased gradually until 6 hr after pupa formation (APF). From 6 hr APF to 48 hr APF, Fru^M expression accumulated dramatically and reached the highest level at 48 hr APF (Figures 1A-B). Then the Fru^M expression decreased. One-day and five-day adult flies showed significantly decreased Fru^M expression (Figures 1A-B). Fluorescent signals of Fru^M and V5 antibodies in the ventral nerve cord (VNC) were largely overlapped except for some nonspecific staining of the Fru^M antibody (Figure S1A). The variation of Fru^M expression in VNC during the development was similar to that in the brain (Figure S1B). These results demonstrate the dynamic expression of Fru^M from larval to adult stages, which is crucial for the development of sex circuitry and sexual behaviors in *Drosophila* males (Figure 1C).[4]

JH signaling inhibits larval Fru^M expression through Met/Gce

The ring gland is an important endocrine organ in the larva. It contains the corpus allatum (CA) and prothoracic gland (PG) which synthesize and secret JH and ecdysone respectively (Figure 2A). During development, the titer of JH is high in the middle larval stage and decreases at the late larval stage, while 20-hydroxyecdysone (20E) titer increases before pupariation, peaks at the pupal stage and thereafter declines,[19] which is quite similar to the temporal expression mode of Fru^M (Figure 2A). Thus, we wondered if the temporal expression patterns of Fru^M during developmental stages were related to JH and 20E signaling pathways.

To investigate whether JH signaling modulates Fru^M expression at the larval stage, we firstly checked the *fru^M* mRNA level in the double mutant males for JH receptors (*Met^{27}, gce$^{2.5K}$*) at 108 hr AEL by quantitative real-time PCR (qPCR). The mutant males showed significantly increased *fru^M* mRNA levels compared with wild-type Canton-S (*WTCS*) or *w^{1118}* males (Figure S2A). We further examined the changes in Fru^M protein levels in the mutant using *fru^{V5}* knock-in flies. The expression level of Fru^M in *Met^{27}, gce$^{2.5K}$* mutant males was not significantly different from the control at 96 hr AEL (Figure 2B), but was much higher than that in the control at 108 hr AEL (Figure 2C). These results indicated that JH signaling might regulate the expression of *fru^M* in a limited period of development. JH acid methyltransferase (JHAMT) is an enzyme responsible for JH biosynthesis.[20,36] We found that the *jhamt^2* mutant exhibited higher Fru^M expression than *WTCS* males at 108 hr AEL (Figure 2D), while there was no significant difference at pupal or adult stages (Figure S3). We further examined whether other component of JH signaling, the main JH-response gene (*Kr-h1*) was crucial for the expression of Fru^M and found that *Kr-h1* mutant male also exhibited increased Fru^M levels at 108 hr AEL (Figure 2E). These results indicate that JH signaling suppresses Fru^M expression through Met/Gce in male wandering larvae (Figure 2F).

20E signaling promotes pupal Fru^M expression

Given the titer change of 20E is consistent with the expression change of *fru^M* (Figure 2A), we set out to test how 20E signaling would regulate *fru^M* expression. It is known that the *EcR* gene encodes three isoforms (*EcR-A*, *EcR-B1*, and *EcR-B2*) that contain common hormone- and DNA-binding domains but different N-terminal regions.[37-40] We first tested how EcR isoforms would interact with Fru^M. When the dominant-negative isoform of *EcR* (*EcR.B1^{DN}* or *EcR.B2^{DN}*) was expressed in the eyes driven by *GMR-GAL4*, flies displayed rough eyes with necrotic areas compared with the normal eyes, while the ectopic expression of *EcR.A^{DN}* led to lethality (Figures S4A & C). We found that overexpression of *fru^{MB}* not only partially rescued the necrotic parts of the rough eyes of *GMR>EcR.B1^{DN}* and *GMR>EcR.B2^{DN}* males, but also rescued the lethality of *GMR>EcR.A^{DN}* males (Figure S4D). However, the small and rough eyes caused by the overexpression of *fru^{MB}* did not change (Figures S4B & D). These results suggest that Fru^M may function downstream of EcR isoforms.

To further test whether 20E regulates *fru^M* expression through EcR, we examined the mRNA and protein levels of *fru^M* in different EcR^{DN} (EcR.A^{DN}, EcR.B1^{DN} or EcR.B2^{DN}) expressed males driven by *fru^{GAL4}*. The results showed that *fru^M* mRNA levels were significantly decreased in male flies expressing different EcR^{DN}s at 24 hr APF (Figure S2B). Similarly, anti-Fru^M signals also decreased significantly in the brains of different EcR^{DN} expressed males compared to their GAL4 heterozygous control at 24 hr APF (Figures 3A-B). These results indicate that 20E signaling promotes Fru^M expression at pupal stage. To find out the critical period during which 20E signaling increased Fru^M expression, we used *tub-GAL80^{ts}* to restrict EcR.A^{DN} expression in *fru^M* neurons during 0~24 hr or 24~48 hr APF. We found that Fru^M expression was decreased in the brain only when EcR.A^{DN} was expressed in *fru^M* neurons during 0~24 hr APF (Figures 3C & E), but not significantly altered if EcR.A^{DN} was expressed during 24~48 hr APF (Figures 3D-E). These results indicate that 20E signaling promotes *fru^M* expression mainly during the first 24 hr APF.

JH signaling suppresses larval Fru^M expression by inhibiting 20E signaling

To investigate whether JH directly regulates *fru^M* expression, we visualized JH signaling with a LacZ reporter *JHRR-LacZ*,[29] but did not find obvious overlap of LacZ and Fru^M in brains from larval to adult stages (Figures S5A-C). In contrast, EcR and Fru^M were expressed in a large number of overlapped neurons from larval to adult *WTCS* males (Figures S5D-F). Considering JH and 20E signaling regulate each other's biosynthesis antagonistically within the ring gland in *Drosophila*,[41] we suspected that JH may act on Met/Gce in PG to regulate ecdysone biosynthesis and thus regulate *fru^M* expression indirectly (Figure 3F). To test this hypothesis, we checked the protein levels of *fru^M* at 108 hr AEL after knocking down *Met* and *Gce* simultaneously in PG or the central nervous system (CNS) using *UAS-Met::Gce-IR* driven by *Phm-GAL4* or *R57C10-GAL4* respectively. The efficiency of *Met/Gce* knockdown was confirmed by qPCR (Figure S2C). We found that Fru^M expression increased significantly after knocking down *Met*/*Gce* in PG but not in CNS (Figure 3G and 3H). These results suggest that JH signaling inhibits larval Fru^M expression through repressing 20E signaling.

To further validate that 20E signaling promotes Fru^M expression, we fed 96 hr AEL larva with 20E or the control solvent DMSO for 10~13 hr or 24~27 hr. Anti-Fru^M signals revealed that 20E feeding enhanced Fru^M expression compared with the control (Figure S6). We next decreased 20E signaling in *fru^M* neurons by expressing different EcR^{DN} driven by *fru^{GAL4}* and found that *fru^M* expression in all these male larvae decreased significantly compared with their GAL4 heterozygous control at 108 hr AEL (Figures 3I-J & S2D). These results indicate that JH and 20E signaling antagonistically regulate Fru^M expression at the larval stage.

Taken together, these results indicate that JH signaling inhibits 20E signal-

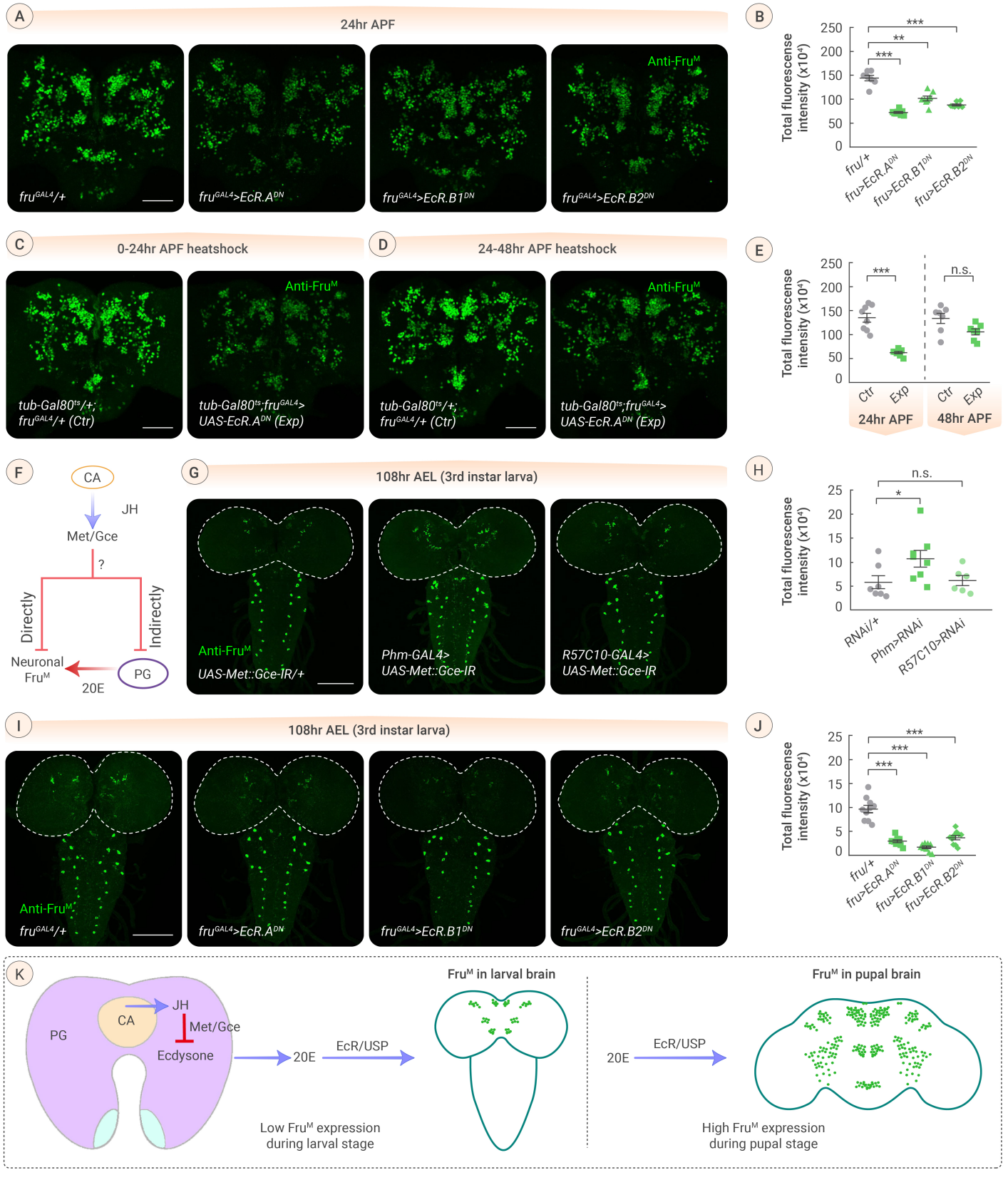


Figure 3. 20E signaling promotes larval and pupal Fru^{M} expression (A, B) Anti-Fru^{M} signals (green) in male brains after expressing different dominant negative isoforms of EcR (EcR^{DN}) in *fru^{M}* neurons at 24 hr APF (A), which are quantified in (B). n = 7-8. **p < 0.01, ***p < 0.001, Mann-Whitney *U*-test. (C) Anti-Fru^{M} signals (green) in *tub-Gal80^{ts}/UAS-EcR.A^{DN}; fru^{GAL4}/+*(Exp) and *tub-Gal80^{ts}/+; fru^{GAL4}/+*(Ctr) male brains at 24 hr APF after heat shock (30℃) during 0-24 hr APF. (D) Anti-Fru^{M} signals (green) in *tub-Gal80^{ts}/UAS-EcR.A^{DN}; fru^{GAL4}/+*(Exp) and *tub-Gal80^{ts}/+; fru^{GAL4}/+*(Ctr) male brains at 48 hr APF after heat shock (30℃) during 24-48 hr APF. (E) Quantification of anti-Fru^{M} signals in (C) and (D). n = 7-8. n.s., not significant, ***p < 0.001, Mann-Whitney *U*-test. (F) A schematic for the possible mechanisms by which JH regulates larval *fru^{M}* expression. (G, H) Anti-Fru^{M} signals (green) in male brains after knocking down *Met* and *Gce* in PG or CNS at 108 hr AEL (G), which are quantified in (H). n = 6-7. n.s., not significant, *p < 0.05, Mann-Whitney *U*-test. The brains are outlined by the white dotted line. (I, J) Anti-Fru^{M} signals (green) in male brains after expressing different EcR^{DN}s in *fru^{M}* neurons at 108 hr AEL (I), which are quantified in (J). n = 9-10. ***p < 0.001, unpaired *t*-test. (K) A schematic for hormonal regulation of Fru^{M} expression in male brain during larval and pupal stages. CA (orange), corpus allatum. PG (purple), prothoracic gland. Scale bars in (A), (C) and (D), 50 μm. Scale bars in (G) and (I), 100 μm. Error bars indicate SEM.

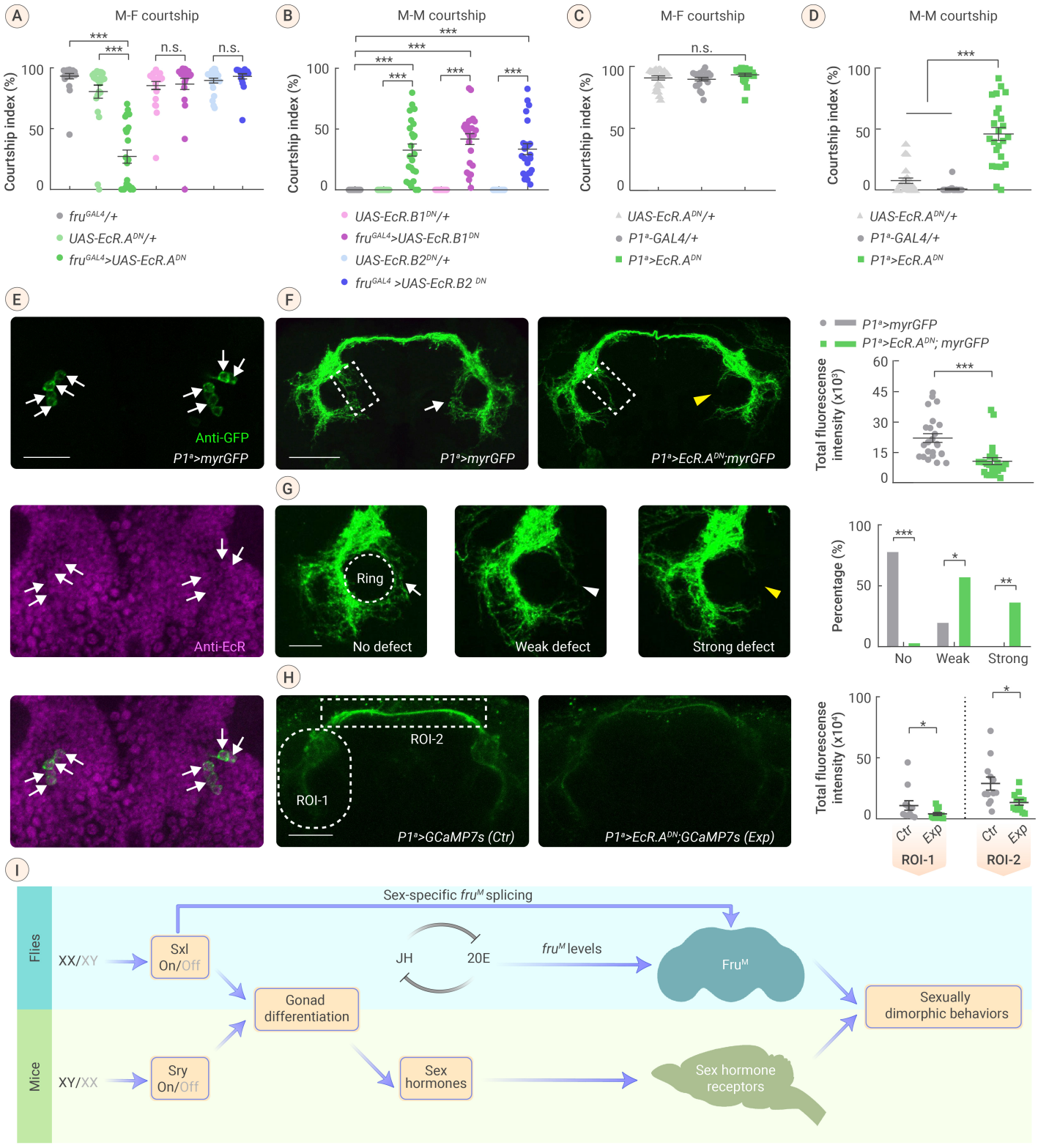


Figure 4. 20E signaling regulates the morphology and activity of P1^a neurons to alter male sexual orientation (A, B) Courtship behaviors performed by males expressing different EcR^{DN}s in *fru^M* neurons. For male-female courtship (A), n = 19-24. For male-male courtship (B), n = 24 for each. n.s., not significant, ***p < 0.001, Mann-Whitney *U*-test. (C, D) Courtship behaviors performed by males expressing EcR.A^{DN} in P1^a neurons. For male-female courtship (C), n = 22-24. For male-male courtship (D), n = 24 for each. n.s., not significant, ***p < 0.001, Mann-Whitney *U*-test. (E) Co-labelling of anti-EcR (magenta) and anti-GFP signals (green) in P1^a neurons driven by *P1^a-splitGAL4*. (F) Expression of EcR.A^{DN} induced morphological defects in P1^a neurons at 96 hr APF. The dashed rectangles showed the region of interest (ROI) where the fluorescent intensities were quantified. n = 24 for each. ***p < 0.001, Mann-Whitney *U*-test. The white arrow indicates the normal morphology of P1^a neurons. The yellow arrowhead indicates the innervation defect. (G) Different levels of defects after expressing EcR.A^{DN} in P1^a neurons at 96 hr APF. White arrow, white and yellow arrowheads indicate no, weak and strong defects, respectively. n = 24 for each. *p < 0.05, **p < 0.01, ***p < 0.001, Fisher's exact test. (H) Calcium activities indicated by GCaMP7s signals of P1^a neurons in 4d adult males with or without expressing EcR.A^{DN} in P1^a neurons. The dashed ellipse and rectangle showed ROI-1 and ROI-2, respectively. n = 12 for each. *p < 0.05, Mann-Whitney *U*-test. Scale bar in (E) and (G), 20 μm. Scale bar in (F) and (H), 50 μm. (I) Comparison of hormonal regulation on sexually dimorphic behaviors in flies and mice.

ing to maintain a low level of Fru^{M} expression during larval stage, while 20E signaling continues to promote Fru^{M} expression during pupal stage (Figure 3K).

20E signaling influences the morphology and activity of P1 neurons to alter male sexual orientation

We next examined the hormonal effects on male courtship behavior. Expressing EcR.A^{DN} in *fru^{M}* neurons reduced courtship of males toward females (Figure 4A). In addition, expressing any of the three different EcR^{DN}s induced strong male-male courtship (Figure 4B). These results indicate that 20E signaling plays a crucial role in male sexual behaviors.

Previous studies have revealed that a subset of *fru^{M}*-expressing interneurons termed P1 neurons are command neurons initiating male courtship.[42-44] Thus, we next assayed whether reducing 20E signaling in P1 neurons would impair male courtship behaviors. We expressed EcR.A^{DN} in a subset of P1^{a} neurons and found that these males displayed regular male-female courtship but elevated male-male courtship behaviors (Figures 4C-D). These results suggest that 20E-EcR signaling functions in P1^{a} neurons to inhibit male-male courtship.

To further confirm the role of EcR in P1^{a} neurons, we first examined EcR expression and observed anti-EcR signals in P1^{a} neurons (Figure 4E). We then checked the morphology of P1^{a} neurons after expressing EcR.A^{DN} in P1^{a} neurons. The immunostaining results showed that expressing EcR.A^{DN} led to fewer or/and shorter nerve fibers near the ring-like arborization of P1^{a} neurons (Figures 4F-G). To further examine whether the 20E signaling would regulate physiological function of P1^{a} neurons, we checked the calcium activity of P1^{a} neurons after expressing EcR.A^{DN} and found reduced activity of P1^{a} neurons in both regions of interest (ROI-1 and ROI-2, Figure 4H). These results indicate that 20E signaling regulates both the development and physiological function of P1^{a} neurons and affects sexual orientation in adult males.

DISCUSSION

In this study, we find that JH and 20E signaling regulate the dynamic expression of *fru^{M}* during development. 20E signaling promotes the expression of *fru^{M}* in both larval and pupal stages, while JH suppresses larval *fru^{M}* expression through inhibiting 20E signaling. We further show that 20E signaling affects the morphology and physiological function of command-like P1 neurons and regulates sexual orientation in male flies.

How does 20E signaling regulate the expression of Fru^{M}? We propose that both the 20E titer change as well as the dynamic expression of the different isoforms of EcR may contribute to such regulation. Previous studies have suggested that different EcR isoforms are expressed at different times and locations, and play distinct roles.[14,37-40,45,46] EcR-B1 and EcR-B2 are involved in larval molting and the pruning of larval neurons, while EcR-A is required for the outgrowth of adult-specific neurons during metamorphosis.[40,46] Here we used three isoform-specific dominant negative EcRs to interfere with the 20E signaling. However, there is no obvious isoform-specific difference, which is probably because these dominant negative EcRs have different mutations in the ligand-binding domain but the same DNA-binding domain.[46,47] Furthermore, the N-terminal domain of EcR-A and EcR-B showed inhibitory and activation functions separately.[48,49] We proposed that EcR.A^{DN} might not only interfere with the endogenous EcR, but also inhibit the expression of *fru^{M}*, such that EcR.A^{DN} expressed (*fru^{GAL4}>EcR.A^{DN}*) males showed a maximum reduction of Fru^{M} expression and impaired male courtship behavior toward females.

An intriguing phenotype by interfering 20E signaling is the induction of strong male-male courtship. This may be primarily due to impaired EcR function in the male-specific P1^{a} neurons, as expressing EcR.A^{DN} in P1^{a} neurons resulted in similarly high levels of male-male courtship compared to expression of EcR.A^{DN} in all *fru^{M}* neurons. However, expressing EcR.A^{DN} in P1^{a} neurons did not affect male-female courtship, suggesting the involvement of other *fru^{M}* neurons influenced by 20E signaling, such as a subset of GABAergic mAL neurons.[11] In addition to regulating the development of P1^{a} neurons, 20E signaling also regulates the basal activity of P1^{a} neurons. There are at least two possibilities. First, the abnormal projection of P1^{a} neurons after expressing EcR.A^{DN} may affect the connections from upstream neurons to P1^{a} neurons, resulting in less input from excitatory neurons or/and more input from inhibitory neurons. Second, the reduction of 20E signaling in P1^{a} neurons may lead to aberrant expression of certain ion channel proteins that normally ensure appropriate basal activity of neurons. Further studies are still needed to distinguish between these scenarios and to explore how the dysfunction of P1^{a} neurons leads to in male-male courtship behaviors.

Our results suggest that the hormonal signaling in *Drosophila* plays a conserved and crucial role like steroid sex hormones in vertebrates in regulating sexual development and behaviors (Figure 4I). In flies, the *Sxl* gene controls the *fru^{M}* splicing patterns, while JH and 20E hormone signaling pathways coordinate to regulate the expression level of *fru^{M}*. Taken together, they lead to the dynamic expression pattern of *fru^{M}* that controls most aspects of male sexual behaviors. In mice, the *Sry* gene determines the differentiation of gonads that synthesize and secret sex hormones into circulation. The sex hormones then act on their receptors in the brain to organize the neural substrate of sexually dimorphic behaviors during development and activate these circuitries for sex-specific behaviors in adults.

Although hormones may share conserved functions in regulating sexual behavior in insects and mammals, they still function in different ways. First, unlike sex hormones in mammals, 20E itself is not sexually dimorphic,[50] but instead, it regulates expression of sex-specific isoforms of *fru* gene to alter sex-specific behaviors. Second, the proportion of hormones in regulating sexual behavior differs in insects and mammals. In mammals, sex hormones are the decisive factor in regulating the behavior and the formation of corresponding neural circuitry,[51] while in insects, hormones only modulate rather than initiate the expression level of the key sex determination gene. Third, hormones may function at different periods. Sex hormones regulate mammals throughout their entire life, not only regulating the formation of the neural circuit during development but also controlling behaviors in adulthood, which makes their behaviors more flexible.[51] In contrast, the hormone 20E, which we found to play a key regulatory role in flies, has a peak titer mainly during the pupal stage. Moreover, EcR/USP activity is rarely detectable in the CNS of 5 or 10 days old adult flies.[52] Combined with previous studies,[4,14] we suggest that hormones mainly function during development in insects, although several studies indicated that hormones also have a certain regulatory function in adult flies.[14,18] This type of hormonal regulation in insects may facilitate the formation of fixed behavioral circuits that are more conducive to survival and reproduction.

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AUTHOR CONTRIBUTIONS

X.J., S.Li and Y.P. conceived the study. X.J., L.W., X.L., and S.Liu performed experiments and analyzed the data. X.J., S.Li and Y.P. wrote the manuscript. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

Not applicable.

DATA AND CODE AVAILABILITY

All data are provided in the figures and supplemental figures. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon reasonable request. This paper does not report original code.

SUPPLEMENTAL INFORMATION

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