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The function of juvenile-adult transition axis in female sexual receptivity of *Drosophila melanogaster*

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Abstract

Female sexual receptivity is essential for reproduction of a species. Neuropeptides play the main role in regulating female receptivity. However, whether neuropeptides regulate female sexual receptivity during the neurodevelopment is unknown. Here we found the peptide hormone prothoracicotropic hormone (PTTH), which belongs to the insect PG axis, negatively regulated virgin female receptivity through ecdysone during neurodevelopment in *Drosophila melanogaster*. We identified PTTH neurons as doublesex-positive neurons, they regulated virgin female receptivity before the metamorphosis during the 3rd-instar larval stage. PTTH deletion resulted in the increased EcR-A expression in the whole newly formed prepupae. Furthermore, the ecdysone receptor EcR-A in pC1 neurons positively regulated virgin female receptivity during metamorphosis. The decreased EcR-A in pC1 neurons induced abnormal morphological development of pC1 neurons without changing neural activity. Among all subtypes of pC1 neurons, the function of EcR-A in pC1b neurons was necessary for virgin female copulation rate. These suggested that the changes of synaptic connections between pC1b and other neurons decreased female copulation rate. Moreover, female receptivity significantly decreased when the expression of PTTH receptor Torso was reduced in pC1 neurons. This suggested that PTTH not only regulates female receptivity through ecdysone but also through affecting female receptivity associated neurons directly. The PG axis has similar functional strategy as the HPG axis in mammals to trigger the juvenile–adult transition. Our work suggests a general mechanism underlying which the neurodevelopment during maturation regulates female sexual receptivity.

eLife assessment

The aim of this **valuable** study is to uncover developmental roles of the neuropeptide prothoracicotropic hormone (PTTH) and ecdysone, which later regulate female receptivity of Drosophila melanogaster. The work combines spatially and temporally restricted genetic manipulation with behavior quantification to explore these molecular pathways and the neuronal substrates participating in the control of female sexual receptivity. At present, the implication of both signaling pathways in this process is **convincing** but the strength of the evidence is **incomplete** to support the main claim that PTTH pathway controls female sexual receptivity through the function of ecdysone in pC1 neurons.

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Introduction

The success of copulation is important for the reproduction of a species. *Drosophila melanogaster* provides a powerful system to investigate the neuronal and molecular mechanism of sexual behaviors. Females decide to mate or not according to their physiological status and the environmental condition (Dickson, 2008). Sexually mature adult virgin females validate males after sensing the courtship song and male-specific sex pheromone, receive courtship with pausing and opening the vaginal plate (Ferveur, 2010; Greenspan et al., 2000; Hall, 1994; Wang et al., 2021). If the female is not willing to mate, she may kick her legs, flick her wings, or extrude the ovipositor to deter males (Connolly et al., 1973). Mated females reject males for several days after mating mainly through more ovipositor extrusion and less opening the vaginal plate (Fuyama et al., 1997; Wang et al., 2021). These options need the establishment of neural circuits for female sexual receptivity. However, the associated mechanism of neural maturation and the effect of neural maturation on female sexual receptivity is little known.

doublesex (*dsx*) and *fruitless* (*fru*) are the terminal genes in sex determination regulatory hierarchy. They specify nearly all aspects of somatic sexual differentiation, including the preparation for sexual behaviors (Dickson, 2008; Manoli et al., 2013; Manoli et al., 2006; Mellert et al., 2012; Pavlou et al., 2013; Siwicki et al., 2009; Yamamoto, 2007; Yamamoto et al., 2013). In males, expression of male-specific Fru^M (Billeter et al., 2006; Demir et al., 2005; Hall, 1978; Manoli et al., 2005; Stockinger et al., 2005) and male-specific Dsx^M (Kohatsu et al., 2011; Pan et al., 2014; Pan et al., 2011; Rideout et al., 2010) are important for male courtship behaviors. In females, although functional Fru protein is not translated, neurons with Dsx^F or *fru* P1 promoter regulate some aspects of the female sexual behaviors (Kvitsiani et al., 2006; Rideout et al., 2010). *Fru and dsx* are involved in regulating the sexual dimorphism during neurodevelopment (Yamamoto et al., 2013). For instance, the sexual dimorphism of P1 and mAL neurons which are all associated with male courtship and aggression behaviors (Clowney et al., 2015; Hoopfer et al., 2015; Kimura et al., 2008; Kohatsu et al., 2011; Pan et al., 2012; Sengupta et al., 2022; von Philipsborn et al., 2011) is the result of regulation by Dsx and/or Fru (Ito et al., 2012; Kimura et al., 2008). In the cis-vaccenyl acetate (cVA) pathway, which induces the courtship inhibiting in males (Kurtovic et al., 2007; Wang et al., 2010), the first-order to the fourth-order components are all fru-Gal4-positive neurons and are either male-specific or sexually dimorphic (Ruta et al., 2010). However, the role of Dsx^F in neurodevelopment associated with female sexual behaviors is little understood.

During postembryonic development, the PG axis triggers the juvenile–adult transition, similar to the function of hypothalamic–pituitary–gonadal (HPG) axis in mammals (Herbison, 2016; Pan et al., 2019). Hormones of the PG axis act to transform the larval nervous system into an adult version (Truman et al., 2023). Ecdysone belonging to the PG axis is the prime mover of insect molting and metamorphosis and is involved in all phases of neurodevelopment, including neurogenesis, pruning, arbor outgrowth, and cell death (Truman et al., 2023). The neurons read the ecdysteroid titer through two isoforms of the ecdysone receptor, EcR-A and EcR-B1, according to spatial and temporal conditions in the central nervous system (Riddiford et al., 2000; Truman et al., 1994). EcR-A is required in fru P1-expressing neurons for the establishment of male-specific neuronal architecture, and ecdysone receptor deficient males display increased male–male courtship behavior (Dalton et al., 2009; Ganter et al., 2007). However, how ecdysone regulates the neurodevelopment associated with female sexual receptivity, especially the fru^+ and dsx^+ neurons, is unknown.

Much of studies to understand female sexual receptivity has focused on its regulation. How a female respond to males is highly dependent on whether or not she has previously mated. In virgin females, dsx^+ pCd neurons respond to the cVA, while dsx^+ pC1 neurons also respond to male courtship song (Zhou et al., 2014). The receptive females open the vaginal plate (VPO) through activation of the dsx^+ vpoDN neurons (Wang et al., 2021). After mated, sex peptide in the seminal fluid binds to the fru^+ dsx^+ sex peptide sensory neurons (SPSNs) in the female uterus. Then neuronal activity in the dsx^+ sex peptide abdominal ganglion (SAG) neurons of the ventral nerve cord and in the pC1 neurons is reduced (Avila et al., 2011; Feng et al., 2014; Häsemeyer et al., 2009; Kubli, 2003; Wang et al., 2020b; Yang et al., 2009; Zhou et al., 2014). Therefore, the sexual receptivity is reduced with less VPO and more ovipositor extrusion (OE) which is controlled by dsx^+ DpN13 neurons (Wang et al., 2020a). In addition, neuropeptides and monoamines play a critical role in regulation of the female receptivity. The neuropeptides Drosulfakinin, myoinhibitory peptides and SIFamide are involved in female sexual receptivity (Jang et al., 2017; Terhzaz et al., 2007; Wang et al., 2022). As monoamines, dopamine, serotonin and octopamine are pivotal to female sexual behaviors (Ishimoto et al., 2020; Ma et al., 2022; Neckameyer, 1998; Rezával et al., 2014). So far, the identified neuropeptides and monoamines modulating female sexual receptivity all function during the adult stage. However, whether neuropeptides or monoamines regulate the establishment of neural circuits for female sexual receptivity is unknown.

To explore the factors that regulate *Drosophila* virgin female receptivity especially during neurodevelopment, we did a knock-out screen including most of CCT members. We discovered a requirement for the prothoracicotropic hormone (PTTH) during postembryonic development for virgin female receptivity. We also found that PTTH neurons expressing PTTH are dsx^+ neurons. PTTH, a brain derived neuropeptide hormone, is the primary promoter of the synthesis of steroid hormone 20-hydroxyecdysone (20E) (McBrayer et al., 2007; Rewitz et al., 2009). Indeed, the enhanced virgin female receptivity due to the loss of PTTH could be rescued through feeding 20E to the 3^{rd}-instar larvae. Because 20E acts through its receptor EcR (Riddiford et al., 2000), we then tested the function of EcR in pC1 neurons which encode the mating status of females (Zhou et al., 2014). The reduced EcR-A expression in pC1 neurons resulted in the abnormal anatomical pattern of pC1 neurons and the reduced female copulation rate. This may be explained by the increased EcR-A in newly formed prepupae resulted from the PTTH deletion. Furthermore, the decreased female copulation rate was due to the reduced EcR-A in pC1b neurons. Besides, we detected the inhibited female receptivity when PTTH receptor torso was decreased in pC1 neurons, suggested the direct function of PTTH on other dsx^+ neurons to regulate female receptivity. Thus, in addition to demonstrating the function of PTTH in virgin female receptivity during neurodevelopment, our study identified the necessary role of the normal pC1b neural morphology in virgin female receptivity.

Results

PTTH modulates virgin female receptivity

In *Drosophila*, neuropeptides and monoamines, belonging to the chemoconnectome (CCT, the entire set of neurotransmitters, neuromodulators, neuropeptides, and their receptors underlying chemotransmission) (Deng et al., 2019), play a critical role in regulation of the female receptivity. To explore the factors that regulate virgin female receptivity especially during neurodevelopment, we screened 108 chemoconnectome (CCT) knock-out lines generated by the CRISPR-Cas9 system (Deng et al., 2019) (unpublished data). The result showed that prothoracicotropic hormone (PTTH) might regulate virgin female receptivity. The deletion mutant $Ptth^{Delete}$ removed part of the 5' UTR and almost all coding sequence and is a protein null (**Figure 1A**). We confirmed the PTTH knock-out flies by using PCR analysis at the PTTH locus in genomic DNA samples (**Figure 1B**), by using RT-PCR to identify the loss of PTTH transcripts in cDNA samples (**Figure 1C**) and by detecting the immunoreactivity of PTTH in the central brain (**Figure 1—figure supplement 1A**). Primers used are listed in Supplementary File 1. PTTH immunoreactivity was found in the brain of wild-type and heterozygous flies (**Figure 1—figure supplement 1A1 and 1A3**), but was absent in homozygous $Ptth^{Delete}$ flies (**Figure 1—figure supplement 1A2**). As the previous study, the $Ptth^{Delete}$ larvae lacking PTTH undergo metamorphosis with about 1 day delay compared with the wild type control (Shimell et al., 2018) (data not shown). Besides, the $Ptth^{Delete}$adult male and female flies had the significant increased weight than wild type flies (**Figure 1—figure supplement 1B**). This is also consistent with that PTTH regulates developmental timing and body size in *Drosophila* (Mcbrayer et al., 2007; Shimell et al., 2018).

To confirm the function of PTTH, we tested virgin female receptivity of $Ptth^{Delete}$ female flies. We found that the virgin female losing PTTH had significantly higher copulation rate and shorter latency to copulation than wild type flies (**Figure 1D-G**). In addition, the $Ptth^{Delete}$ flies had higher copulation rate and lower latency to copulation compared to heterozygous null mutant females within 2 days (**Figure 1D-E**) and within 3 days, respectively (**Figure 1D-F**). The enhanced virgin female receptivity had no relationship either with the attractivity or with the locomotion activity of virgin females (**Figure 1—figure supplement 1C-E**). These results suggested that PTTH deletion regulates virgin female receptivity in a dose-dependent manner. Female receptivity increases with the increase of age after eclosion, not only for wild type flies but also PTTH mutants. At the first day after eclosion (**Figure 1D**), maybe the loss of PTTH in $PTTH^{Delete}$/+ flies is not enough for sexual precocity as $PTTH^{Delete}/PTTH^{Delete}$. At the second day after eclosion and after (**Figure 1E-G**), the loss of PTTH in $PTTH^{Delete}$/+ flies is enough for sexual precocity compared with wild type flies. However, After the 2nd day of adult, female receptivity of all genotype flies increases sharply. At the 3rd day of adult and after, female receptivity of $PTTH^{Delete}/PTTH^{Delete}$ reaches the peak and the receptivity of $PTTH^{Delete}$/+ reaches more nearly to $PTTH^{Delete}/PTTH^{Delete}$ when flies get older (**Figure 1F-G**). However, the overexpression through PTTH-Gal4>UAS-PTTH is also not sufficient to change female receptivity (**Figure 1—figure supplement 2A**). Similarly, decreased expression of PTTH through PTTH-Gal4>UAS-PTTH-RNAi or dsx-Gal4>UAS-PTTH-RNAi did not result in the similar phenotype to that of $PTTH^{Delete}/PTTH^{Delete}$ (**Figure 1—figure supplement 2B-C**). It is possible that both decreasing and increasing PTTH expression are not sufficient to change female receptivity.

Furthermore, we carried out genetic rescue experiments to further confirm the function of PTTH in modulating virgin female receptivity. We used the pan-neuronal driver elav-Gal4 to drive UAS-PTTH expression in PTTH mutant background, although elav-Gal4 did not express in PTTH neurons (**Figure 1—figure supplement 2D**). We detected the PTTH signals using PTTH antibody in the rescued female brains (**Figure 1—figure supplement 1A4**). We found that neuron-

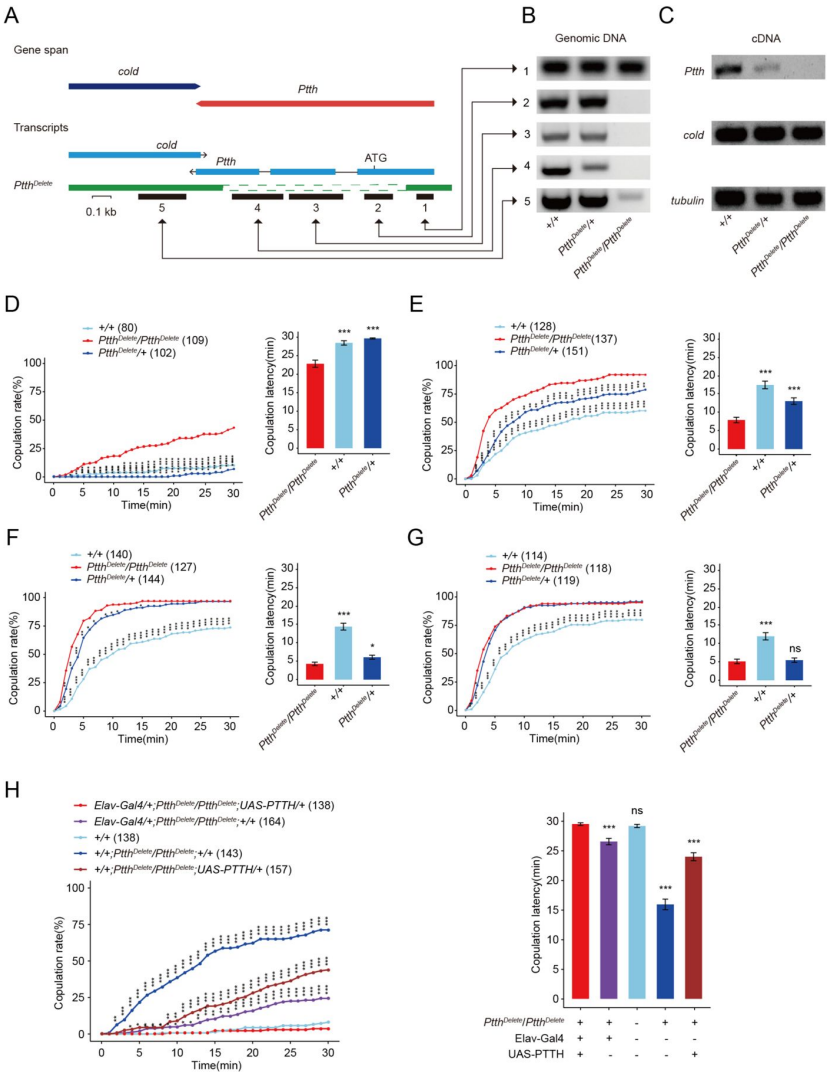


Figure 1

*P*tth null mutants have increased virgin female receptivity.

(A-C) Generation and validation of a 974 bp deletion mutant of the *Ptth* gene. The 5' UTR and almost all coding sequence were deleted. The deletion was confirmed through PCR analysis at the PTTH locus in genomic DNA samples (B), and through RT-PCR to identify the loss of PTTH transcripts in cDNA samples of wandering larvae (C). (D-G) Virgin female receptivity of *Ptth* null mutants on the 1st (D), 2nd (E), 3rd (F) and 6th day (G) respectively. The comparison referred to *Ptth^{Delete}/Ptth^{Delete}*. (H) Enhanced virgin female receptivity of *ΔPtth* null mutants was rescued by elav-Gal4 driving UAS-PTTH. The increased copulation rate and decreased latency to copulation on the 1st day after eclosion were rescued to the comparable level of control. The comparison referred to *elav-Gal4/+; Ptth^{Delete}/Ptth^{Delete};UAS-PTTH/+*. The copulation latency *and copulation rate* of *elav-Gal4/+; Ptth^{Delete}/Ptth^{Delete}* is higher and lower than *Ptth^{Delete}/Ptth^{Delete};UAS-PTTH/+ respectively*. The number of female flies paired with wild type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For the latency to copulation, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests are applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

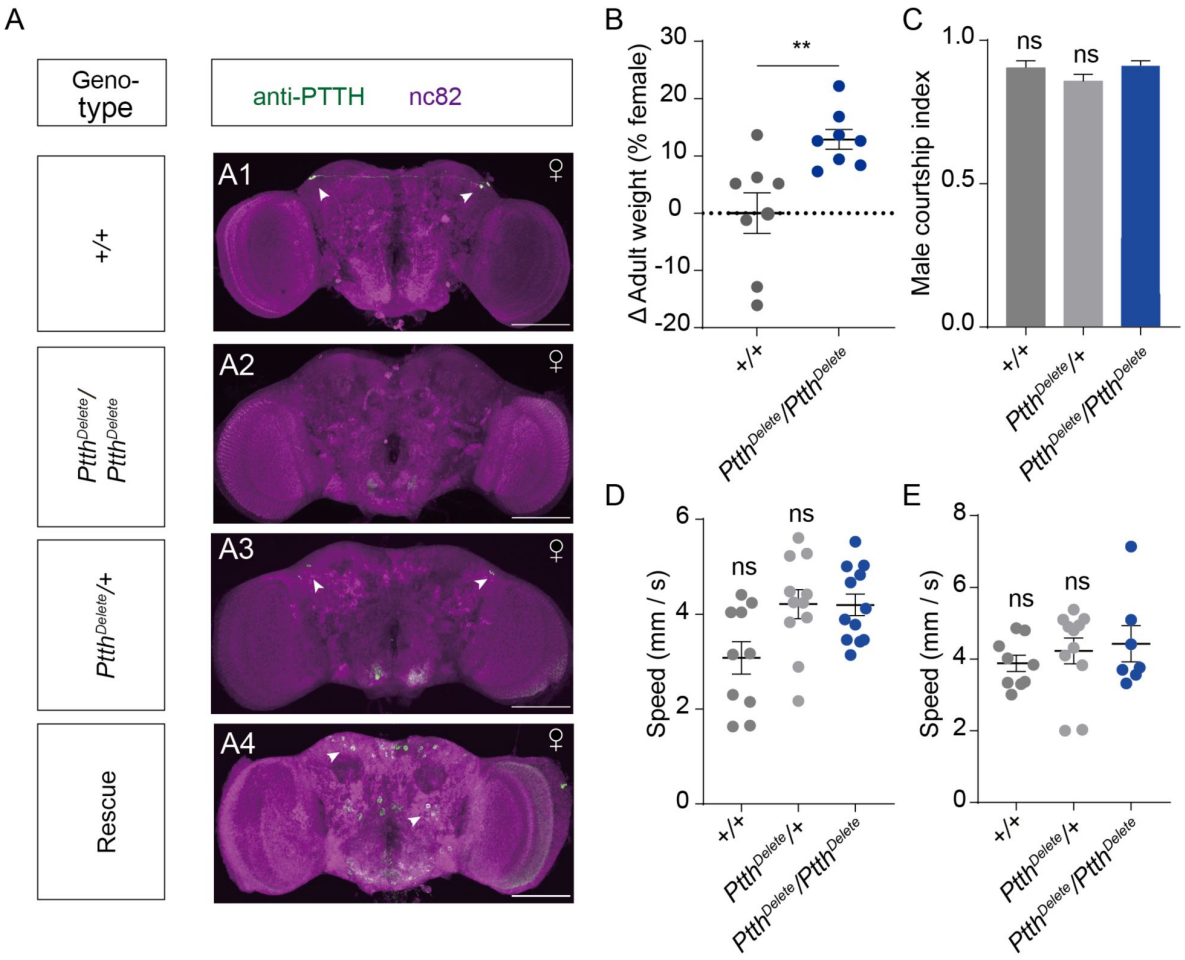


Figure 1–figure supplement 1

PTTH expression, weight, attractiveness and locomotion behavior of *Ptth* null mutant virgin females.

(A) Brain of indicated genotype, immunostained with anti-PTTH antibody (green) and counterstained with nc82 (magenta). Arrows show signals (green) stained with anti-PTTH antibody. Female flies were within 10 hours after eclosion. Scale bars, 50 μm. (B) The weights of 24h-old adult $Ptth^{Delete}/Ptth^{Delete}$ null mutant females were significantly higher than that of wild type females (Mann-Whitney U test, n=8 groups, 10 flies in each group). (C) Courtship index of wild-type males during the first 5 min of courtship towards a female with the indicated genotype (Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests, n = 7-9). (D-E) Mean velocity had no significant change in $Ptth^{Delete}/Ptth^{Delete}$ null mutant females on the 1st day (D) and the 6th day (E) compared with control females (Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests, n = 7-12). The comparison referred to $Ptth^{Delete}/Ptth^{Delete}$. Female flies for behavioral assay were 4-6-days-old adults. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.

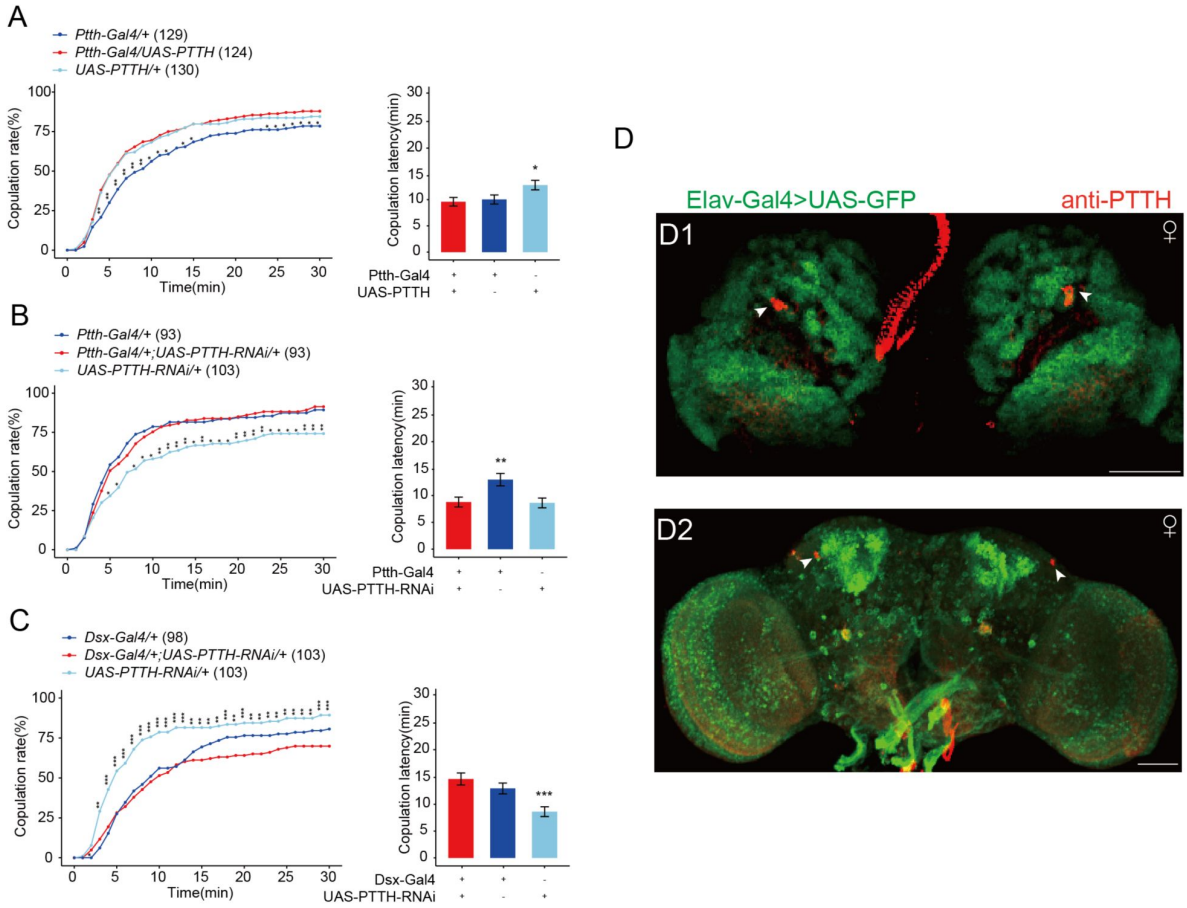


Figure 1–figure supplement 2

Effect of the expression of PTTH on female receptivity.

(A) Overexpressing PTTH in PTTH neurons. The comparison referred to flies *Ptth-Gal4/+;UAS-PTTH/+.* Female flies for behavioral assay were 4-6 days-old. (B-C) Decreasing PTTH in PTTH neurons. The comparison referred to flies *Ptth-Gal4/+;UAS-PTTH-RNAi/+* and *Dsx-Gal4/+;UAS-PTTH-RNAi/+.* Female flies for behavioral assay were 2-days-old adults. (D) The fly brain of elav-Gal4>UAS-GFP were stained with PTTH antibody. The larvae flies were the wandering ones. The adult flies were within 10 hours after eclosion. Representative of 5 female brains. Scale bars, 50 μm.

specific expression of PTTH could restore the enhanced copulation rate and shorter latency to copulation in $PTTH^{Delete}/PTTH^{Delete}$virgin females (**Figure 1H**). Except for the projection of axons to PG gland, PTTH also carries endocrine function to regulate light avoidance of larvae (Yamanaka et al., 2013). The overexpressed PTTH in other neurons through elav-Gal4>UAS-PTTH may act on the PG gland through endocrine function and then induce the ecdysone synthesis and release. In summary, these results suggested that PTTH regulates virgin female receptivity through ecdysone.

Dsx^+ PTTH neurons regulate virgin female receptivity

We used new Ptth-Gal4 and Ptth-LexA which inserts Gal4 or LexA sequence before the stop codon of the *Ptth* gene (Deng et al., 2019) to label and manipulate PTTH neurons expressing PTTH. The labeled neurons were the same as reported before (McBrayer et al., 2007; Yamanaka et al., 2013), a pair of bilateral neurosecretory cells in the brain directly innervating the prothoracic gland during the larval stage (**Figure 2A** and **Figure 2—figure supplement 1A**). The newly emerged flies had the similar anatomical pattern with that of the larval stage (**Figure 2B** and **Figure 2—figure supplement 1B**). However, while the prothoracic gland cells are gradually degenerating during pharate adult development (Dai et al., 1991; Roy et al., 2018), the pattern of PTTH neurons labeled by Ptth-Gal4 > UAS-mCD8GFP gradually could not be found before the 10^{th} hour after eclosion (**Figure 2—figure supplement 2**).

Most identified neurons associated with female sexual behaviors express *doublesex* gene. We asked whether PTTH neurons are a part of the doublesex circuitry or not. Double labeling of dsx-LexA and Ptth-Gal4 neurons (LexAop-tomato,UAS-stinger-GFP/Ptth-LexA;dsx-Gal4/+) revealed that PTTH neurons are all doublesex-positive (**Figure 2C**). We then used an intersectional strategy to visualize overlapped expression between dsx-LexA and Ptth-Gal4 (*UAS* > *stop* > *myrGFP/+;LexAop2-FlpL,dsx-LexA/Ptth-Gal4*). We observed all PTTH neurons with GFP signals (**Figure 2D**). These results suggested that PTTH neurons are dsx^+ neurons. Furthermore, we wanted to know whether Dsx^F regulates female receptivity in PTTH neurons. We decreased the Dsx^F expression in PTTH neurons and did not detect significantly changed female receptivity (**Figure 2—figure supplement 1C**). We supposed that PTTH neurons have some relationship with other Dsx^F-positive neurons which regulate female receptivity.

We then analyzed whether PTTH neurons are involved in the modulation of virgin female receptivity. First, we activated PTTH neurons transiently in adult virgin females by driving the temperature-sensitive activator dTrpA1 (Hamada et al., 2008) using Ptth-Gal4. PTTH neurons were activated at 29°C compared with the control treatment at 23°C. No significantly different copulation rate or latency to copulation was detected (**Figure 2—figure supplement 3A-C**). This suggested that PTTH neurons do not regulate virgin female receptivity during the adult stage.

To identify the detail time for the function of PTTH neurons in virgin female receptivity, we inactivated PTTH neurons through kir2.1 under the control of the temporal and regional gene expression targeting system (McGuire et al., 2004). When the experiment was done at the pupal stage is the only situation when the controls were both different from the experimental. (**Figure 2—figure supplement 1D**). However, when PTTH neurons were inactivated during whole pupal or whole adult stages, virgin female copulation rate did not change significantly (**Figure 2—figure supplement 1D**). Furthermore, we activated PTTH neurons at different stages overlapping the postembryonic larval developmental time using dTrpA1 (**Figure 3A**). Stage 1 was from the 1^{st}-instar larvae to six hours before the 3^{rd}-instar larvae. Stage 2 was from six hours before the 3^{rd}-instar larvae to the end of the wandering larvae (the start of prepupa stage). Stage 3 was from the start of prepupa stage to the end of the 2^{nd} day of the pupal stage. Stage 4 was from the end of the 2^{nd} day of the pupal stage to the eclosion of adults. The copulation rate did not change significantly when activating PTTH neurons during the stage 1, stage 3 or stage 4 (**Figure 3B, 3D and 3E**). However, we found the significant lower copulation rate and the longer latency to

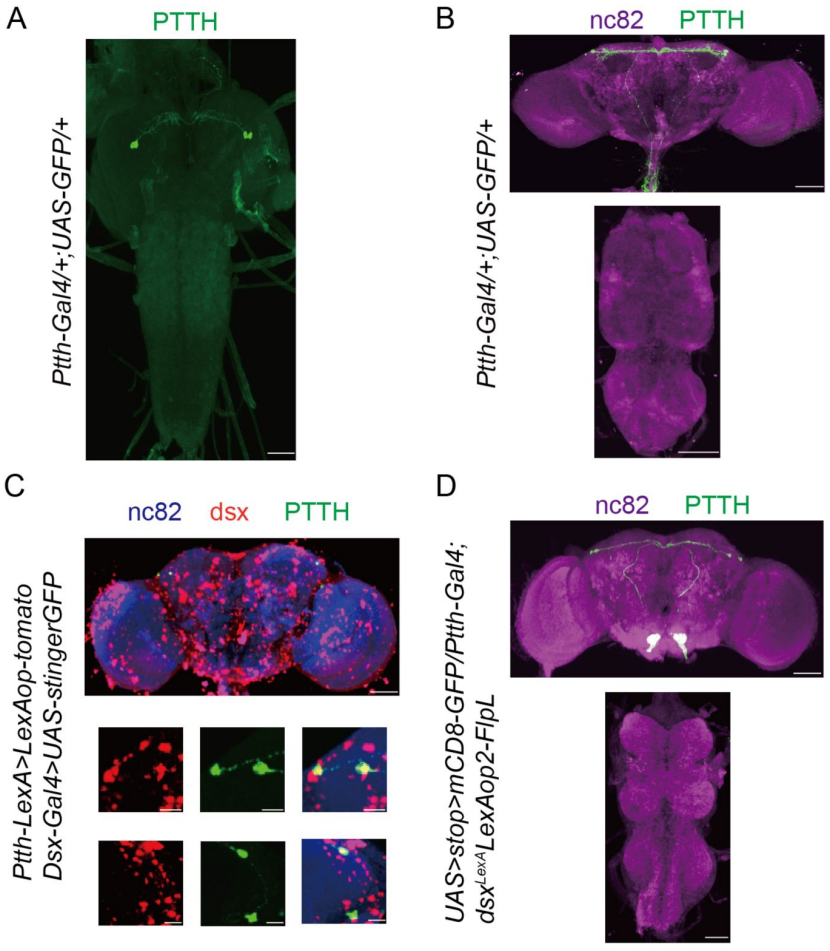


Figure 2

PTTH neurons are doublesex-positive neurons.

(A-B) Expression pattern of Ptth-Gal4 revealed by anti-GFP in larvae central nervous system (CNS) (A) and adult brain (B). Representative of five female flies. Scale bars, 50 μm. (C) All PTTH neurons were colabeled by dsx-Gal4 driving UAS-GFP-Stinger (red) and Ptth-LexA driving LexAop-tomato (green). Representative of five female brains. Scale bars, 50 μm and 5 μm (zoom-in). (D) All PTTH neurons were *Ptth* and *Dsx* co-expressing, labeled by intersectional strategy. The larvae flies were the wandering ones. The adult flies were within-10 hours-old adults. Representative of 5 female brains. Scale bars, 50 μm.

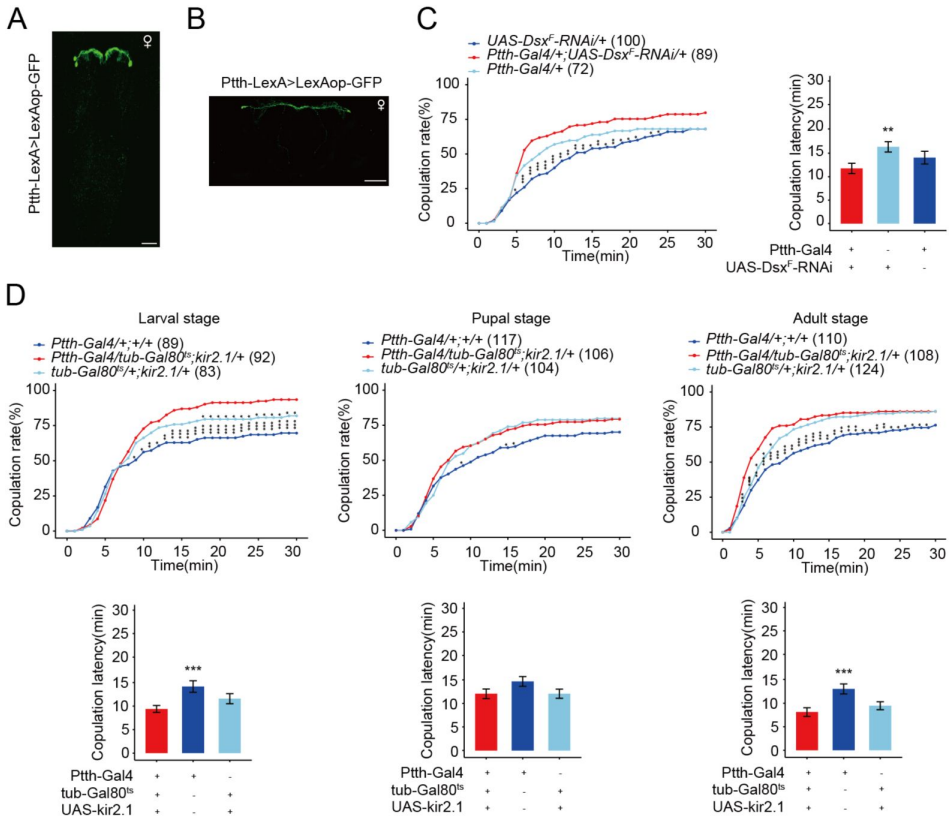


Figure 2–figure supplement 1.

The function of PTTH neurons in female receptivity.

Expression pattern of Ptth-LexA in the brain revealed by anti-GFP (green) in wandering larvae CNS (A) and within-10-hours adult brain (B). Representative of five female flies. Scale bars, 50 μm. (C) The female receptivity when deceasing the expression of Dsx^F in PTTH neurons. The comparison referred to *Ptth-Gal4/+;UAS-Dsx^F-RNAi/+*. Female flies were 2-days-old adults. (D) PTTH neurons were inactivated during the whole larval, pupal and adult stages respectively by kir2.1, restricted by shifts from 18°C to 30°C. When the experiment was done at the larval stage is the only situation when the controls were both different from the experimental. The comparison referred to *Ptth-Gal4/tub-Gal80ts;kir2.1/+*. Female flies were 2-days-old adults. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

Figure 2–figure supplement 2.

The anatomical pattern of PTTH neurons expressing PTTH at different developmental stages.

Expression pattern of Ptth-Gal4 in the brain revealed by anti-GFP from the 3rd larval stage to the 4th day after eclosion. Arrows show PTTH signals (green) stained with anti-GFP antibody. L3, the 3rd -instar larvae; wander, the wandering larvae; P4, the 4th day of the pupal stage; A0h, the 1st hour of the adult stage; A3h, the 3rd hour of the adult stage; A6h, the 6th hour of the adult stage; A9h, the 9th hour of the adult stage; A12h, the 12th hour of the adult stage; A4d, the 4th day of the adult stage. Representative of five female flies. Scale bars, 50 μm.

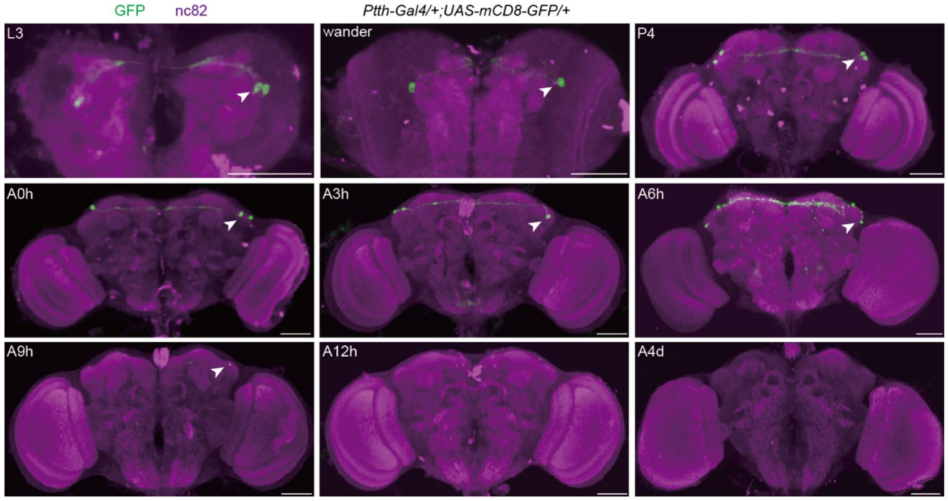
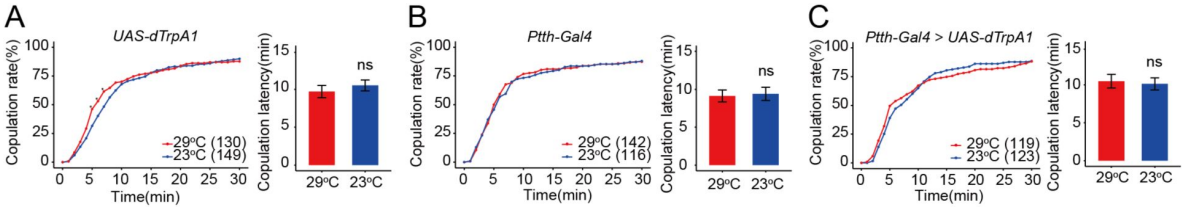


Figure 2–figure supplement 3.

PTTH neurons expressing PTTH do not regulate virgin female copulation rate during adult stage.

PTTH neurons were activated during adult stage by dTrpA1 at 29°C. The female copulation rate and the latency to copulation did not change significantly. The number of female flies paired with wild-type males is displayed in parentheses. Female flies were 4-days-old adults. For the copulation rate, chi-square test is applied. For the latency to copulation, Mann-Whitney U test is applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.



copulation only when PTTH neurons were activated during the stage 2 (**Figure 3C** and **Figure 3F**). The defected copulation was not due to a lower locomotion activity of virgin females (**Figure 3G**). Taken together, our findings indicated that the activity of dsx^{+} PTTH neurons negatively regulate virgin female receptivity during the stage from the start of the 3rd-instar to the end of wandering stage.

PTTH modulates virgin female receptivity through ecdysone

The 3rd-intar larval stage is the critical stage for the initiation of metamorphosis involving the synthesis of ecdysone (Imura et al., 2020; Lavrynenko et al., 2015; Shimell et al., 2018). To test whether PTTH regulates virgin female receptivity through regulating the synthesis of ecdysone, we rescued the enhanced female receptivity by feeding 20E to the 3rd-intar larval $Ptth^{Delete}$ flies. The enhanced copulation rate and shorter latency to copulation of the $Ptth^{Delete}$flies were rescued to the comparable level of wild type females (**Figure 4**). Furthermore, the wild type females fed by 20E had no significantly different copulation rate and latency to copulation compared with the wild type females fed by the same volume of 95% ethanol which is the solvent of 20E (**Figure 4**). This suggested that PTTH regulates virgin female receptivity through the titer of ecdysone.

Ecdysone receptor EcR-A in pC1 neurons regulates virgin female copulation rate

Given that PTTH regulates virgin female receptivity through ecdysone which acts on its receptor EcR, we then asked whether ecdysone regulates the function of neurons associated with virgin female receptivity through EcR. pC1 and vpoDN neurons are two main dsx^{+} neurons involved in virgin female receptivity (Wang et al., 2021; Zhou et al., 2014). pC1 neurons encode the mating status of female flies, vpoDN neurons regulate the opening of vaginal plate when females attend to accept males. EcR-A and EcR-B1 are the two prominently expressed ecdysone receptors in the CNS (Riddiford et al., 2000). First, we tested the expression of EcR-A and EcR-B1 in these two neurons on the 2nd day of the pupal stage when ecdysone functions as the main mover in the metamorphosis (Dalton et al., 2009; Truman et al., 1994). The GFP signals labeled by pC1-ss2-Gal4 and vpoDN-ss1-Gal4 were merged well with the signals of both EcR-A and EcR-B1 antibodies respectively (**Figure 5—figure supplement 1**). This revealed that EcR-A and EcR-B1 express in both pC1 and vpoDN neurons. We then tested the function of EcR in pC1 and vpoDN neurons through reducing the expression of EcR-A and EcR-B1 respectively. We used the split-Gal4 for pC1 and vpoDN neurons to drive the UAS-EcR-RNAi. First, we reduced the expression of all EcR isoforms in pC1 neurons, this decreased the copulation rate and prolonged the latency to copulation significantly (**Figure 5—figure supplement 2A**). Furthermore, we reduced the expression of EcR-A in pC1 neurons. The virgin female had the significant lower copulation rate and longer latency to copulation (**Figure 5A**). The reduced copulation rate had no relationship with the attractivity (**Figure 5E**) and the locomotion activity of virgin females **Figure 5—figure supplement 2B**). When reducing the expression of EcR-B1 in pC1 neurons, virgin females had the significant longer latency to copulation but the comparable copulation rate to controls (**Figure 5B**). However, reducing the expression of EcR-A (**Figure 5—figure supplement 3A–C**) and EcR-B1 (**Figure 5—figure supplement 3D–F**) using three split vpoDN-Gal4s in vpoDN neurons all did not affect virgin female receptivity. This suggested that the expression of EcR-A in pC1 neurons regulates virgin female copulation rate, but EcR isoforms in vpoDN neurons do not modulate virgin female receptivity.

Two split-Gal4 drivers for pC1 neurons had been obtained previously. pC1-ss1-Gal4 labels pC1-a, c and -e neurons, and pC1-ss2-Gal4 labels all pC1-a, -b, -c, -d and -e neurons (Wang et al., 2020b). We also tested virgin female receptivity when EcR-A or EcR-B1 were reduced in pC1-a, -c and -e neurons simultaneously using pC1-ss1-Gal4 respectively. While the copulation rate or the latency

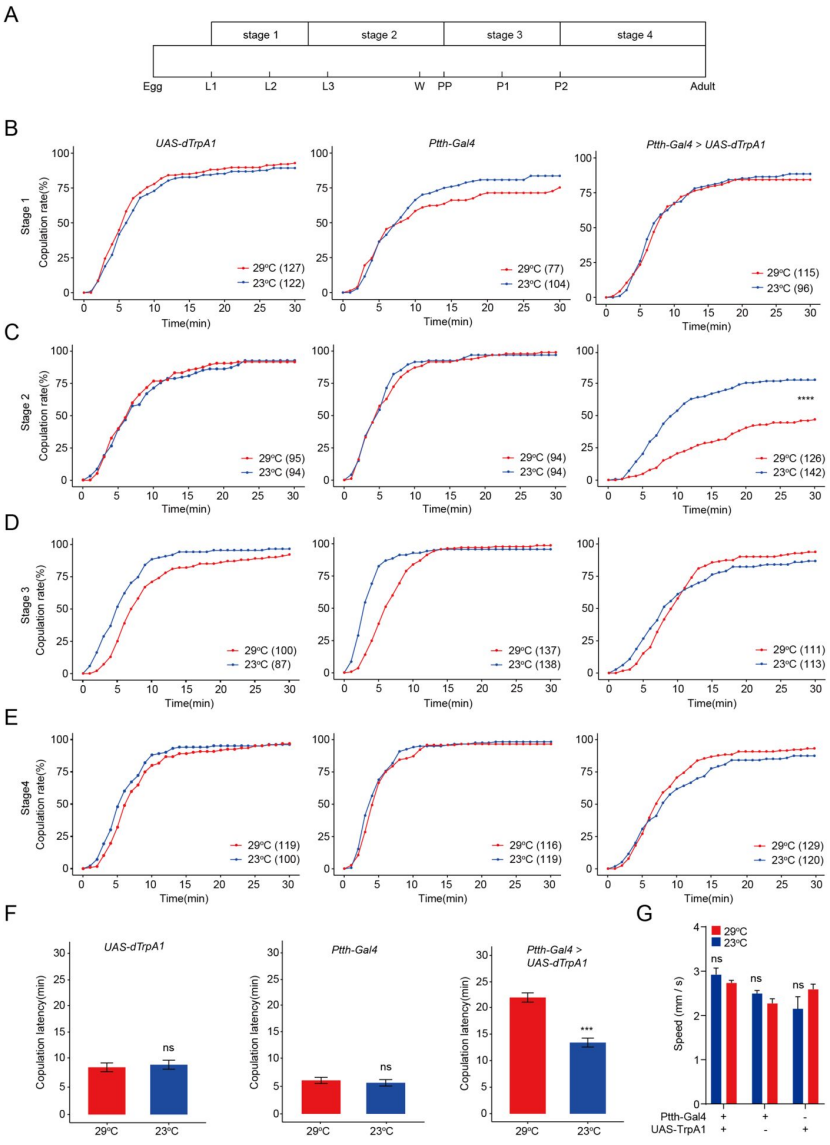


Figure 3

Activation of PTTH neurons expressing PTTH during the 3rd-instar larvae inhibits virgin female receptivity.

(A) Four developmental stages of *Drosophila* before eclosion when PTTH neurons were thermogenetic activated by dTrpA1. L1, L2, and L3: start of three larval stages, W: start of wandering stage, Pp: puparium formation, P1 and P2: start of the 1st and 2nd day of pupal stage. (B-E) Ptth-Gal4 driving UAS-dTrpA1 activated PTTH neurons at 29°C. Activation of PTTH neurons at the stage 2 significantly decreased copulation rate (C), but not at the stage 1 (B), stage 3 (D) and stage 4 (E). (F) Activation of PTTH neurons at the stage 2 significantly increased the latency to copulation. (G) Mean velocity had no significant change when PTTH neurons were activated during the stage 2 compared with control females (ns = not significant, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests, mean ± SEM, n = 8-12). The comparison referred to *Ptth-Gal4/UAS-dTrpA1*. Female flies were 4-days-old adults. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For the latency to copulation, Mann-Whitney U test is applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

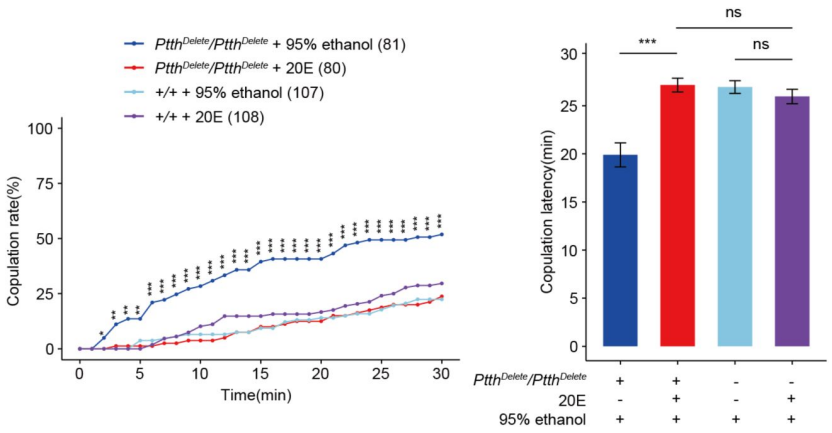


Figure 4

Feeding 20E restores virgin female receptivity of *Ptth* null mutant flies.

(A-B) The increased copulation rate and decreased latency to copulation of the 24h-old *ΔPtth* flies were rescued to the comparable level of wild type females by feeding 20E to the 3rd-instar larval *ΔPtth* flies. The wild type larval females fed by 20E had no significantly different copulation rate and latency to copulation compared with the wild type females fed by the same volume of 95% ethanol which is the solvent of 20E. The comparison referred to $Ptth^{Delete}/Ptth^{Delete}$ + 20E. Female flies were 1-day-old. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For the latency to copulation, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests are applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

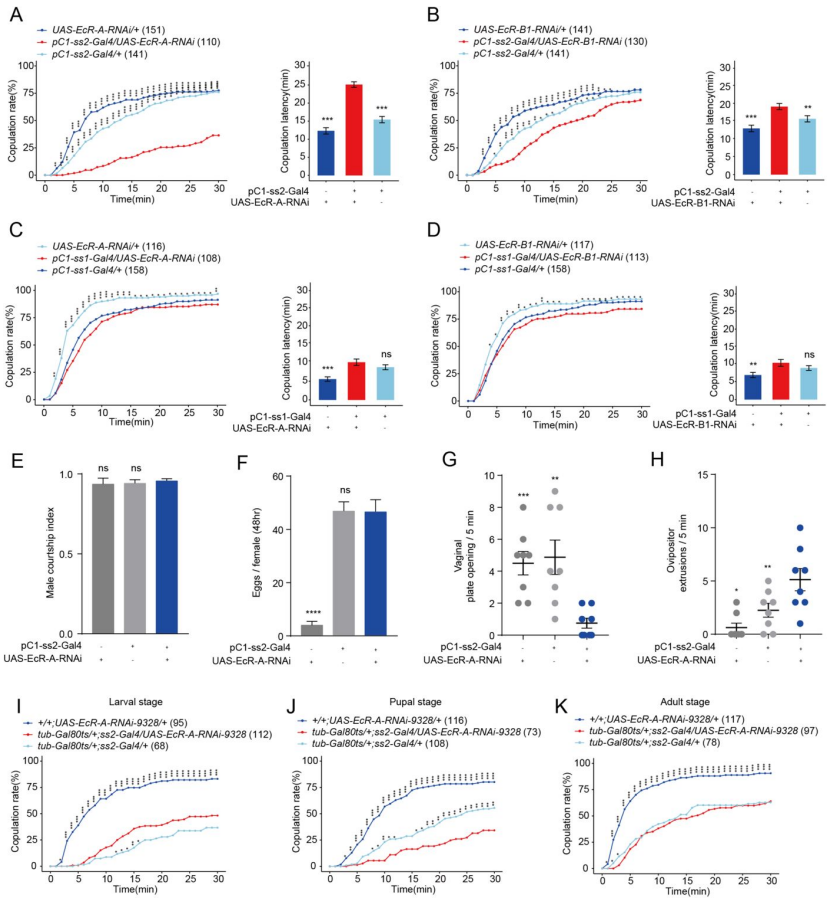


Figure 5

Virgin females with reduced EcR-A in pC1 neurons have reduced sexual receptivity.

(A) Knock-down of EcR-A in pC1 neurons driven by pC1-ss2-Gal4 significantly decreased the copulation rate and increased the latency to copulation. (B) Knock-down of EcR-B1 in pC1 neurons driven by pC1-ss2-Gal4 significantly prolonged the latency to copulation. (C-D) Knock-down of EcR-A (C) or EcR-B1 (D) in pC1 neurons driven by pC1-ss1-Gal4 did not affect the copulation rate or the latency to copulation. (E) Courtship index of wild-type males towards a female with the indicated genotype (n = 8). (F) The number of eggs laid by virgin females during the 3rd - 4th day after eclosion when EcR-A was knocked down in pC1 neurons (n = 17-36). The UAS-EcR-A-RNAi control causes a massive decrease in female fertility. (G) Knock-down of EcR-A in pC1 neurons decreased the opening of vaginal plate of virgin females compared with controls (n = 8). (H) Knock-down of EcR-A in pC1 neurons increased the ovipositor extrusion of virgin females compared with controls (n = 8). (I-K) Virgin female copulation rate when EcR-A was knocked down in pC1 neurons temporally restricted by shifts from 18°C to 30°C. EcR-A was knocked down during the whole larval (I), pupal (J) and adult (K) stages respectively. When the experiment was done at the pupal stage is the only situation when the controls were both different from the experimental (J). The comparison referred to flies with decreased EcR isoform in pC1 neurons. Female flies for behavioral assay were 4-6-days-old adults. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For other comparisons, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests are applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

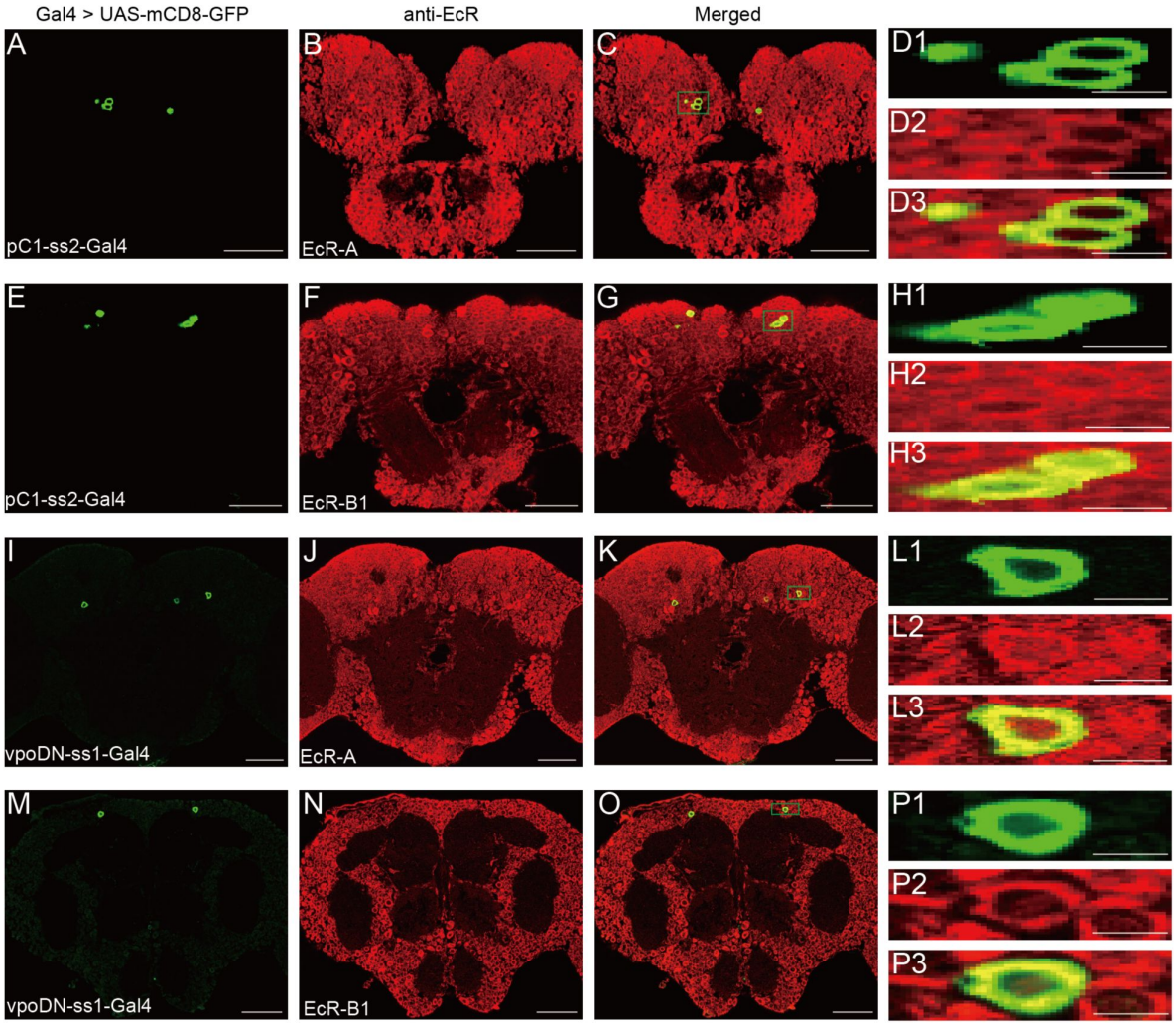


Figure 5–figure supplement 1.

Expression of EcR-A and EcR-B1 in pC1 and vpoDN neurons.

(A-C) pC1 neurons were colabeled by pC1-ss2 driving UAS-mCD8-GFP (green, A) and EcR-A antibodies (red, B). Magnification of green boxed region in (C) is shown in (D1– D3). (E-G) pC1 neurons were colabeled by pC1-ss2 driving UAS-mCD8-GFP (green, E) and EcR-B1 antibodies (red, F). Magnification of green boxed region in (G) is shown in (H1–H3). (I-K) vpoDN neurons were colabeled by vpo-ss1 driving UAS-mCD8-GFP (green, I) and EcR-A antibodies (red, J). Magnification of green boxed region in (K) is shown in (L1–L3). (M-O) vpoDN neurons were colabeled by vpo-ss1 driving UAS-mCD8-GFP (green, M) and EcR-B1 antibodies (red, N). Magnification of green boxed region in (O) is shown in (P1–P3). Female flies were during prepupae stage. Scale bars for magnified regions are 5 μm, for others are 50 μm.

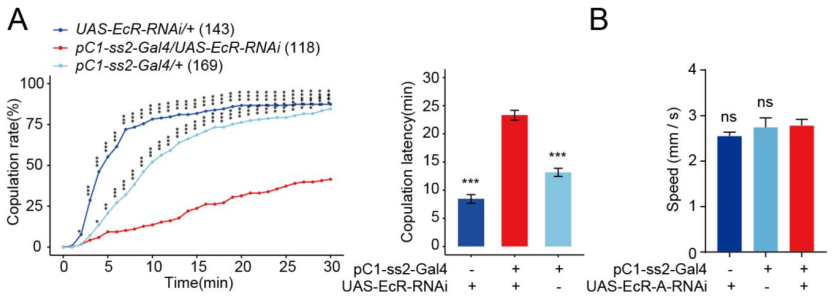


Figure 5–figure supplement 2.

Reduced EcR in pC1 neurons reduces virgin female receptivity.

(A) Knock-down of EcR in pC1 neurons driven by pC1-ss2-Gal4 significantly decreased the copulation rate and increased the latency to copulation. (B) Mean velocity had no significant change when EcR-A was knocked down in pC1 neurons compared with controls (Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests, n = 8-11). The comparison referred to pC1-ss2-Gal4/UAS-EcR-RNAi (A) and pC1-ss2-Gal4/UAS-EcR-A-RNAi (B). Female flies were 4-6-days-old adults. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For the latency to copulation, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests are applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

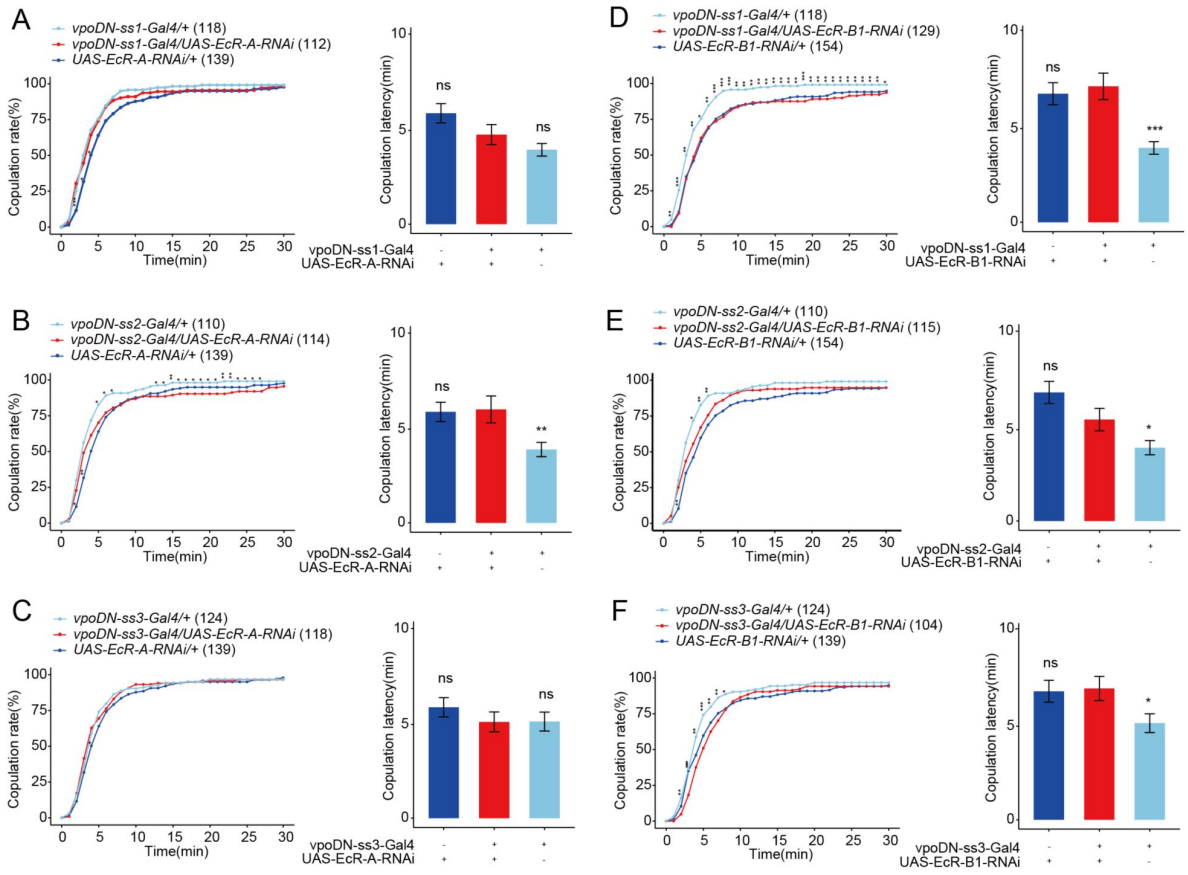


Figure 5–figure supplement 3.

Reduced EcR in vpoDN neurons has no effect on virgin female receptivity.

(A-C) Knock-down of EcR-A in vpoDN neurons driven by vpoDN-ss1-Gal4, vpoDN-ss2-Gal4 and vpoDN-ss3-Gal4 had no effect on virgin female receptivity. (D-F) Knock-down of EcR-B1 in vpoDN neurons driven by vpoDN-ss1-Gal4, vpoDN-ss2-Gal4 and vpoDN-ss3-Gal4 had no effect on virgin female receptivity. The comparison referred to flies with decreased EcR isoform in vpoDN neurons. Female flies were 4-6-days-old adults. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For the latency to copulation, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests are applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

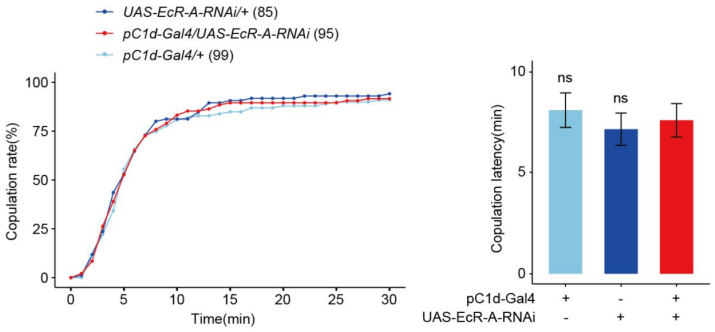


Figure 5–figure supplement 4.

Reduced EcR-A in pC1d neurons has no effect on virgin female receptivity.

(A) Knock-down of EcR-A in pC1d neurons had no effect on virgin female copulation rate and latency to copulation. The comparison referred to pC1d-Gal4/UAS-EcR-A-RNAi. Female flies were 4-6-days-old adults. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For the latency to copulation, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests are applied. Error bars indicate SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

to copulation did not change significantly (**Figure 5C-D**). This suggested that, pC1b only, or both pC1b and pC1d neurons is necessary for the functions of EcR-A and EcR-B1 in pC1 neurons on virgin female receptivity. Whether pC1d is involved in the regulation of female receptivity is uncertain (Deutsch et al., 2020; Schretter et al., 2020; Taisz et al., 2023). However, when reducing EcR-A in pC1d neurons alone using the specific split-Gal4 SS56987 (Schretter et al., 2020), virgin female receptivity including copulation rate and latency to copulation did not change significantly compared with controls (**Figure 5—figure supplement 4**). These results suggested that the function of EcR-A in pC1b neurons is necessary for virgin female copulation rate.

As recently mated females may reduce sexual receptivity and increase egg laying (Avila et al., 2011; Kubli, 2003). we asked whether the decreased copulation rate induced by EcR-A could be a post-mating response and correlate with elevated egg laying. To address this, we examined the number of eggs laid by virgin females when EcR-A was reduced in pC1 neurons. We found that manipulation of EcR-A did not enhance egg laying significantly in virgin females (**Figure 5F**), although the UAS-EcR-A-RNAi control causes a massive decrease in female fertility. Meanwhile, we further analyzed whether reduction of EcR-A in pC1 neurons regulates the opening of vaginal plate (VPO) or the ovipositor extrusion (OE). We found that reducing the EcR-A expression in pC1 neurons lead to the significantly less VPO and more OE (**Figure 5G-H**). These results suggested that reduced EcR-A expression in pC1 neurons results in the similar phenotype to that of mated females.

EcR-A participates in the morphological development of pC1 neurons

EcR isoforms have distinct temporal and spatial expression patterns in the CNS (Riddiford et al., 2000; Truman et al., 1994). It is unknown when EcR-A functions in pC1 neurons for virgin female receptivity. Thus, we examined virgin female receptivity when EcR-A expression was conditionally reduced through RNAi via the pC1-ss2-Gal4 under the control of the temporal and regional gene expression targeting system (McGuire et al., 2004). EcR-A was reduced during the whole larval, pupal and adult stage respectively (**Figure 5I-K**). When the experiment was done at the pupal stage is the only situation when the controls were both different from the experimental (**Figure 5J**). The result suggested that EcR-A in pC1 neurons plays a role in virgin female receptivity during metamorphosis. This is consistent with that PTTH regulates virgin female receptivity before the start of metamorphosis.

We then tested how EcR-A functions in pC1 neurons to modulate virgin female receptivity. First, we tested the morphology of pC1 neurons when reducing the expression of EcR-A in pC1 neurons. We found that the morphology of pC1-ss2-Gal4 expressing neurons appeared after the formation of the white pupa (**Figure 6A1** 1). The reduced EcR-A expression induced the more elaborated morphologies of the pC1-d/e cells, especially the extra vertical projection (EVP) near the midline of brains (**Figure 6 B-D**) (Deutsch et al., 2020). These changes exhibited from the second day of the pupal stage (**Figure 6B1-B2 and 6E**) and maintained at the adult stage (**Figure 6 D1-D2 and 6F**). Meanwhile, the number of pC1 cell bodies in adult flies when EcR-A was reduced were the same as that of wild type flies (**Figure 6G**). Previous studies suggested that pC1d cells serve as a hub within the central brain for dsx^+ and fru^+ neurons (Deutsch et al., 2020). Thus, the abnormal development of pC1d neurons may induce the changes between pC1d neurons and other dsx^+ and fru^+ neurons to affect associated behaviors.

Furthermore, we asked whether reduced female copulation rate was due to that EcR-A expression affected the activity of pC1 neurons. Because all pC1 cells characterized so far project to the lateral junction of the lateral protocerebral complex (LPC) (Kimura et al., 2015; Rezával et al., 2016; Scheffer et al., 2020; Wang et al., 2020b; Wu et al., 2019; Zhou et al., 2014), we expressed GCamp6s in all pC1 neurons and tested the calcium signals in the lateral junction of LPC when

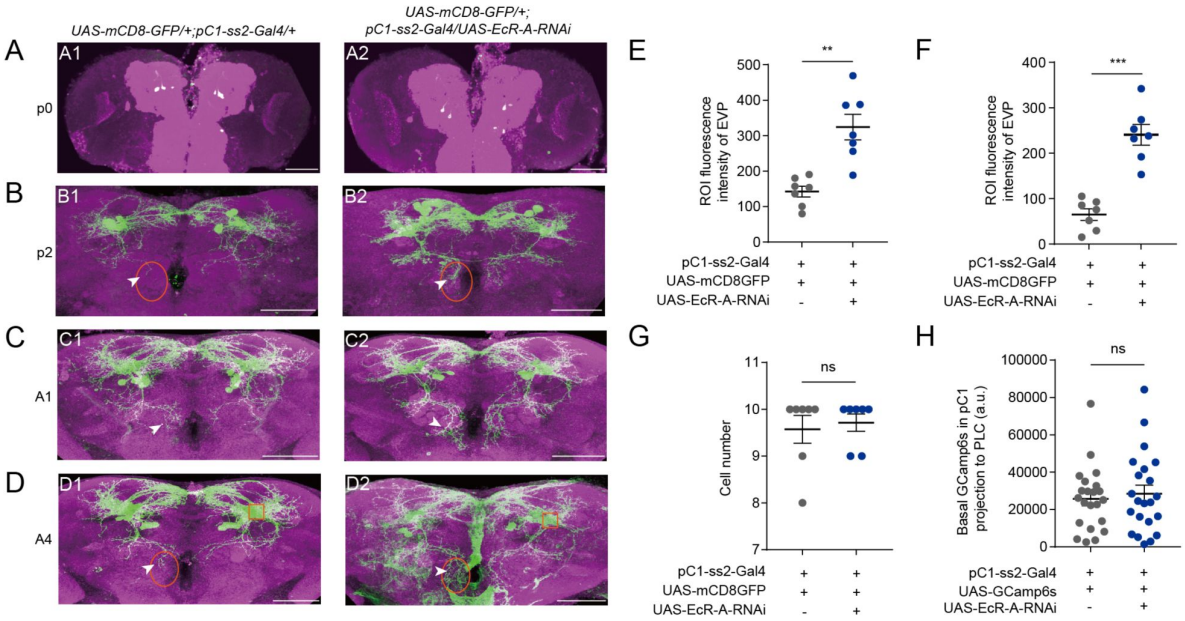


Figure 6

Reduced EcR-A in pC1 neurons induces the morphological changes.

(A1-A2) pC1-ss2-Gal4 expressing neurons appeared at the start of the pupal stage. (B-D) Reduced EcR-A in pC1 neurons induced more elaborated morphologies of pC1d axons, especially the extra vertical projection (EVP). The EVP regions of pC1d neurons was indicated by arrows. The morphological changes appeared on the 2nd day of the pupal stage (B1-B2) and retained to the adult stage including the 1st day (C1-C2) and the 4th day (D1-D2) of the adult stage. p0, the 1st day of the pupal stage; p2, the 2nd day of the pupal stage; A1, the 1st day of the adult stage; A4, the 4th day of the adult stage. (E-F) Fluorescence intensity of EVP in pC1d neurons on the 2nd day of the pupal stage (E) and the 4th day of the adult stage (F) was quantified when EcR-A was reduced in pC1 neurons (n=7). The quantified EVP regions were marked in (B) and (D) with orange ellipses. (G) pC1 neurons of the 4th day adults had comparable cell body number when EcR-A was reduced in pC1 neurons or not (n = 7). (H) Basal GCaMP6s signals in the LPC region of pC1 neurons when EcR-A was reduced in pC1 neurons (n = 22). LPC regions, the neurites extending from pC1 cell bodies, were marked with orange square in (D1) and (D2). The comparison referred to flies with decreased EcR isoform in pC1 neurons. Female flies in (G-H) were 4-days-old adults. Scale bars are 50 μm. For all comparisons, Mann-Whitney U test is applied. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

EcR-A was knocked down (**Figure 6 D1-D2**). Reduced EcR-A did not induce significantly different calcium responses in the LPC (**Figure 6H**). Thus, our results suggested that the decreased female copulation rate induced by reduced EcR-A in pC1 neurons was mainly due to the morphological changes of pC1b neurons, which then modulate the connections of pC1b neurons with other neurons.

The function of PTTH on EcR-A and pC1 neurons

As newly formed prepupae, the ptth-Gal4 > UAS-Grim flies display similar changes in gene expression to the genetic control flies to response to a high-titer ecdysone pulse. These genes include the repression of EcR (McBrayer et al., 2007). According to the contradictory functions of PTTH deletion and EcR-A reduction in pC1 neurons, we wanted to know whether there is a similar feedforward relationship between PTTH and EcR-A. We quantified the EcR-A expression in the whole pupa body during the start of prepupa stage, when the pC1-ss2-Gal4 expressing neurons appear. Indeed, PTTH -/- induced upregulated EcR-A compared with PTTH -/+ flies (**Figure 7A**). This suggested the feedforward relationship between PTTH and EcR-A expression during the start of prepupa stage. Consistent with this, when PTTH was deleted, pC1 neurons exhibited the contradictory pattern to that when EcR-A was decreased in pC1 neurons (**Figure 7B-C**). These suggested the feedforward relationship between PTTH and EcR-A, and may explain the contradictory functions of PTTH deletion and EcR-A reduction in pC1 neurons for female receptivity.

Furthermore, PTTH neurons are *dsx*-positive, almost all neurons regulating female receptivity express Dsx^F. We wanted to know whether PTTH neurons affect other dsx^+ neurons, including pC1 neurons. Indeed, we detected the slightly overlap of dsx-LexA>LexAop-RFP and torso-Gal4>UAS-GFP during larval stage (**Figure 7D**). Furthermore, decreasing torso expression in pC1 neurons significantly inhibit female receptivity (**Figure 7E**). The inhibited virgin female receptivity had no relationship either with the locomotion activity of virgin females (**Figure 7F**). These results suggest that, PTTH regulates female receptivity not only through ecdysone, but also may through regulating other neurons especially Dsx^F-positive neurons associated with female receptivity directly.

Discussion

In this study, we found that peptide hormone PTTH negatively modulates virgin female receptivity through ecdysone. PTTH neurons are doublesex-positive and regulate virgin female receptivity during neural development. PTTH deletion resulted in the increased ecdysone receptor EcR-A expression in newly formed prepupae. Furthermore, EcR-A functions in pC1 neurons to positively regulate virgin female receptivity during metamorphosis mainly through modulating the anatomical morphology of pC1b neurons. Additionally, decreasing the expression of PTTH receptor torso in pC1 neurons inhibited female receptivity. Taken together, our results revealed the contrary functions of PTTH deletion and reduction of EcR-A in pC1 neurons during neurodevelopment. In addition, EcR-A in pC1 neurons regulates virgin female copulation rate during metamorphosis mainly through modulating the morphology of pC1b neurons.

Most of neurons regulating sexual behaviors in female flies are dsx-positive. Our results showed that PTTH neurons are also dsx^+ neurons. This suggested that PTTH neurons have relationships with other dsx^+ neurons and the juvenile–adult transition is regulated by *doublesex* gene. Indeed, we detected the overlap between torso-Gal4 signal and dsx-LexA signal at the larval stage. Furthermore, when PTTH receptor torso was decreased in dsx^+ pC1 neurons, female receptivity was inhibited. This suggested that PTTH functions in pC1 neurons through neuronal projection or endocrine pathway directly to regulate female receptivity. However, we did not detect the change

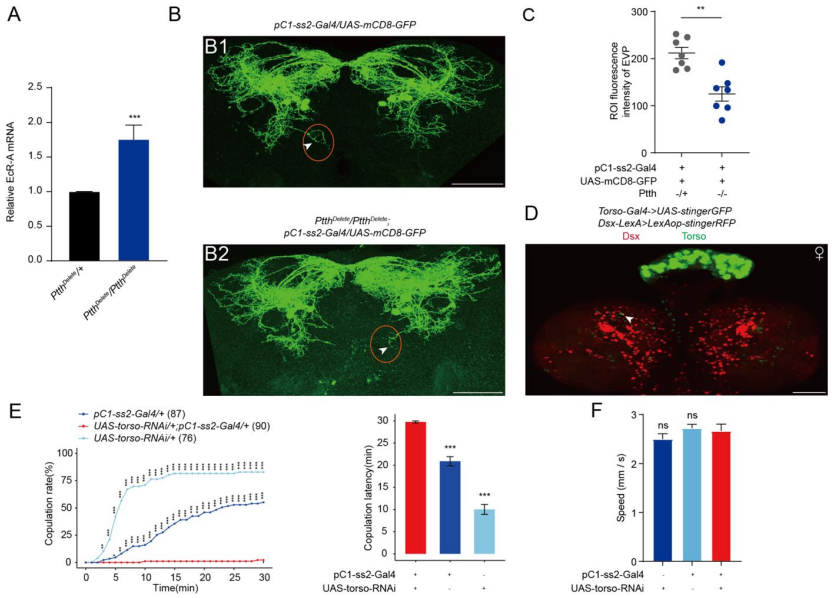


Figure 7

The function of PTTH on EcR-A and pC1 neurons.

(A) qRT-PCR for EcR-A when PTTH was deleted. Bars represent mean ± SEM. p values are from Mann-Whitney U test (n = 8 for $Ptth^{Delete}$/+ and n = 6 for $Ptth^{Delete}/Ptth^{Delete}$, each sample contains about 10 bodies of newly formed prepupae). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference. (B) Deletion of PTTH induced less elaborated morphologies of pC1d axons, especially the extra vertical projection (EVP). The EVP regions of pC1d neurons was indicated by arrows. Female flies were 4-days-old adults. (C) Fluorescence intensity of EVP in pC1d neurons on the 4th day was quantified when PTTH was deleted (n=7). The quantified EVP regions were marked in (B1) and (B2) with red ellipses. (D) Torso-Gal4 and Dsx-LexA were labeled by stinger GFP and stingerRFP respectively. Arrows indicated the overlap of GFP and RFP signals. Representative of five female brains. Scale bars, 50 μm. (E-F) Knock-down of Torso in pC1 neurons inhibited virgin female copulation rate and enhanced latency to copulation. Females had similar locomotion speeds between groups (F). The pC1-ss2-Gal4 control causes a massive decrease in female receptivity. The comparison referred to pC1-ss2-Gal4/UAS-torso-RNAi. Female flies were 4-6-days-old adults. The number of female flies paired with wild-type males is displayed in parentheses. For the copulation rate, chi-square test is applied. For the latency to copulation, Kruskal-Wallis ANOVA and post hoc Mann-Whitney U tests are applied. Error bars indicate SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significant difference.

of female receptivity when Dsx^F was decreased in PTTH neurons. This suggested that Dsx^F functions in PTTH neurons on other aspects, such as the development of PTTH neurons or the synthesis and release of PTTH to regulate development.

PTTH regulates virgin female receptivity in an ecdysone-dependent manner before metamorphosis. Ecdysone functions through its receptor EcR which is involved in all phases of the nervous system development. In our study, reduced EcR-A expression in all pC1 neurons, which encode the mating status of females, lead to the decreased copulation rate. While, reduced EcR-A in pC1-a, c and e simultaneously did not reduce the copulation rate significantly. This suggested that EcR-A plays the critical role in pC1-b or/and -d neurons for regulating virgin female receptivity. Our results revealed that reduced EcR-A induced the more elaborated morphologies of pC1d neurons. Previous studies detected the synaptic connections between the axons of pC1d and the dendrites of DNp13 neurons (Deutsch et al., 2020; Mezzera et al., 2020). DNp13 neurons are command neurons for ovipositor extrusion. When females extruded, the ovipositor physically impedes copulation (Mezzera et al., 2020; Wang et al., 2020a). However, reduced EcR-A expression in pC1d neurons did not affect virgin female receptivity (**Figure 5—figure supplement 4**). This might be due to three possibilities. First, the more elaborated morphologies of pC1d neurons did not affect synaptic connections between pC1d and DNp13 neurons. Second, the unchanged pC1 neural activity could not affect the neural activity of DNp13 neurons. Third, the morphological change of pC1d neurons is not sufficient for the decreased copulation rate. To sum, these suggest that the morphological change of pC1b neurons is necessary for the decreased copulation rate. However, due to the lack of pC1b drivers, we could not rule out morphological changes in pC1b neurons when EcR-A was reduced.

In our study, PTTH negatively regulates female receptivity, while EcR-A positively regulates female receptivity. Previous study revealed that, expression of EcR is repressed in newly formed prepupae to response to high-titer ecdysone pulse (Mcbrayer et al., 2007). We detected the similar feedforward relationship between PTTH and EcR-A in newly formed prepupae. Consistent with this, we detected the contrary pattern of pC1d neurons when PTTH was deleted, compare with that when EcR-A was decreased in pC1 neurons. However, it is not sure that PTTH deletion could result in the increased expression of EcR-A in pC1 neurons. In addition, PTTH deletion must affect the development of almost other neurons, but not only pC1 neurons. This maybe the reason for that reduction of Torso in pC1 neurons had contrary effect on female receptivity to that when PTTH is deleted. So, the feedforward relationship between PTTH and EcR-A in newly formed prepupae is one possible reason for the contrary functions of PTTH deletion and reduction of EcR-A in pC1 neurons on female receptivity.

Previous studies have demonstrated that the development of fru^+ neurons need EcR-A in male *Drosophila melanogaster*. Furthermore, reduced EcR-A in fru^+ neurons induced the male-male courtship (Dalton et al., 2009). We also detected the male-male courtship behavior when PTTH was deleted (data not shown) as previous study (Mcbrayer et al., 2007). This remits to the impact of ecdysone on dsx^+ or fru^+ neurons. Similarly, PTTH deletion may also affect the development of other neurons and further other behaviors such as the female fecundity and the body size (Mcbrayer et al., 2007; Rewitz K F 2009; Shimell et al., 2018), although there is no sufficient evidence for the effects of fecundity and the body size on female receptivity. So, we could not exclude all effects of other aspects on female receptivity.

Our results suggested a regulatory role of PTTH in virgin female receptivity. Even though insects and mammals represent highly diverged classes, insects have evolved a similar strategy for triggering the juvenile–adult transition (Herbison, 2016; Pan et al., 2019). The juvenile–adult transition involves the hypothalamic–pituitary–gonadal (HPG) axis in mammals and the PG axis in insects. Among the neurons belonging to the axis, PTTH neurons and GnRH neurons have the similar function to stimulate the PG gland and pituitary gland to release hormones which trigger

maturation, respectively. It will be interesting to study the function of GnRH neurons on the mammal sexual behaviors. This work extends the understanding of how neurodevelopmental processes regulate adult sexual behavior.

Materials and methods

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
antibody	Mouse anti-Bruchpilot antibody (nc82)	Developmental Studies Hybridoma Bank	Cat# nc82, RRID: AB_2314866	IHC (1:40)
antibody	Mouse Anti-Drosophila ecdysone receptor (EcR-A) Monoclonal Antibody	Developmental Studies Hybridoma Bank	Cat# 15G1a (EcR-A), RRID: AB_528214	IHC (1:10)
antibody	mouse Anti-Drosophila ecdysone receptor (EcR-B1) Antibody	Developmental Studies Hybridoma Bank	Cat# AD4.4(EcR-B1), RRID: AB_2154902	IHC (1:10)
antibody	Rabbit polyclonal anti-GFP	Thermo Fisher Scientific	Cat# A-11122, RRID: AB_221569	IHC (1:1000)
antibody	chicken polyclonal anti-GFP	Thermo Fisher Scientific	Cat# A10262, RRID: AB_2534023	IHC (1:1000)
antibody	Goat anti-rabbit, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-11034, RRID: AB_2576217	IHC (1:500)
antibody	Goat anti-chickent, Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11039; RRID: AB_2534096	IHC (1:500)
antibody	Goat anti-mouse, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-11029, RRID: AB_2534088	IHC (1:500)
antibody	Goat anti-rabbit, Alexa Fluor 546	Thermo Fisher Scientific	Cat# A-11010, RRID: AB_2534077	IHC (1:500)
antibody	Rabbit polyclonal anti-RFP	Thermo Fisher Scientific	Cat# R10367, RRID: AB_10563941	IHC (1:500)
antibody	Goat anti-mouse, Alexa Fluor 647	Thermo Fisher Scientific	Cat# A-21235, RRID: AB_2535804	IHC (1:500)
antibody	Rabbit polyclonal anti-PTTH	Zhou Lab, Chinese Academy of Sciences, this paper	N/A	IHC (1:1300)
chemical compound, drug	Paraformaldehyde (PFA)	Electron Microscopy Sciences	Cat#15713	8% PFA diluted in 1XPBS at 1:4 or 1:2
chemical compound, drug	DPX Mountant	Sigma-Aldrich	Cat#44581	
chemical compound, drug	Normal goat serum	Sigma-Aldrich	Cat#G9023	
chemical compound, drug	20-hydroxyecdysone	Cayman	Cat#16145	dissolved in 95% ethanol, 0.2 mg/ml
chemical compound, drug	TRIzol	Ambion	Cat#15596018	
Genetic reagent (*D.melanogaster*)	*LexAop2-mCD8::GFP*	Bloomington Stock Center	BL#32203	
Genetic reagent (*D.melanogaster*)	*;;UAS-mCD8::GFP*	Bloomington Stock Center	BL#32194	
Genetic reagent (*D.melanogaster*)	*;UAS-mCD8::GFP;*	Bloomington Stock Center	BL#5137	
Genetic reagent (*D.melanogaster*)	*UAS-dTrpA1/cyo*	Garrity Lab, Brandeis University	N/A	

Genetic reagent (*D.melanogaster*)	*UAS-Kir2.1*	Bloomington Stock Center	BL#6595	
Genetic reagent (*D.melanogaster*)	*Ptth-Gal4*	Rao Lab, Peking University	N/A	
Genetic reagent (*D.melanogaster*)	*PtthLexA*	Rao Lab, Peking University	N/A	
Genetic reagent (*D.melanogaster*)	*⊿PTTH*	Rao Lab, Peking University	N/A	
Genetic reagent (*D.melanogaster*)	*UAS-PTTH*	Zhou Lab, Chinese Academy of Sciences, this paper	N/A	
Genetic reagent (*D.melanogaster*)	*isoCS*	Rao Lab, Peking University	N/A	
Genetic reagent (*D.melanogaster*)	*elav-Gal4*	Rao Lab, Peking University	N/A	
Genetic reagent (*D.melanogaster*)	*UAS-GFPStinger*	Janelia Research Campus	N/A	
Genetic reagent (*D.melanogaster*)	*LexAop-tomato*	Janelia Research Campus	N/A	
Genetic reagent (*D.melanogaster*)	*LexAop2-FlpL*	Janelia Research Campus	N/A	
Genetic reagent (*D.melanogaster*)	*UAS > stop > mCD8-GFP*	Janelia Research Campus	N/A	
Genetic reagent (*D.melanogaster*)	*Dsx-Gal4*	Janelia Research Campus	N/A	
Genetic reagent (*D.melanogaster*)	*Dsx-LexA*	Janelia Research Campus	N/A	
Genetic reagent (*D.melanogaster*)	*tub-Gal80^{ts}*	Pan Lab, Southeast University	BL#7018	
Genetic reagent (*D.melanogaster*)	*pC1-ss1-Gal4*	Wang Lab, Lingang Laboratory	N/A	
Genetic reagent (*D.melanogaster*)	*pC1-ss2- Gal4*	Wang Lab, Lingang Laboratory	N/A	
Genetic reagent (*D.melanogaster*)	*vpoDN-ss1-Gal4*	Wang Lab, Lingang Laboratory	N/A	
Genetic reagent (*D.melanogaster*)	*vpoDN-ss2-Gal4*	Wang Lab, Lingang Laboratory	N/A	
Genetic reagent (*D.melanogaster*)	*vpoDN-ss3-Gal4*	Wang Lab, Lingang Laboratory	N/A	
Genetic reagent (*D.melanogaster*)	*UAS-EcR-RNAi*	Bloomington Stock Center	BL#9327	
Genetic reagent (*D.melanogaster*)	*UAS-EcR-A-RNAi*	Bloomington Stock Center	BL#9328	
Genetic reagent (*D.melanogaster*)	*UAS-EcR-B1-RNAi*	Bloomington Stock Center	BL#9329	
Genetic reagent (*D.melanogaster*)	*pC1d-Gal4*	Bloomington Stock Center	BL#86847	

Genetic reagent (*D.melanogaster*)	*UAS-PTTH-RNAi*	VDRC	V102043	
Genetic reagent (*D.melanogaster*)	*UAS-Dsx^{F}-RNAi*	Pan Lab	N/A	
Genetic reagent (*D.melanogaster*)	*UAS-Torso-RNAi*	Liu Lab	BL#33627	
recombinant DNA reagent	pBSK-attP-3P3-RFP-loxP	Bowen Deng et al., 2019	N/A	
recombinant DNA reagent	pBSK-attB-loxP-myc-T2A-Gal4Gal4-GMR-miniwhite	Bowen Deng et al., 2019	N/A	
recombinant DNA reagent	pBSK-attB-loxP-V5-T2A-LexA::p65-GMR-miniwhite	Bowen Deng et al., 2019	N/A	
software, algorithm	MATLAB	MathWorks, Natick, MA	https://www.mathworks.com/products/matlab.html	
software, algorithm	ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/	
software, algorithm	Prism 7	GraphPad	https://www.graphpad.com/	
software, algorithm	R 4.1.3	RStudio	https://www.r-project.org	

Fly stocks

Flies were reared on standard cornmeal-yeast medium under a 12 hr:12 hr dark:light cycle at 25°C and 60% humidity. All the knock-out lines in this study for screening have been published [49]. The following strains were obtained from Dr. Yi Rao: *isoCS* (wild-type), *ΔPtth, Ptth-Gal4, Ptth-LexA, elav-Gal4 and UAS-Kir2.1* (BL#6595). UAS-dTrpA1 was a gift from Dr. Paul Garrity. *UAS-GFPStinger*, *LexAop-tomato, LexAop2-FlpL, UAS > stop > mCD8-GFP, dsx-Gal4* and *dsx-LexA* (Mellert et al., 2010) have been described previously (Pfeiffer et al., 2008; Pfeiffer et al., 2010) and are obtained from Janelia Research Campus. *tub-Gal80^{ts}* (BL#7018) was provided by Dr. Yufeng Pan. *pC1-ss1-Gal4, pC1-ss2-Gal4, vpoDN-ss1-Gal4, vpoDN-ss2-Gal4* and *vpoDN-ss3-Gal4* were provided by Dr. Kaiyu Wang. *Torso-RNAi* (BL#33627) was a gift from Suning Liu. The following lines were obtained from the Bloomington Drosophila Stock Center: *UAS-EcR-RNAi* (BL#9327), *UAS-EcR-A-RNAi* (BL#9328), *UAS-EcR-B1-RNAi* (BL#9329), *UAS-mCD8::GFP* (BL#32194), *LexAop2-mCD8::GFP* (BL#32203), *UAS-mCD8::GFP* (BL#5137) and *pC1d-Gal4* (BL#86847). *UAS-PTTH-RNAi* (v102043) was from Vienna Drosophila Resource Center (VDRC).

Behavioral assays

Flies were reared at 25°C and 60% humidity under a 12 hr light:12 hr dark cycle. Virgin females and wild-type males were collected upon eclosion, placed in groups of 12 flies each and aged 4–6 days (except for the assays for PTTH mutant on different days after eclosion, and the molecular rescue assay for the 24h-old females) before carrying out behavioral assay except for the transient thermogenetic experiments. Female receptivity assays were conducted as previously described (Wang et al., 2022; Zhou et al., 2014). A virgin female of defined genotype and a wild type male were gently cold anesthetized and respectively introduced into two layers of the courtship chambers separated by a removable transparent film. The flies were allowed to recover for at least 45 min before the film was removed to allow the pair of flies to contact. The mating behavior was recorded using a camera (Canon VIXIA HF R500) for 30 min at 17 fps for further analysis.

For transient activation experiment by dTrpA1 in adult stage, flies were reared at 23°C. Flies were loaded into courtship chamber and recovered for at least 30 min at 23°C, then were placed at 23°C (control group) or 29°C (experimental group) for 30 min prior to removing the film and videotaping. For activation experiment by dTrpA1 during development, flies were reared at 29°C

during the specific stages compared with the controls who were reared at 23°C all the time. Flies were loaded into courtship chamber and recovered for at least 45 min at 23°C prior to removing the film and videotaping.

Quantification and statistical analysis of female receptivity behavior

Two parameters including copulation rate and latency to copulation were used to characterize receptivity and we got the data sets of two parameters from the same flies. The time from removing the film to successful copulation was measured for each female. The number of females that had engaged in copulation by the end of each 1 min interval within 30 min were summed and plotted as a percentage of successful copulation. The latency to copulation was the time from removing the film to successful copulation. All the time points that female successfully copulated were manually analyzed and the data of unhealthy flies were discarded.

Temporally restricted RNAi

tub-Gal80^{ts} crosses were reared at either 18°C for control groups or 30°C for experimental groups. Virgin females were collected at eclosion and were placed in groups of 12 flies each and aged 4–6 days before carrying out behavior assay. Assays were tested at 23°C.

Male courtship index

Courtship index was defined as the proportion of time the male followed, oriented toward and attempted to copulate the female within 5 min of courtship initiation, marked by the initial orientation toward and following the female.

Vaginal plate opening and ovipositor extrusion

A virgin female of defined genotype and a wild type male were aspirated into the courtship chambers and respectively introduced into two layers of the courtship chambers separated by a removable transparent film. The flies were allowed to recover for 30 min before the film was removed. To allow visualization of vaginal plate opening, we recorded uncompressed image sequences at 896 x 896 pixels and 50 frames per second using a Photron Mini AX camera (Photron) with an AF-S VR Micro-Nikkor 105mm lens (Nikon). Instances of vaginal plate opening and ovipositor extrusion were scored blind to genotype from frame by-frame playback during the first 5 min of courtship or until copulation if it occurred within 5 min. Courtship initiation was defined as the male orienting toward and beginning to follow the female. Rare trials with fewer than 30 s of total courtship were discarded.

Locomotion assays

The rearing and experimental conditions in locomotion assays were the same as that in the corresponding female receptivity assays, excepting that individual females were loaded in the chambers without males. Spontaneous movements of the flies were recorded with a camera (Canon VIXIA HF R500) for 30 min at 30 fps for further analysis. The activity of flies during the middle 10 min was analyzed to calculate the average walking speed using Ctrax software.

Egg Laying

Virgin females were collected upon eclosion and one fly was housed on standard medium at 25°C, 60% relative humidity, 12 hr light:12 hr dark and allowed to lay eggs in single vials. Each fly was transferred into new food tube every 24 hr. The number of eggs was counted at the end of each 24 hr period. The numbers during the 3rd and 4th day were summed for statistics and plot.

Immunohistochemistry

Whole brains of flies were dissected in 1x PBS and fixed in 2% paraformaldehyde diluted in 1x PBS for 55 min at room temperature. The samples were washed with PBT (1x PBS containing 0.3% Triton X-100) for 1 hour (3 x 20 min), followed by blocking in 5% normal goat serum (Blocking solution, diluted in 0.3% PBT) for 1 hour at room temperature. Then, the samples were incubated in primary antibodies (diluted in blocking solution) for 18–24 hours at 4°C. Samples were washed with 0.3% PBT for 1 hour (3 x 20 min), then were incubated in secondary antibodies (diluted in blocking solution) for 18–24 hours at 4°C. Samples were washed with 0.3% PBT for 1 hour (3 x 20 min), then were fixed in 4% paraformaldehyde for 4 hours at room temperature. After washed with 0.3% PBT for 1 hour (3 x 20 min), brains were mounted on poly-L-lysine-coated coverslip in 1x PBS. The coverslip was dipped for 5 min with ethanol of 30%→50%→70%→95%→100% sequentially at room temperature, and then dipped for 5 min three times with xylene. Finally, brains were mounted with DPX and allowed DPX to dry for 2 days before imaging. Primary antibodies used were: chicken anti-GFP (1:1000; Life Technologies #A10262), rabbit anti-GFP (1:1000; Life Technologies #A11122), rabbit anti-RFP (1:1000; Life Technologies #R10367), rabbit anti-PTTH antibody (1:1300), mouse anti-nc82 (1:40; DSHB), mouse anti-EcR-A (1:10; AB_528214) and mouse anti-EcR-B1 (1:10; AB_2154902). Secondary antibodies used were: Alexa Fluor goat anti-chicken 488 (1:500; Life Technologies #A11039), Alexa Fluor goat anti-rabbit 488 (1:500; Life Technologies #A11034), Alexa Fluor goat anti-rabbit 546 (1:500; Life Technologies #A11010), Alexa Fluor goat anti-mouse 647 (1:500; Life Technologies #A21235), and Alexa Fluor goat anti-mouse 488 (1:500; Life Technologies #A11029).

Confocal microscopy and image analysis

Confocal imaging was performed under an LSM 710 inverted confocal microscope (ZEISS, Germany), with a Plan-Apochromat 20×/0.8 M27 objective or an EC Plan-Neofluar 40x/1.30 oil DIC M27 objective, and later analyzed using Fiji software.

Generation of anti-PTTH antibody

The antisera used to recognize PTTH peptide were raised in New Zealand white rabbits using the synthetic peptide N'-TSQSDHPYSWMNKDQPWQFKC -C'. The synthesis of antigen peptide, the production and purification of antiserum were performed by Beijing Genomics Institute (BGI).

Generation of UAS-PTTH

pJFRC28-5XUAS-IVS-GFP-p10 (#12073; Fungene Biotechnology, Shanghai, China) was used for the generation of the pJFRC28-UAS-PTTH construct. The pJFRC28-10XUAS-IVS-GFP-p10 plasmid was digested with NotI and XbaI to remove the GFP coding sequence, and then the cDNA of PTTH was cloned into this plasmid by Gibson Assembly. The Kozak sequence was added right upstream of the ATG. UAS-PTTH constructs were injected and integrated into the attP2 site on the third chromosome through phiC31 integrase mediated transgenesis. The construct was confirmed using DNA sequencing through PCR. The primers used for cloning PTTH cDNA were as follows:

UAS-PTTH-F ATTCTTATCCTTTACTTCAGGCGGCCGCAAAATGGATATAAAAGTATGGCGACTCC UAS-PTTH-R GTTATTTTAAAAACGATTCATTCTAGATCACTTTGTGCAGAAGCAGCCG

Genomic DNA extraction and RT-PCR

Genomic DNA was extracted from 10 whole bodies of wandering flies using MightyPrep reagent for DNA (Takara #9182). Whole body RNA was extracted from 10 whole bodies of wandering flies using TRIzol (Ambion #15596018). cDNA was generated from total RNA using the Prime Script

reagent kit (Takara #RR047A). Candidates of *ΔPtth* were characterized by the loss of DNA band in the deleted areas through PCR on the genomic DNA and cDNA. Primer sequences used in **Figure 1** are listed in Table S1.

Measurements of pupariation timing and adult mass

The flies were reared at 25°C and 60% humidity under a 12 hr light:12 hr dark cycle. Two-hour time collections of embryos laid on standard food vials. Each vial contained 20-30 eggs. The range of time for pupariation were recorded for each vial. Sexed adults of 24h-old were weighted in groups of ten flies using a NENVER-TP-214 microbalance at the same time.

Identification of sex in *Drosophila* larvae

Third instar larvae can be sexed (True et al., 2014). Gonads are translucent and visible in side view in the posterior third of the larva. The male gonads are about five times bigger than the female gonads. The identified wandering female and male larvae were reared in different vials for the subsequent experiments.

Rescue by 20-hydroxyecdysone feeding

Thirty freshly ecdysed *ΔPtth* L3 larvae, grown at 25°C and 60% humidity under a 12 hr light:12 hr dark cycle, were washed with water and transferred to normal food for additional aging. After 20 h, larvae were washed and transferred to a vial supplemented with either 20-hydroxyecdysone (20E, Cayman #16145, dissolved in 95% ethanol, final concentration 0.2 mg/ml) or 95% ethanol (same volume as 20E). The wild type larvae were directly transferred to vials supplemented with 20E or 95% ethanol upon L3 ecdysis. Once seeded with L3 larvae, the vials were returned to 25°C and 60% humidity under a 12 hr light:12 hr dark cycle.

Quantification of fluorescence intensity

The fluorescence intensity was quantified using Fiji software. The areas of interest (ROI) were marked in the slices including the interested regions and quantified using the “plot z-axis profile” function. The fluorescence intensity in each slice was summed for statistics and plot. The parameters used for confocal imaging of each brain were the same.

Calcium imaging

Flies aged 4-6-days were immobilized on ice for ~30 s. The brain was then dissected out in extracellular solution (ECS) that contains (in millimoles): 108 NaCl, 5 KC1, 5 trehalose, 5 sucrose, 5 HEPES, 26 $NaHCO_3$, 1 NaH_2PO_4, 2 $CaCl_2$, and 1.5 $MgCl_2$ [pH 7.1 – 7.3 when bubbled with 95% (vol/vol) O_2/5% (vol/vol) CO_2, ~290 mOsm] and mounted on a poly-D-lysine coated coverslip. The samples were continuously perfused with ECS.

Calcium imaging was performed at 21°C on a customized two-photon microscope equipped with a resonant scanner (Nikon), a piezo objective scanner (Nikon) and a 40x water-immersion objective (Nikon). GCaMP6s was excited at 920 nm.

Analysis of calcium imaging data was done offline with NIS-Elements AR 5.30.01. Briefly, the square region of interest (ROIs), 25 pixels on the pC1 neurons in the center of lateral junction, was chosen for measurements. For each frame, the average fluorescence intensity of pixels within ROIs was calculated blind to genotype. The average fluorescence intensity of ROIs in each frame covering pC1 neurons were summed for statistics and plot.

qRT-PCR

Total RNA was extracted from about 10 flies using TRIzol (Ambion #15596018). The cDNA was synthesized using Prime Script reagent kit (Takara #RR047A). Quantitative PCR was performed on Thermo Piko Real 96 (Thermo) using SYBR Green PCR Master Mix (Takara #RR820A). The mRNA expression level was calculated by the $2^{-\Delta\Delta Ct}$ method and the results were plotted by using tubulin as the reference gene. Primers are listed in Table S1. All reactions were performed in triplicate. The average of four biological replicates ± SEM was plotted.

Statistical analysis

Statistical analyses were carried out using R software version 3.4.3 or Prism7 (GraphPad software). For the copulation rate, chi-square test is applied. The Mann-Whitney U test was applied for analyzing the significance of two columns. Kruskal-Wallis ANOVA test followed by post-hoc Mann-Whitney U test, was used to identify significant differences between multiple groups.

Data, Materials, and Software Availability

All study data are included in the main text and supporting information, except for the sequence data of RNAseq (Supplementary File 3) has been deposited in the Genome Sequence Archive under the accession number CRA012130 in PRJCA018750. This study does not involve new code. Fly stocks and reagents used in this study are available from the corresponding author upon reasonable request.

Author Contributions

Jing Li, Conceptualization, Resources, Data curation, Writing -original draft, review and editing, Funding acquisition; Chao Ning and Yaohua Liu, Data curation; Bowen Deng, Resources; Bingcai Wang, Kai Shi and Rencong Wang, Software; Ruixin Fang, Data curation; Chuan Zhou, Conceptualization, Funding acquisition.

Competing Interest Statement

The authors declare no competing interest.

Impact statement

Prothoracicotropic hormone and ecdysone belonging to the insect PG axis modulate virgin female sexual receptivity.

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Editors

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Reviewer #1 (Public Review):

Summary

This article delves into the role of Ecdysone in regulating female sexual receptivity in Drosophila. The researchers discovered that PTTH, a positive regulator of Ecdysone production, hurts the receptivity of adult virgin females. Specifically, the researchers found that losing larval PTTH before metamorphosis significantly increases female receptivity immediately after adult eclosion. In addition, Ecdysone, through its receptor EcR-A, is necessary during metamorphic neurodevelopment for the proper development of P1 neurons, as its silencing leads to morphological changes associated with reduced adult female receptivity. Furthermore, Torso enhances receptivity in the adult stage. The molecular mechanisms linking each molecule to female receptivity have yet to be fully understood; therefore, the involvement of the juvenile-to-adult hormonal pathway (PTTH/Torso/ecdysone) in female receptivity is not proven.

Strengths

(1) Robust Methodology and Experimental Design: The study employs a comprehensive and well-structured experimental approach, combining genetic manipulations, behavioral assays, and molecular analyses. This multi-faceted methodology allows for a thorough investigation of the role of PTTH and Ecdysone in regulating female sexual receptivity in Drosophila. The use of specific gene knockouts, RNA interference, and overexpression techniques provides strong evidence supporting the findings.
(2) Clear and Substantial Findings: The authors provide compelling data showing that PTTH negatively regulates female receptivity during the larval stage, which is rescued by Ecdysone feeding. Instead, metamorphic Ecdysone has a positive role during neurodevelopment. The experiments demonstrate this dual and temporally distinct role of PTTH/Ecdysone, shedding light on a complex hormonal regulation mechanism.
(3) Clarification of Experimental Details: In response to the initial review, the authors have clarified important experimental details, such as the precise timing of genetic manipulations and the specific developmental stages examined. This clarification enhances the reproducibility and understanding of the study.

Weaknesses

(1) Unresolved Contradictions and Complexity in Results: Despite the detailed responses, the paper still presents complex and somewhat contradictory findings regarding the roles of PTTH, Torso, and Ecdysone. The observed increase in EcR-A expression in PTTH mutants and the nuanced explanation regarding the feedforward relationship, while insightful, do not fully resolve the initial confusion about the differing effects of PTTH and Ecdysone manipulations on female receptivity. This required more exploration.
(2) Insufficient Exploration of Mechanistic Pathways: The potential mechanisms underlying the role of PTTH/Torso-Ecdysone across different developmental stages remain underexplored. While the authors suggest a feedforward relationship and possible interaction with other neurons, these hypotheses are not thoroughly tested or elaborated upon, leaving gaps in the mechanistic understanding.
(3) Limited Scope of Validation Experiments: While the authors addressed some reviewer concerns about validation, the scope remains somewhat limited. The lack of existing PTTH mutants and the challenges in manipulating PTTH expression without affecting receptivity suggests that further work is needed to validate these pathways robustly. The inability to fully replicate the PTTHdelete phenotype through other means leaves some questions unanswered.
(4). Complexity in Interpretation of dsx-Positive Neurons: The relevance of dsx-positive neurons in the context of PTTH's effects on female receptivity remains ambiguous. Although

the authors provide some context, the biological significance of these observations is not fully clarified.

Conclusion
The manuscript presents a well-conceived study with significant findings that advance the understanding of hormonal regulation of female receptivity in Drosophila. However, complexities in the data and unresolved mechanistic questions suggest that further work is needed to clarify the exact pathways and interactions involved. The authors' responses to feedback have strengthened the paper, but additional experiments and more thorough mechanistic exploration would enhance the robustness and clarity of the conclusions.

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Reviewer #2 (Public Review):

Summary:

The authors tried to identify novel adult functions of the classical Drosophila juvenile-adult transition axis (i.e. ptth-ecdysone). Surprisingly, larval ptth-expressing neurons expressed the sex-specific doublesex gene, thus belonging to the sexual dimorphic circuit. Lack of ptth during late larval development caused enhanced female sexual receptivity, effect rescued by supplying ecdysone in the food. Among many other cellular players, pC1 neurons control receptivity by encoding the mating status of females. Interestingly, during metamorphosis a subtype of pC1 neurons required Ecdysone Receptor A in order to regulate such female receptivity. A transcriptomic analysis using pC1-specific Ecdyone signaling down-regulation gives some hints of possible downstream mechanisms.

Strengths:

The manuscript showed solid genetic evidence that lack of ptth during development caused enhanced copulation rate in female flies, which includes ptth mutant rescue experiments by over-expressing ptth as well as by adding ecdysone-supplemented food. They also present elegant data dissecting the temporal requirements of ptth-expressing neurons by shifting animals from non-permissive to permissive temperatures, in order to inactivate neuronal function (although not exclusively ptth function). They showed that EcR-A is up-regulated in ptth mutant background. By combining different drivers together with EcR-A RNAi and torso RNAi lines authors also identified the Ecdysone receptor and torso requirements of a particular subtype of pC1 neurons during metamorphosis. Convincing live calcium imaging showed no apparent effect of EcR-A in neural activity, although some effect on morphology is uncovered. Finally, bulk RNAseq shows differential gene expression after EcR-A down-regulation.

Weaknesses:

The paper has three main weaknesses. The first one refers to temporal requirements of ptth and ecdysone signaling. Whereas ptth is necessary during larval development, ecdysone effect appears during pupal development. ptth induces ecdysone synthesis during larval development but there is no published evidence about a similar role for ptth during pupal stages. The down-regulation of EcR-A by RNAi requires at least 8 h to be complete, whereas the activation of ptth neurons in larva stages is immediate. Furthermore, larval and pupal ecdysone functions are different (triggering metamorphosis vs tissue remodeling). The second caveat is the fact that ptth and ecdysone/torso loss-of-function experiments render opposite effects (enhancing and decreasing copulation rates, respectively). The most plausible explanation is that both functions are independent of each other, also suggested by differential temporal requirements. Finally, in order to identify the effect in the

transcriptional response of down-regulating EcR-A in a very small population of neurons, a scRNAseq study should have been performed instead of bulk RNAseq.

In summary, despite the authors providing convincing evidence that ptth and ecdysone signaling pathways are involved in female receptivity, the main claim that ptth regulates this process through ecdysone is not supported by results. More likely, they'd rather be independent processes.

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Reviewer #3 (Public Review):

Summary:

This manuscript shows that mutations that disable the gene encoding the PTTH gene cause an increase in female receptivity (they mate more quickly), a phenotype that can be reversed by feeding these mutants the molting hormone, 20-hydoxyecdysone (20E). The use of an inducible system reveals that inhibition or activation of PTTH neurons during the larval stages increases and decreases female receptivity, respectively, suggesting that PTTH is required during the larval stages to affect the receptivity of the (adult) female fly. Showing that these neurons express the sex-determining gene dsx leads the authors to show that interfering with 20E actions in pC1 neurons, which are dsx-positive neurons known to regulate female receptivity, reduces female receptivity and increases the arborization pattern of pC1 neurons. The work concludes by showing that targeted knockdown of EcRA in pC1 neurons causes 527 genes to be differentially expressed in the brains of female flies, of which 123 passed a false discovery rate cutoff of 0.01; interestingly, the gene showing the greatest down-regulation was the gene encoding dopamine beta-monooxygenase.

This reviewer appreciates the effort that was done to revise the manuscript and address the various comments made by the reviewers. Nevertheless, I feel that the main concerns remain. These are not necessarily due to an unwillingness on the part of the authors to address them, but rather to difficulties that are inherent to trying to assign specific roles to EcR and pC1 neurons at a time when major changes are occurring (or are about to occur) in the nervous system, and do so using tools that are currently not sharp or specific enough. Many of the conclusions are supported by the results and those that may have alternative interpretations can remain more speculative until better tools become available. It is, nevertheless, an interesting and provocative piece of work.

Strengths

This is an interesting piece of work, which may shed light on the basis for the observation noted previously that flies lacking PTTH neurons show reproductive defects ("... females show reduced fecundity"; McBrayer, 2007; DOI 10.1016/j.devcel.2007.11.003).

Weaknesses:

There are some results whose interpretation seem ambiguous and findings whose causal relationship is implied but not demonstrated.

(1) At some level, the findings reported here are not at all surprising. Since 20E regulates the profound changes that occur in the central nervous system (CNS) during metamorphosis, it is not surprising that PTTH would play a role in this process. Although animals lacking PTTH (rather paradoxically) live to adulthood, they do show greatly extended larval instars and a corresponding great delay in the 20E rise that signals the start of metamorphosis. For this reason, concluding that PTTH plays a SPECIFIC role in regulating female receptivity seems a little misleading, since the metamorphic remodeling of the entire CNS is likely altered in

PTTH mutants. Since these mutants produce overall normal (albeit larger--due to their prolonged larval stages) adults, these alterations are likely to be subtle. Courtship has been reported as one defect expressed by animals lacking PTTH neurons, but this behavior may stand out because reduced fertility and increased male-male courtship (McBrayer, 2007) would be noticeable defects to researchers handling these flies. By contrast, detecting defects in other behaviors (e.g., optomotor responses, learning and memory, sleep, etc) would require closer examination. For this reason I would ask the authors to temper their statement that PTTH is SPECIFICALLY involved in regulating female receptivity.
(2) The link between PTTH and the role of pC1 neurons in regulating female receptivity is not clear. Again, since 20E controls the metamorphic changes that occur in the CNS, it is not surprising that 20E would regulate the arborization of pC1 neurons. And since these neurons have been implicated in female receptivity, it would therefore be expected that altering 20E signaling in pC1 neurons would affect this phenotype. However, this does not mean that the defects in female receptivity expressed by PTTH mutants are due to defects in pC1 arborization. For this the authors would at least have to show that PTTH mutants show the changes in pC1 arborization shown in Fig. 6. And even then the most that could be said is that the changes observed in these neurons "may contribute" to the observed behavioral changes. Indeed, the changes observed in female receptivity may be caused by PTTH/20E actions on different neurons.
(3) Some of the results need commenting on, or refining, or revising:
(a) For some assays PTTH behaves sometimes like a recessive gene and at other times like a semi-dominant, and yet at others like a dominant gene. For instance, in Fig. 1D-G, PTTH[-]/+ flies behave like wildtype (D), express an intermediate phenotype (E-F), or behave like the mutant (G). This may all be correct but merits some comment.
(b) Some of the conclusions are overstated. i) Although Fig. 2E-G does show that silencing the PTTH neurons during the larval stages affects copulation rate (E) the strength of the conclusion is tempered by the behavior of one of the controls (tub-GAL80[ts]/+, UAS-Kir2.1/+) in panels F and G, where it behaves essentially the same as the experimental group (and quite differently from the PTTH-GAL4/+ control; blue line).(Incidentally, the corresponding copulation latency should also be shown for these data.). ii) For Fig. 5I-K, the conclusion stated is that "Knock-down of EcR-A during pupal stage significantly decreased the copulation rate." Although strictly correct, the problem is that panel J is the only one for which the behavior of the control lacking the RNAi is not the same as that of the experimental group. Thus, it could just be that when the experiment was done at the pupal stage is the only situation when the controls were both different from the experimental. Again, the results shown in J are strictly speaking correct but the statement is too definitive given the behavior of one of the controls in panels I and K. Note also that panel F shows that the UAS-RNAi control causes a massive decrease in female fertility, yet no mention is made of this fact.

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Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *Summary: This article explores the role of Ecdysone in regulating female sexual receptivity in Drosophila. The researchers found that PTTH, throughout its role as a positive regulator of ecdysone production, negatively affects the receptivity of adult virgin females. Indeed, loss of larval PTTH before metamorphosis significantly increases female receptivity right after adult eclosion and also later. However, during metamorphic neurodevelopment, Ecdysone, primarily through its receptor EcR-A, is required to properly develop the P1 neurons since its silencing led to morphological changes*

> *associated with a reduction in adult female receptivity. Nonetheless, the result shown in this manuscript sheds light on how Ecdysone plays a dual role in female adult receptivity, inhibiting it during larval development and enhancing it during metamorphic development. Unfortunately, this dual and opposite effect in two temporally different developmental stages has not been highlighted or explained.*
>
> *Strengths: This paper exhibits multiple strengths in its approach, employing a well-structured experimental methodology that combines genetic manipulations, behavioral assays, and molecular analysis to explore the impact of Ecdysone on regulating virgin female receptivity in Drosophila. The study provides clear and substantial findings, highlighting that removing PTTH, a positive Ecdysone regulator, increases virgin female receptivity. Additionally, the research expands into the temporal necessity of PTTH and Ecdysone function during development.*
>
> *Weaknesses:*
>
> *There are two important caveats with the data that are reflecting a weakness:*
>
> *(1) Contradictory Effects of Ecdysone and PTTH: One notable weakness in the data is the contrasting effects observed between Ecdysone and its positive regulator PTTH. PTTH loss of function increases female receptivity, while ecdysone loss of function reduces it. Given that PTTH positively regulates Ecdysone, one would expect that the loss of function of both would result in a similar phenotype or at least a consistent directional change.*

A1. As newly formed prepupae, the ptth-Gal4>UAS-Grim flies display similar changes in gene expression to the genetic control flies to response to a high-titer ecdysone pulse. These include the repression of EcR (McBrayer et al.,2007). We tested whether there is a similar feedforward relationship between PTTH and EcR-A. We quantified the EcR-A mRNA level of PTTH -/- and PTTH -/+ in the whole body of newly formed prepupae. Indeed, PTTH -/- induced increased EcR-A expression in the whole body of newly formed prepupae compared with PTTH -/+ flies. Because of the function of EcR-A in gene expression, this suggests that PTTH -/- disturbs the regulation of a serious of gene expressions during metamorphosis. However, it is not sure that the EcR-A expression in pC1 neurons is increased compared with genetic controls when PTTH is deleted. Furthermore, PTTH -/- must affect development of other neurons rather than only pC1 neurons. So, the feedforward relationship between PTTH and EcRA at the start of prepupal stage is one possible cause for the contradictory effects of PTTH -/- and EcR-A RNAi in pC1 neurons.

> *(2) Discordant Temporal Requirements for Ecdysone and PTTH: Another weakness lies in the different temporal requirements for Ecdysone and PTTH. The data from the manuscript suggest that PTTH is necessary during the larval stage, as shown in Figure 2 E-G, while Ecdysone is required during the pupal stage, as indicated in Figure 5 I-K. Ecdysone is a crucial developmental hormone with precisely regulated expression throughout development, exhibiting several peaks during both larval and pupal stages. PTTH is known to regulate Ecdysone during the larval stage, specifically by stimulating the kinetics of Ecdysone peaking at the wandering stage. However, it remains unclear whether pupal PTTH, expressed at higher levels during metamorphosis, can stimulate Ecdysone production during the pupal stage. Additionally, given the transient nature of the Ecdysone peak produced at wandering time, which disappears shortly before the end of the prepupal stage, it is challenging to infer that larval PTTH will regulate Ecdysone production during the pupal stage based on the current state of knowledge in the neuroendocrine field.*
>
> *Considering these two caveats, the results suggest that the authors are witnessing distinct temporal and directional effects of Ecdysone on virgin female receptivity.*

A2. First of all, it is necessary to clarify the detailed time for the manipulation of *Ptth* gene and PTTH neurons. In Figure 3, activation of PTTH neurons during the stage 2 inhibited the female receptivity. The “stage 2” is from six hours before the 3rd-instar larvae to the end of the wandering larvae (the start of prepupae). In Figure 5, The “pupal stage” is from the prepupal stage to the end of pupal stage. This “pupal stage” includes the forming of prepupae when the ecdysone peak is not disappeared. The time of manipulating *Ptth* and EcR-A in pC1 neurons are continuous. In addition, the pC1-Gal4 expressing neurons appear also at the start of prepupal stage. So, it is possible that PTTH regulates female receptivity through the function of EcR-A in pC1 neurons.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *In light of the significant caveat previously discussed, I will just make a few general suggestions:*
>
> *(1) The paper primarily focuses on robust phenotypes, particularly in PTTH mutants, with a well-detailed execution of several experiments, resulting in thorough and robust outcomes. However, due to the caveat previously presented (opposite effect in larva and pupa), consider splitting the paper into two parts: Figures 1 to 4 deal with the negative effect of PTTH-Ecdysone on early virgin female receptivity, while Figures 5 to 7 focus on the positive metamorphic effect of Ecdysone in P1 metamorphic neurodevelopment. However, in this scenario, the mechanism by which PTTH loss of function increases female receptivity should be addressed.*

A3. It is a good suggestion that splitting the paper into two parts associated with the PTTH function and EcR function in pC1 neurons separately, if it is impossible that PTTH functions in female receptivity through the function of EcR-A in pC1 neurons. However, because of the feedforward relationship between PTTH and EcR-A in the newly formed prepupae, and the time of manipulating *Ptth* and EcR-A in pC1 neurons is continuous, it is possible that these two functions are not independent of each other. So, we still keep the initial edition.

> *(2) Validate the PTTH mutants by examining homozygous mutant phenotypes and the dose-dependent heterozygous mutant phenotype using existing PTTH mutants. This could also be achieved using RNAi techniques.*

A4. We did not get other existing PTTH mutants. We instead decreased the PTTH expression in PTTH neurons and dsx+ neurons, but did not detect the similar phenotype to that of PTTH -/-. Similarly, the overexpression through PTTH-Gal4>UAS-PTTH is also not sufficient to change female receptivity. It is possible that both decreasing and increasing PTTH expression are not sufficient to change female receptivity.

> *(3) Clarify if elav-Gal4 is not expressed in PTTH neurons and discuss how the rescue mechanisms work (hormonal, paracrine, etc.) in the text.*

A5. We tested the overlap of elav-Gal4>GFP signal and the stained PTTH with PTTH antibody. We did not detect the overlap. It suggests that elav-Gal4 is not expressed in PTTH neurons. However, we detected the expression of PTTH (PTTH antibody) in CNS when overexpressed PTTH using elav-Gal4>UASPTTH based on PTTH -/-. Furthermore, this rescued the phenotype of PTTH -/- in female receptivity. Insect PTTH isoforms have similar probable signal peptide for secreting. Indeed, except for the projection of axons to PG gland, PTTH also carries endocrine function acting on its receptor Torso in light sensors to regulate light avoidance of larvae. The overexpressed PTTH in other neurons through elav-Gal4>UASPTTH may act on the PG gland through endocrine function and then induce the ecdysone synthesis and release. So that, although elav-Gal4 is not expressed in PTTH neurons, the ecdysone synthesis

triggered by PTTH from the hemolymph may result in the rescued PTTH -/- phenotype in female receptivity.

> *(4) Consider renaming the new PTTH mutant to avoid confusion with the existing PTTHDelta allele.*

A6. We have renamed our new PTTH mutant as *PtthDelete*.

> *(5) Include the age of virgin females in each figure legend, especially for Figures 2 to 7, to aid in interpretation. This is essential information since wild-type early virgins -day 1- show no receptivity. In contrast, they reach a typical 80% receptivity later, and the mechanism regulating the first face might differ from the one occurring later.*

A7. We have included the age of virgin females in each figure legend.

> *(6) Explain the relevance of observing that PTTH adult neurons are dsx-positive, as it's unclear why this observation is significant, considering that these neurons are not responsible for the observed receptivity effect in virgin females. Alternatively, address this in the context of the third instar larva or clarify its relevance.*

A8. We decreased the DsxF expression in PTTH neurons and did not detect significantly changed female receptivity. Almost all neurons regulating female receptivity, including pC1 neurons, express DsxF. We suppose that PTTH neurons have some relationship with other DsxF-positive neurons which regulate female receptivity. Indeed, we detected the overlap of dsx-LexA>LexAop-RFP and torso-Gal4>UAS-GFP during larval stage. Furthermore, decreasing Torso expression in pC1 neurons significantly inhibit female receptivity.

These results suggest that, PTTH regulates female receptivity not only through ecdysone, but also may through regulating other neurons especially DsxF-positive neurons associated with female receptivity directly.

> ***Reviewer #2 (Public Review):***
>
> *Summary: The authors tried to identify novel adult functions of the classical Drosophila juvenile-adult transition axis (i.e. ptth-ecdysone). Surprisingly, larval ptth-expressing neurons expressed the sex-specific doublesex gene, thus belonging to the sexual dimorphic circuit. Lack of ptth during late larval development caused enhanced female sexual receptivity, an effect rescued by supplying ecdysone in the food. Among many other cellular players, pC1 neurons control receptivity by encoding the mating status of females. Interestingly, during metamorphosis, a subtype of pC1 neurons required Ecdysone Receptor A in order to regulate such female receptivity. A transcriptomic analysis using pC1-specific Ecdyone signaling down-regulation gives some hints of possible downstream mechanisms.*
>
> *Strengths: the manuscript showed solid genetic evidence that lack of ptth during development caused enhanced copulation rate in female flies, which includes ptth mutant rescue experiments by overexpressing ptth as well as by adding ecdysone-supplemented food. They also present elegant data dissecting the temporal requirements of ptth-expressing neurons by shifting animals from non-permissive to permissive temperatures, in order to inactivate neuronal function (although not exclusively ptth function). By combining different drivers together with a EcR-A RNAi line authors also identified the Ecdysone receptor requirements of a particular subtype of pC1 neurons during metamorphosis. Convincing live calcium imaging showed no apparent effect of EcR-A in neural activity, although some effect on morphology is uncovered. Finally, bulk RNAseq shows differential gene expression after EcR-A down-regulation.*

> *Weaknesses: the paper has three main weaknesses. The first one refers to temporal requirements of ptth and ecdysone signaling. Whereas ptth is necessary during larval development, the ecdysone effect appears during pupal development. ptth induces ecdysone synthesis during larval development but there is no published evidence about a similar role for ptth during pupal stages. Furthermore, larval and pupal ecdysone functions are different (triggering metamorphosis vs tissue remodeling). The second caveat is the fact that ptth and ecdysone loss-of-function experiments render opposite effects (enhancing and decreasing copulation rates, respectively). The most plausible explanation is that both functions are independent of each other, also suggested by differential temporal requirements. Finally, in order to identify the effect in the transcriptional response of down-regulating EcR-A in a very small population of neurons, a scRNAseq study should have been performed instead of bulk RNAseq.*
>
> *In summary, despite the authors providing convincing evidence that ptth and ecdysone signaling pathways are involved in female receptivity, the main claim that ptth regulates this process through ecdysone is not supported by results. More likely, they'd rather be independent processes.*

B1. Clarification: in Figure 3, activation of PTTH neurons during the stage 2 inhibited the female receptivity. The “stage 2” is from six hours before the 3rd-instar larvae to the end of the wandering larvae (the start of prepupae). In Figure 5, The “pupal stage” is from the start of prepupal stage to the end of pupal stage. This “pupal stage” includes the forming of prepupae when the ecdysone peak is not disappeared. The time of manipulating *Ptth* and EcR-A in pC1 neurons are continuous. In addition, the pC1-Gal4 expressing neurons appear also at the start of prepupal stage. So, it is possible that PTTH regulates female receptivity through the function of EcR-A in pC1 neurons.

B2. During the forming of prepupae, the ptth-Gal4>UAS-Grim flies display similar changes in gene expression to the genetic control flies to response to a high-titer ecdysone pulse. These include the repression of EcR (McBrayer et al.,2007). We tested whether there is a similar feedforward relationship between PTTH and EcR-A. We quantified the EcR-A mRNA level of PTTH -/- and PTTH -/+ in the whole body of newly formed prepupae. Indeed, PTTH -/- induced increased EcR-A compared with PTTH -/+ flies. Because of the function of EcR-A in gene expression, this suggests that PTTH -/- disturbs the regulation of a serious of gene expressions during metamorphosis. However, it is not sure that the EcR-A expression in pC1 neurons is increased compared with genetic controls when PTTH is deleted. Furthermore, PTTH -/- must affect the development of other neurons rather than only pC1 neurons. So, the feedforward relationship between PTTH and EcR-A at the start of prepupal stage is one possible cause for the contradictory effects of PTTH -/- and EcR-A RNAi in pC1 neurons.

B3. We will do single cell sequencing in pC1 neurons for the exploration of detailed molecular mechanism of female receptivity in the future.

> *Reviewer #2 (Recommendations For The Authors):*
>
> *Additional experiments and suggestions:*
>
> *- torso LOF in the PG to determine whether or not the ecdysone peak regulated by ptth (there is a 1-day delay in pupation) is responsible for the ptth effect in L3. In the same line, what happens if torso is downregulated in the pC1 neurons? Is there any effect on copulation rates?*

B4. Because the loss of phm-Gal4, we could not test female receptivity when decreasing the expression of Torso in PG gland. However, decreasing Torso expression in pC1 neurons significantly inhibit female receptivity. This suggests that PTTH regulates female receptivity

not only through ecdysone but also through regulating dsx+ pC1 neurons in female receptivity directly.

> *- What is the effect of down-regulating ptth in the dsx+ neurons? No ptth RNAi experiments are shown in the paper.*

B5. We decreased PTTH expression in dsx+ neurons but did not detect the change in female receptivity. We also decreased PTTH expression in PTTH neurons using PTTH-Gal4, also did not detect the change in female receptivity. Similarly, the overexpression through PTTH-Gal4>UAS-PTTH is also not sufficient to change female receptivity. It is possible that both decreasing and increasing PTTH expression are not sufficient to change female receptivity.

> *- Why are most copulation rate experiments performed between 4-6 days after eclosion? ptth LOF effect only lasts until day 3 after eclosion (but very weak-fig 1). Again, this supports the idea that ptth and ecdysone effects are unrelated.*

B6. Most behavioral experiments were performed between 4-6 days after eclosion as most other studies in flies, because the female receptivity reaches the peak at that time. Ptth LOF made female receptivity enhanced from the first day after eclosion. This seems like the precocious puberty. Wild type females reach high receptivity at 2 days after eclosion (about 75% within 10 min). We suppose that Ptth LOF effect only lasts until day 3 after eclosion because too high level of receptivity of control flies to exceed.

It is not sure whether the effect of PTTH-/- in female receptivity disappears after the 3rd day of adult flies. So that it is not sure whether PTTH and EcR-A effects in pC1 neurons are unrelated.

> *- The fact that pC1d neuronal morphology changes (and not pC1b) does not explain the effect of EcR-A LOF. Despite it is highlighted in the discussion, data do not support the hypothesis. How do these pC1 neurons look like in a ptth mutant animal regarding Calcium imaging and/or morphology?*

B7. We detected the pattern of pC1 neurons when PTTH is deleted. Consistent with the feedforward relationship between PTTH and expression of EcR-A in newly formed prepupae, PTTH deletion induced less established pC1-d neurons contrary to that induced by EcR-A reduction in pC1 neurons. However, it is not sure that the expression of EcR-A in pC1 neurons is increased when PTTH is deleted. Furthermore, on the one hand, manipulation of PTTH has general effect on the neurodevelopment not only regulating pC1 neurons. On the other hand, the detailed pattern of pC1-b neurons which is the key subtype regulating female receptivity when EcR-A is decreased in pC1 neurons or PTTH is deleted could not be seen clearly. So, the abnormal development of pC1-b neurons, if this is true, is just one of the possible reasons for the effect of PTTH deletion on female receptivity.

> *- The discussion is incomplete, especially the link between ptth and ecdysone; discuss why the phenotype is the opposite (ptth as a negative regulator of ecdysone in the pupa, for instance); the difference in size due to ptth LOF might be related to differential copulation rates.*

B8. We have revised the discussion. We could not exclude the effect of size of body on female receptivity when PTTH was deleted or PTTH neurons were manipulated, although there was not enough evidence for the effect of body size on female receptivity.

> *- scheme of pC neurons may help.*

B9. We have tried to label pC1 neurons with GFP and sort pC1 neurons through flow cytometry sorting, but could not success. This may because the number of pC1 neurons is too low in one brain. We will try single-cell sequencing in the future.

> *- Immunofluorescence images are too small.*

B10. We have resized the small images.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *This manuscript shows that mutations that disable the gene encoding the PTTH gene cause an increase in female receptivity (they mate more quickly), a phenotype that can be reversed by feeding these mutants the molting hormone, 20-hydoxyecdysone (20E). The use of an inducible system reveals that inhibition or activation of PTTH neurons during the larval stages increases and decreases female receptivity, respectively, suggesting that PTTH is required during the larval stages to affect the receptivity of the (adult) female fly. Showing that these neurons express the sex-determining gene dsx leads the authors to show that interfering with 20E actions in pC1 neurons, which are dsx-positive neurons known to regulate female receptivity, reduces female receptivity and increases the arborization pattern of pC1 neurons. The work concludes by showing that targeted knockdown of EcRA in pC1 neurons causes 527 genes to be differentially expressed in the brains of female flies, of which 123 passed a false discovery rate cutoff of 0.01; interestingly, the gene showing the greatest down-regulation was the gene encoding dopamine beta-monooxygenase.*
>
> *Strengths*
>
> *This is an interesting piece of work, which may shed light on the basis for the observation noted previously that flies lacking PTTH neurons show reproductive defects ("... females show reduced fecundity"; McBrayer, 2007; DOI 10.1016/j.devcel.2007.11.003).*
>
> *Weaknesses:*
>
> *There are some results whose interpretation seem ambiguous and findings whose causal relationship is implied but not demonstrated.*
>
> *(1) At some level, the findings reported here are not at all surprising. Since 20E regulates the profound changes that occur in the central nervous system (CNS) during metamorphosis, it is not surprising that PTTH would play a role in this process. Although animals lacking PTTH (rather paradoxically) live to adulthood, they do show greatly extended larval instars and a corresponding great delay in the 20E rise that signals the start of metamorphosis. For this reason, concluding that PTTH plays a SPECIFIC role in regulating female receptivity seems a little misleading, since the metamorphic remodeling of the entire CNS is likely altered in PTTH mutants. Since these mutants produce overall normal (albeit larger--due to their prolonged larval stages) adults, these alterations are likely to be subtle. Courtship has been reported as one defect expressed by animals lacking PTTH neurons, but this behavior may stand out because reduced fertility and increased male-male courtship (McBrayer, 2007) would be noticeable defects to researchers handling these flies. By contrast, detecting defects in other behaviors (e.g., optomotor responses, learning and memory, sleep, etc) would require closer examination. For this reason, I would ask the authors to temper their statement that PTTH is SPECIFICALLY involved in regulating female receptivity.*

C1. We agree with that, it is not surprising that PTTH regulates the profound changes that occur in the CNS during metamorphosis through ecdysone. Also, the behavioral changes induced by PTTH mutants include not only female receptivity. We will temper the statement about the function of PTTH on female receptivity.

We think there are two new points in our text although more evidences are needed in the future. On the one hand, PTTH deletion and the reduction of EcR-A in pC1 neurons during metamorphosis have opposite effects on female receptivity. On the other hand, development of pC1-b neurons regulated by EcR-A during metamorphosis is important for female receptivity.

> *(2) The link between PTTH and the role of pC1 neurons in regulating female receptivity is not clear. Again, since 20E controls the metamorphic changes that occur in the CNS, it is not surprising that 20E would regulate the arborization of pC1 neurons. And since these neurons have been implicated in female receptivity, it would therefore be expected that altering 20E signaling in pC1 neurons would affect this phenotype. However, this does not mean that the defects in female receptivity expressed by PTTH mutants are due to defects in pC1 arborization. For this, the authors would at least have to show that PTTH mutants show the changes in pC1 arborization shown in Fig. 6. And even then the most that could be said is that the changes observed in these neurons "may contribute" to the observed behavioral changes. Indeed, the changes observed in female receptivity may be caused by PTTH/20E actions on different neurons.*

C2. As newly formed prepupae, the ptth-Gal4>UAS-Grim flies display similar changes in gene expression to the genetic control flies to response to a high-titer ecdysone pulse. These include the repression of EcR (McBrayer et al., 2007). We tested whether there is a similar feedforward relationship between PTTH and EcR-A. We quantified the EcR-A mRNA level of PTTH -/- and PTTH -/+ in the whole body of newly formed prepupae. Indeed, PTTH -/- induced upregulated EcR-A in the whole body of newly formed prepupae compared with PTTH -/+ flies. We also detected the pattern of pC1 neurons when PTTH is deleted. Consistent with the feedforward relationship between PTTH and expression of EcR-A in newly formed prepupae, PTTH deletion induced less established pC1-d neurons contrary to that induced by EcR-A reduction in pC1 neurons.

However, it is not sure that the expression of EcR-A in pC1 neurons increases compared with genetic controls when PTTH is deleted. Furthermore, on the one hand, manipulation of PTTH has general effect on the neurodevelopment. On the other hand, the detailed pattern of pC1-b neurons which is the key subtype regulating female receptivity through EcR-A function in pC1 neurons could not be seen clearly. So, the abnormal development of pC1b neurons, if this is true, is just one of the possible reasons for the effect of PTTH deletion on female receptivity.

> *(3) Some of the results need commenting on, or refining, or revising: a- For some assays PTTH behaves sometimes like a recessive gene and at other times like a semidominant, and yet at others like a dominant gene. For instance, in Fig. 1D-G, PTTH[-]/+ flies behave like wildtype (D), express an intermediate phenotype (E-F), or behave like the mutant (G). This may all be correct but merits some comment.*

C3. Female receptivity increases with the increase of age after eclosion, not only for wild type flies but also PTTH mutants. At the first day after eclosion (Figure 1D), maybe the loss of PTTH in PTTH[-]/+ flies is not enough for sexual precocity as in PTTH -/-. At the second day after eclosion and after (Figure 1E-G), the loss of PTTH in PTTH[-]/+ flies is sufficient to enhance female receptivity compared with wild type flies. However, After the 2nd day of adult, female receptivity of all genotype flies increases sharply. At the 3rd day of adult and after, female

receptivity of PTTH -/- reaches the peak and the receptivity of PTTH[-]/+ reaches more nearly to PTTH -/- when flies get older.

> *b - Some of the conclusions are overstated. i) Although Fig. 2E-G does show that silencing the PTTH neurons during the larval stages affects copulation rate (E) the strength of the conclusion is tempered by the behavior of one of the controls (tub-Gal80[ts]/+, UAS-Kir2.1/+) in panels F and G, where it behaves essentially the same as the experimental group (and quite differently from the PTTH-Gal4/+ control; blue line).(Incidentally, the corresponding copulation latency should also be shown for these data.). ii) For Fig. 5I-K, the conclusion stated is that "Knock-down of EcR-A during pupal stage significantly decreased the copulation rate." Although strictly correct, the problem is that panel J is the only one for which the behavior of the control lacking the RNAi is not the same as that of the experimental group. Thus, it could just be that when the experiment was done at the pupal stage is the only situation when the controls were both different from the experimental. Again, the results shown in J are strictly speaking correct but the statement is too definitive given the behavior of one of the controls in panels I and K. Note also that panel F shows that the UAS-RNAi control causes a massive decrease in female fertility, yet no mention is made of this fact.*

C4. i) For all figures in the text, only when all the control groups were significant different from assay group, we say the assay group is significantly different. In Figure 2E-G, the control groups were both different from the assay group only at the larval stage. The difference between two control groups may due to the genetic background. We have described more detailed statistical analysis in the legend. In addition, the corresponding copulation latency has been shown. ii) For Figure 5, we have revised the conclusion in text as “when the experiment was done at the pupal stage is the only situation when the controls were both different from the experimental.” Besides, the UAS-RNAi control causes a massive decrease in female fertility in panel F has been mentioned.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) I am not sure that PTTH neurons should be referred to as "PG neurons". I am aware that this name has been used before but the PG is a gland that does not have neurons; it is not even innervated in all insects.*

C5. Agree. “PG neurons” has been changed into “PTTH neurons”.

> *(2) Fig. 1A warrants some explanation. One can easily imagine what it shows but a description is warranted.*

C6. Explanation has been added.

> *(3) When more than one genotype is compared it would be more useful to use letters to mark the genotypes that are not statistically different from each other rather than simply using asterisks. For instance, in the case of copulation latencies shown in Fig. 1E-G, which result does the comparison refer to? For example, since the comparisons are the result of ANOVAs, which comparison receives "*" in Fig. 1F? Is it PTTH[-]/+ vs PTTH[-]/PTTH[-] or vs. +/+?*

C7. Referred genotypes and conditions were marked in all figure legends.

> *(4) Fig. 1H: Why is copulation latency of PTTH[-]/PTTH[-]+elav-GAL4 significantly different from that of PTTH[-]/PTTH[-]? This merits a comment. Also, why was elav-GAL4 used to effect the rescue and not the PTTH-GAL4 driver?*

C8. We could not explain this phenomenon. This may due to the different genetic backgrounds between controls. We have mentioned this in figure legend.

> *(5) Fig. 2C, the genotype is written in a confusing order, GAL4+UAS should go together as should LexA+LexAop.*

C9. We have revised for avoiding confusion.

> *(6) In Fig. 2, is "larval stage" the same period that is shown in Fig. 3A? Please clarify.*

C10. We have clarified this in text and legends.

> *(7) Fig. 6. The fact that pC1 neurons can be labeled using the pC1-ss2-Gal4 at the start of the pupal stage does not mean that this is when these neurons appear (are born), only when they start expressing this GAL4. Other types of evidence would be needed to make a statement about the birthdate of these neurons.*

C11. We have revised the description for the appearance of pC1-ss2-Gal4>GFP. The detailed birth time of pC1 neurons will be tested in future.

> *(8) The results shown in Fig. 7 are not pursued further and thus appear like a prelude to the next manuscript. Unless the authors have more to add regarding the role of one of the differentially expressed genes (e.g., dopamine beta-monooxygenase, which they single out) I would suggest leaving this result out.*

C12. We have leave this out.

> *(9) Female flies lacking PTTH neurons were reported to show lower fecundity by McBrayer et al. (2007) and should be cited.*

C13. This important study has been cited in the first manuscript. In this revision, we have cited it again when mentioning the lower fecundity of female flies lacking PTTH neurons.

> *(10) Line 230: when were PTTH neurons activated? Since they are dead by 10h post-eclosion it isn't clear if this experiment even makes sense.*

C14. Yes, we did this for making sure that PTTH neurons do not affect female receptivity at adult stage again.

> *(11) Line 338: the statements in the figures say that PTTH function is required during the larval stages, not during metamorphosis*

C15. This has been revised as “The result suggested that EcR-A in pC1 neurons plays a role in virgin female receptivity during metamorphosis. This is consistent with that PTTH regulates virgin female receptivity before the start of metamorphosis.”

> *(12) Did the authors notice any abnormal behavior in males? McBrayer et al. (2007) mention that males lacking PTTH neurons show male-male courtship. This may remit to the impact of 20E on other dsx[+] neurons.*

C16. Yes, we have noticed that males lacking PTTH show male-male courtship. It is possible that PTTH deletion induces male-male courtship through the impact of 20E on other dsx+ or fru+ neurons. We have added the corresponding discussion.

> *(13) Line 145: please define CCT at first use*

C17. CCT has been defined.

> *(14) Overall the manuscript is well written; however, it would still benefit from editing by a native English speaker. I have marked a few corrections that are needed, but I probably missed some.*
>
> *+ Line 77: "If female is not willing..." should say "If THE female is not willing..."*
>
> *+ Line 78 "...she may kick the legs, flick the wings," should say "...she may kick HER legs, flick HER wings,"*
>
> *+ Lines 93-94 this sentence is unclear: "...while the neurons in that fru P1 promoter or dsx is expressed regulate some aspects..."*
>
> *+ Line 108 "...similar as the function of hypothalamic-pituitary-gonadal (HPG).." should say "...similar*
>
> *TO the function of hypothalamic-pituitary-gonadal (HPG).."*
>
> *+ Line 152 "Due to that 20E functions through its receptor EcR.." should say ""BECAUSE 20E ACTS through its receptor EcR.."*
>
> *+ Lines 155, 354 "unnormal" is not commonly used (although it is an English word); "abnormal" is usually used instead.*
>
> *+ Line 273: "....we then asked that whether ecdysone regulates" delete "that" + Sentences lines 306-309 need to be revised.*

C18. Thank you for your suggestions. We have revised as you advise.

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