Dopaminergic neurons mediate male *Drosophila* courtship motivational state

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Abstract

Motivational states are important determinants of behavior. In *Drosophila melanogaster*, courtship behavior is robust and crucial for race continuation. However, motivation of courtship behavior remains untouched. We first find the phenomena that courtship behavior is modulated by motivational state. Male fly courts to male fly when it firstly courts to decapitated female fly, however male-male courtship behavior rarely happens under normal condition. Male fly with satisfied sexual behavior shows decreased male-female sex drive. So in this phenomena male fly's courtship motivational state is induced by its exposure to female fly. Blocking dopaminergic neurons by expressing TNTe decreases motivational state induced male-male courtship behavior without affecting male-female courtship behavior. Vision cue is another key in sexual driven male-female courtship behavior. Such we identify a base theory that inner motivational state could decide fly's behavior eventually.

Introduction

Motivation provides behavior with purpose and intensity. A detailed neurobiological mechanisms underlying state-dependent changes in behavior is lacking. To understand how the motivational state neural circuits is organized in the brain and how they impact the neural circuit that direct behavior is a major question in neuroscience.

Studies on motivation in insects start from food-seeking behavior in the blowfly *Phormia regina* [1]. It was noted that although exposing gustatory receptor neurons on the proboscis to sugar always generated an electrophysiological response, the blowfly didn't consistently respond by extending the proboscis. However, a food-deprived blowfly was more likely respond with proboscis extension. Recently, a neural circuit that participates in motivational control of appetitive memory is found. In fruit flies, appetitive memory expression is constrained by satiety and promoted by hunger. They found that stimulation of neurons that express Neuropeptide F (dNPF), mimicks food-deprivation and promote memory performance in satiated flies. Robust appetitive memory performance requires the dNPF receptor expressed in dopaminergic neurons that innervate a different region of the mushroom bodies. Blocking these dopaminergic neurons releases memory performance in satiated flies whereas stimulation suppresses memory performance in hungry flies [2].

Courtship behavior, as an innate behavior, has been widely studied since Seymour Benzer's opened up the field of *Drosophila* neurogenetics at Caltech forty years ago. On one hand, male *Drosophila* performs the same ritual: orients towards a female, taps with its forelegs, sings a courtship song, licks the female's genital and attempts copulation [3] after access the auditory, mechanosensory, visual and chemosensory signal from the target [4]. And female fly decides whether to accept male fly based on the song he sings [5] and pheromone he emits [6]. Neural circuits of courtship behavior are densely investigated based on fruitless. fruitless, a master regulator of sexuality in fly [7], identified in 1963 by K.S.Gill and cloned by Daisuke Yamamoto [8] and separately by the group effect of Hall, Bruce Bake and Barbara Taylor [9, 10]. Male courtship behavior can be induced in chromosomally female fly by expressing the male-specific isofrom of *fru* in female's brain. It demonstrated it's a key of male courtship behavior. Courtship circuitry based on fruitless gene has been mapped structurally from sensory input to motor output [11]. This provides the possibility to understand how motivational state circuits direct motor output.

In this study, we build up a paradigm to study male courtship motivational state. Male fly firstly courts to decapitated female fly, then it exposes to male fly. Thus we modulate male fly's sex motivation to a high level. Sex driven male-male courtship behavior was induced comparing with male fly without pervious experience. While no significance enhanced male-female courtship was observed and it might due to ceiling effect. On the contrary, sex satisfied male fly shows reduced courtship behavior to a new encounter female fly. Sex driven male-male courtship behavior dependent mostly on vision cue input of male fly. Blocking dopaminergic neuron transmission decrease sex driven male-male courtship behavior. Whether increased dopamine level could also rescue low sex motivation level caused by satisfied courtship behavior needs to be explored.

Results

Male fly under high sexual motivation displays state dependent male-male courtship behavior, sexual satisfied male fly show decreased sex drive to next female fly.

To investigate whether courtship behavior could be modulated by motivational state, we firstly change male fly's sexual desire by exposing male fly to a decapitated female fly. The time male fly court decapitated female fly for 5min is called exposure period (**FIG 1**), and the subsequently placing male fly with another male fly is called test period (**FIG 1**), which is also 5min. In the first exposure period, male fly courts decapitated female fly with high intensity but unable to copulate with female fly, which representing a high sexual drive state has been induced. While in control group, male fly stay alone without any sexual activity. In this situation, male fly will court to female fly vigorously without successful copulation since decapitated female fly isn't able to accept male fly (**FIG 2 (A) (B) (C)**). Here we use two parameters to describe the activity of courtship behavior. One is CI (courtship index), which means the percentage of total time male fly spending on courtship during observation period. The other is latency, which represents the time from start of experiment to time male fly first display courtship behavior. Male-male courtship behavior is observed when male fly is transferred to a different chamber immediately and is exposed with male fly when comparing with male fly without sexual behavior during exposure period (**FIG 2 (D) (E) (F)**). However, male fly rarely courts male fly under normal condition since this behavior is inhibited by male pheromone: 7-tricosone (7-T). Male fly displays male-male courtship behavior mostly in the 1st min and with short latency.

We also test whether sexual primed male fly display enhanced male – female courtship behavior (**FIG 3**). Different with above experiment, male fly paired with intact virgin female instead of male fly (**FIG 3 (A)**). And then we observe male-female courtship behavior during both periods of different groups. Male fly didn't show significantly enhanced male-female courtship behavior, or significantly reduced latency either. It might be ceiling effect of male-female courtship behavior during exposure period.

To explore the contrary side of high sex motivation, sex satisfied male fly then exposes to a new encounter female fly. Decreased courtship index and enhanced latency display a lower sex motivation state of male fly (**FIG 1S (A)(B)(C)**).

Blocking TH neurons abolish motivational state dependent male-male courtship behavior without decrease male-female courtship behavior during exposure period.
Dopamine was shown to modulate male arousal and visual perception during heterosexual courtship [12, 13], locomotor activity [14], female sexual receptivity [15], male courtship conditioning [16], and ethanol-induced courtship inhibition [17] . Moreover, increased and decreased dopamine level induce homosexual of *Drosophila* [18, 19]. So we test the function of dopamine in this experiment. Using TH-gal4 driving TNT expression, we block TH neurotransmission. We find that male fly doesn't display enhanced male-male courtship behavior comparing with control male fly (**FIG 4 (A) (B) (C)**), while male-female courtship behavior remains unaffected during exposure period (**FIG 4 (D) (E) (F)**). Experiment with Gal80ts expressing in the fly could exclude the developmental effect. This motivational state dependent behavior could be transit, so we guess the developmental effect of TNTe might be slight. As a control group, we blocked octopaminergic neurons's activity at test period, and no influence to courtship motivational state at test period (**FIG 5**).

Sex driven male-male courtship behavior depends mostly on vision perception
Besides inner state, sensory stimuli could also induce courtship behavior. So we want to find the mainly sensory which could play a role in sex driven male-male courtship behavior. Due to this male-male courtship behavior could start although there is a long distance between two flies. We focus on olfaction and vision firstly. Male flies with defect olfaction and white eyes (W^{1118} and WCS; **FIG 6 (C) (D)** and **FIG 6 (E) (F)** separately**.** flies are separately tested. We find that male fly with vision defect couldn't induce this kind of behavior (**FIG 6 (A) (B)**), while flies with olfaction defect have no influence**.** Immobile target fly without any pheromone are used in test period (**FIG 6**).

Discussion

Sensory stimuli play important roles for animals output behavior, but the inner state decides the output of animal behavior, the inner sate enable animal behave flexibly, it will make the animal satisfy its needs to maximum extent. And courtship behavior is important for race continuation, and inner state which underlies this behavior is crucial for animal reproduction. The fixed behavior style of courtship behavior, and relative clear neural circuit based on courtship gene *doublesex* and *fru* have attracted lots of researchers [11, 20-24]. While what's the neural circuit underlies the inner state of courtship behavior remains unclear. Our research has filled the blanks.

The questions that internal state influences the courtship behavior have three questions now: 1. whether the inner state of *Drosophila* will influence the behavior of its output. 2.What's the neural circuit underlies the inner state, how it code and influence courtship behavior. 3. How the neural mechanisms interact with decision center, motor center and sensory input to decide the inner state influence the output behavior. Till now, we have answers for the first two questions, and the third question haven't been solved in our research， remains further study.

For the first question, whether the inner state of *Drosophila* will influence the output of *Drosophila* courtship behavior. Fisrtly, we need to change the inner state of *Drosophila*. Through two round courtship behavior paradigm, we have successfully raise the inner state of *Drosophila* courtship behavior. And the difficulty and novelty of this park of work is to how to display the raised courtship inner state. Here male-male courtship behavior is a good standard to display the raised inner courtship state. Because under normal condition, male-male courtship behavior rarely happens. And it will happen when *Drosophila* has increased internal courtship state. It will rescue the ceiling effect of male-female courtship behavior.

On the contrary, the decreased inner courtship state will be displayed by male-female courtship behavior after successfully copulation with the first female fly. This idea is the familiar with a study in which male fly's courtship state will decrease by multiple copulations with female fly [25]. These two methods have make us successfully adjusted the inner state of *Drosophila*. And at the same time, *Drosophila*'s courtship behavior changed while it's inner state have changed.

For the second questions, what's the neural mechanisms underlies *Drosophila* inner state? In mammalian, including human, several researches have prove that Dopamine system is important in adjusting state of behavior. For example, patients intake L-DOPA have increased sexual activity. In *Drosophila*, some researches have proved that dopamine system will take part in modulating sexual behavior. Feeding drosophila with dopamine receptor agonist will increase the male-female courtship behavior [26]. These researches have showed the conservation of dopamine on modulating courtship behavior. But this pharmacology study has unknown effect on the behavior. So we build the paradigm try to in-depth study the question via behavior and genetic methods.

In our research, we found that activity of dopamine neuron will displays the inner state of courtship behavior. Blocking dopaminergic neuron transmission via expressing UAS-TNTe driving by TH-Gal_4 will decrease the raised inner state induced male-male courtship behavior. So whether increased male-male courtship behavior will increase TH release? To answer the questions, specific subtype of TH neurons needed to be identified firstly. This depends on the genetic method.

In *Drosophila melanogaster*, TH neurons have overlapped cells with *fru* circuit, including aSP4 and aSP13. And aSP4 neurons' presynaptic area project with postsynaptic area of P1, which is the commander of courtship circuit neurons, SMPa [11]. aSP4 and PPL2ab neurons dopaminergic neurons are important candidates in this inner state of courtship behavior. aSP4 dopaminergic neurons belong to PAL subtype. In male fly,

courtship behavior will be abolished if feminizing this neurons [27]. Overexpression tyrosine hydroxylase could increase old male fly's courtship intensity [28].

Besides, one strategy is labeling the neurons activated by increased inner courtship state of *drosophila*. Here we tried two methods. One method is using DopR-Tango system, which will express LexA promoter when DopR1 are activated. Then across with LexAop-reporter Protein to make sure the expression of Dopamine dependent fluorescence [29].

However, due to mutant induced by gene insertion caused incomplete wings, the system doesn't suit for our courtship behavior. And the dopamine receptor only includes DopR but not all receptors is a limitation for the research. If we have conquered the two problems, it will be a good system for the research.

Another method, we identify the neurons activated by increased inner courtship state via CaMPARI system [30], which is a calcium binding protein combing with calcium irreversibly and changed its color from green to red under ultra violet rays. We have found some activity labeled neurons via this method. This needs further study. And if red light will replace ultra violet rays. The system is more suitable for fly since it couldn't sense red light.

For the third questions, how dopamine neurons changed the inner state? In the *drosophila* courtship behavior, some *fru* positive neurons project to *drosophila* sensory integration and behavior output center. P1 inter neurons are important for triggering the courtship behavior. They receive the sexual sensory information from female fly [31, 32], and project to motor neurons [32, 33]. Activation of P1 neurons alone will induce male courtship behavior to a fake female like rubber [34]. Whether dopamine neurons will direct influence motor output neurons to induce male-male courtship behavior and via which dopamine receptor?

Our research found that after male fly raised inner courtship state, the vision cue plays important roles on male-male courtship behavior. Whether dopamine neurons will sensitize vision input or the vision cue as a dependent cue to paly a role remains to be explored. It depends on the detailed relationship of vision input circuits and dopamine neurons.

Sensory neurons have provided the ability for male fly to identity the target, while the center brain's state modulates the output of *drosophila* output behavior finally. The relationship between dopamine neurons and vision sensory circuits remains unknown. They might play a role dependently in courtship motivational state behavior or the inner state might sensitize the vision input circuit. Which specific TH subtype modulates inner state induced male-male courtship behavior remains unknown. Whether increased dopamine level will rescue the vision deprivation induced motivational state induced male-male courtship deficiency. These questions remain further study.

Author Contributions

Guangxia Wang wrote manuscript, designed, performed and analysed all the behavior experiment. Aike Guo for discussion of experiment design. Sheng Wang performed

experiment for desire-reduced experiment in supplemental materials. There is no interest conflict in this paper.

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Bangyu Zhou wrote the Video imprinter software for behavior analysis, which works well especially on windows system. Ying Wang assist for the fly preparation. Tong Liu for discuusion of the work. This work was supported by 973 Program Grant 2011CBA00400 (to A.G.); Natural Science Foundation of China Grants 30921064, 90820008, and 31130027 (to A.G.).

Methods

Fly strains

Fly strains used in this experiments contains: CS, W^{1118} and WCS, UAS-TNTe, TH-Gal4 [13]. All the flies are raised up via Bloomington standard food recipe. Then they are placed in 23~25°C, 50～70% humility, 12h light/12h dark rooms and incubators. All female flies and male flies in the following experiments are virgin, they are picked up in the condition of CO_2 anaesthetization. After that female virgin flies are raised as a group of 10 and virgin male fly raised single in small tube, and potassium sorbate replaces propionic acid as the food preservatives. Male fly is 4~8 days old and female fly are 4~10 days old. All the target flies are wild CS. They are both the first generation of cross unless otherwise stated.

Background washing

Virgin females flies in the experiments (TH-GaL4, TDC2(2)-Gal4, UAS-TNTe [14]) across with male fly W^{1118} , then pick the next generation virgin female fly cross with male W^{1118} for six generation, after that we across the fly with double balancer to get *Drosophila melanogaster* with pure and same genetic background.

All the flies are virgin unless otherwise stated. And male flies are raised in single and female fly are raised in group of 10 flies. All the target fly are CS.

Behavior experiment

All the experiments are performed in a 4 layer courtship chamber, and in the self-made transparent cube (about 100cm X 50cm X 80cm) to make sure constant temperature and humidity. During the experiment, keep 23°C, 60% humidity, except in the experiment changes temperature. And in the cube, wide-angle earthquake-proof DVs (SONY) are fixed to record the whole experiments and after that we analysed the video data with self-coding Video imprinter.

During the experiment, we designed double blind method to exclude subjective deviation the results. The procedures of the experiments are as follows: The target male fly and female fly are under frozen anaesthetization, then transfer them on the temperature-adjustable 4°C copper plate. The female fly is decapitated as the target fly during the

exposure period to make sure induce sex desire, the male is wing-cut via fine scissors as the the target fly during test period to separate with the test male fly. They are placed in adjacent but different layers of courtship chamber. The active test male fly freely moves into the one layer of chamber and next to the female fly.

During exposure period, after the female fly has recover, transfer it into female fly's chamber for 5 min, and quickly and softly move the male fly into another chamber with wing-cut male fly inside it for 5 min, and this is the test period. And control group, during the first period, male fly is transferred into an empty chamber for 5 min. The negative control experiment, during exposure period, male fly is exposed to decapitated male fly. And during test period, they both exposed to normal wing-cut male fly.

Experiment for desire satisfaction: place male fly and two normal virgin females in 3 different layers of courtship chamber. And place the male with the first female first, after successfully copulation with the first female fly, the male fly is transferred with the second virgin female fly without delay and then it will court to the second female fly. After that, courtship index and latency for the first 5 min of the two courtship behavior are separately calculated to indicate the strength of courtship desire.

Olfaction deprivation experiment

Flies are place on 4°C copper plate after frozen anaesthetization, then remove the antenna and maxillary palp with tweezers. After that male fly is transferred back to its tube, recovery 2 days to perform experiment.

Data analysis

Videos recorded are analysed by self-coding Video imprinter software running specially under Windows system. We tracked each step of courtship behavior, including orientation, following, sing courtship song, lick female genitals, attempt copulation, copulation. Then we analyse the script of tracking data, draw picture via R code. Courtship Index is defined as the percentage of the occurrence time of courtship behavior to the total observation time. Courtship latency is defined as the time required from the beginning of the experiment to the initiation of courtship behavior. Because the data are non-normal distribution, Wilson rank test for statistical test is used for significance difference detection. Adobe illustrator are used as phototypesetting software.

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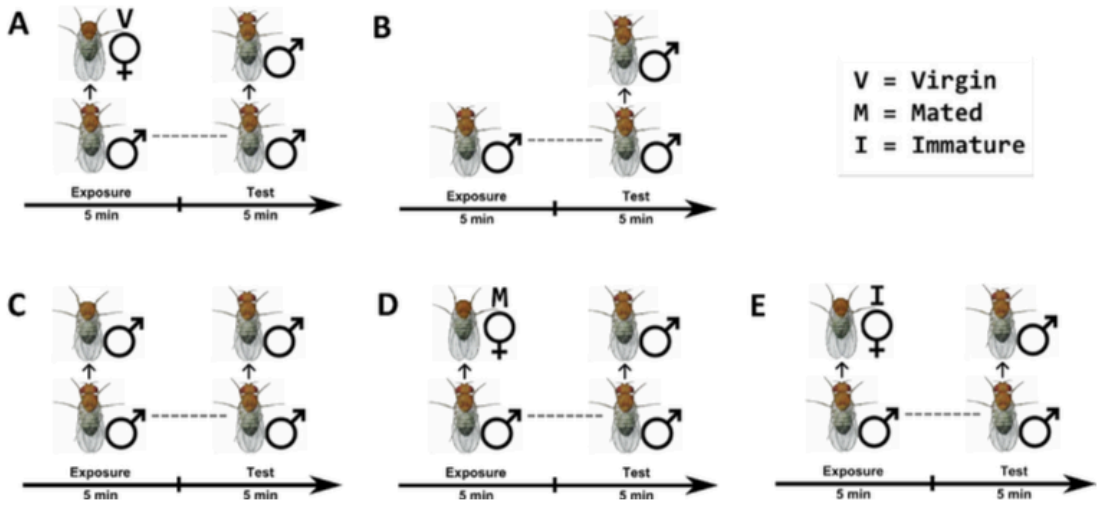

FIG1 Paradigm for studying the male *Drosophila* courtship motivation—two round courtship behavior

During exposure period, male fly is exposed to virgin female fly**(A)**, virgin male fly**(C)**, mated female fly**(D)**, immature female fly **(E)** for 5 min separately. Then transfer the male fly to another courtship chamber with a wing-cut male fly inside it for 5 min, this period is called test period. **(B)**. Male fly stay alone during exposure period for 5 min, and then transfer the male fly to another chamber with a wing-cut male fly inside it for 5 min. Paradigm (A) and (B) are mainly experimental group and control group separately.

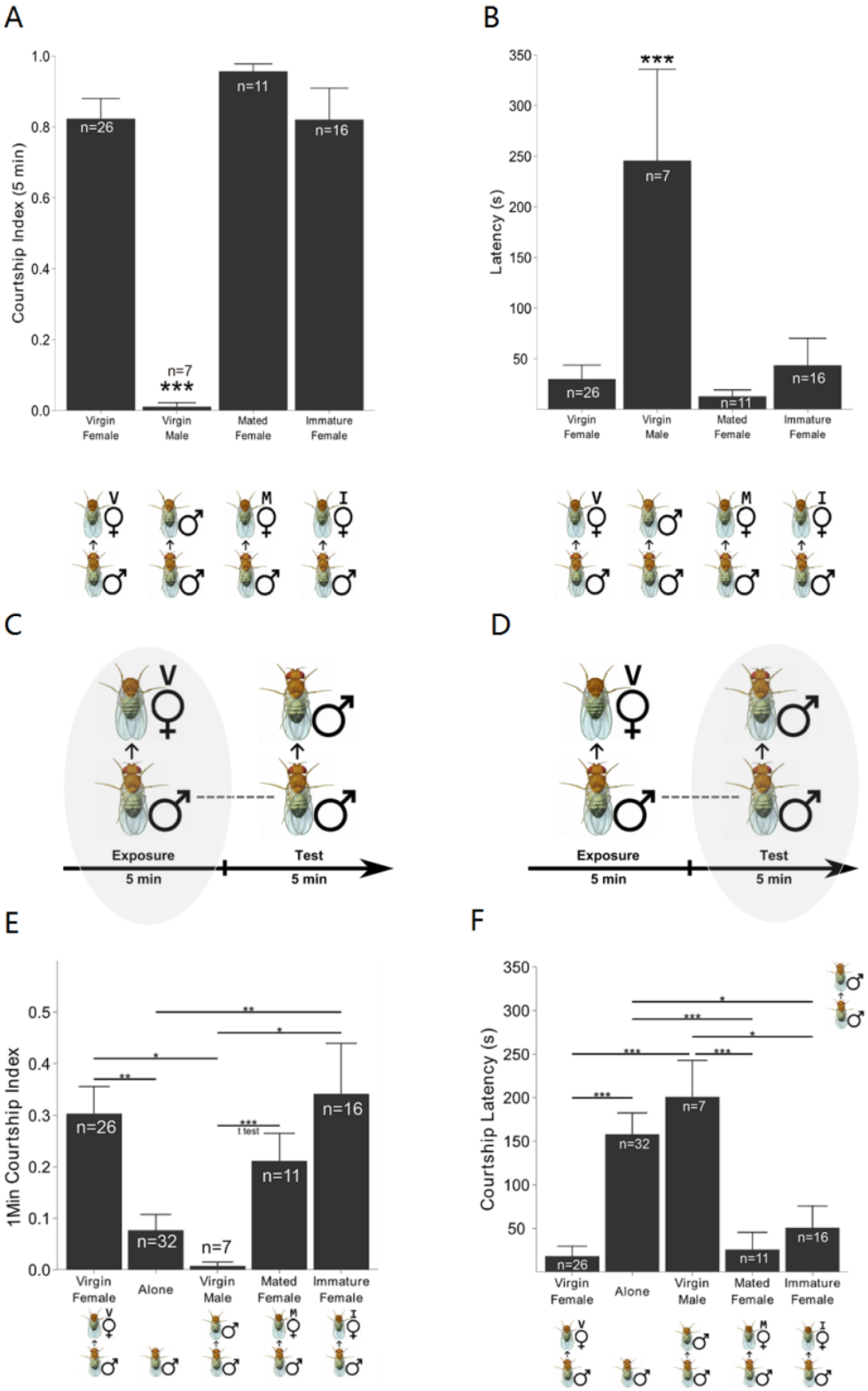

FIG2 Male-male courtship behavior is induced after male fly courts with decapitated female fly intensely during exposure period. (A). **(B)**. The wild type male fly in

experimental group courts with female fly intensely during exposure period. **(A)**. Courtship index of male fly to female fly. **(B)**. Courtship latency of male fly to female fly. **(C)**. The experimental illustration indicates that data of A and B come from exposure period of experimental group. **(D)**. The experimental illustration indicates that data of E and F display male-male courtship behavior of test period. **(E)**. During the first one minute of the test period in the experimental group, the wild-type male fly displays a significantly increased motivational male-male courtship index, compared with the control group. **(F)**. During the first one minute of the test period in the experimental group, the wild-type male fly displays a significantly decreased motivational courtship latency, compared with the control group. n =7~23. Mean ± s.e.m. Standard deviation is illustrated in FIGure. ** indicates P<0.01, *** indicates that P < 0.001。

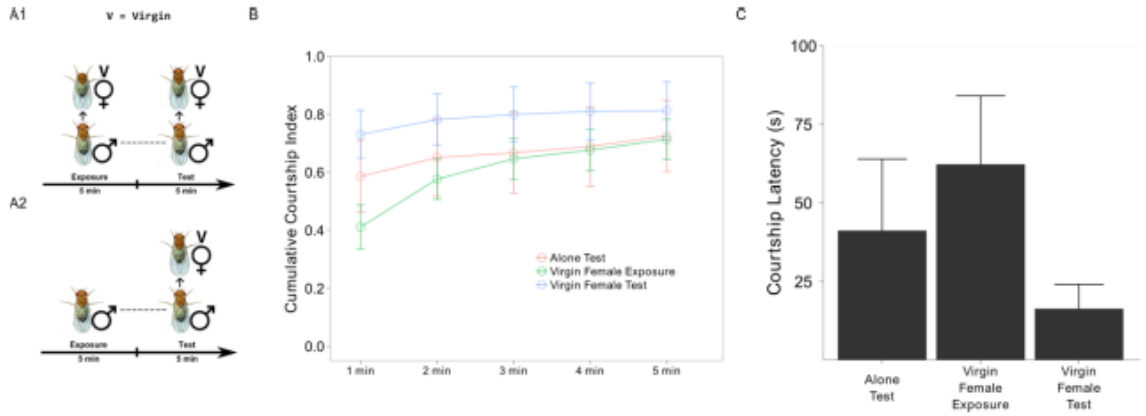

FIG3 There is no increased male-female courtship behavior after male fly courts intensely with the first female fly during test period. (A1).(A2). Behavior paradigm for the state-driven male female courtship behavior. In this paradigm, the male fly is exposed within intact female fly during test period which is the only difference from previous paradigm. **(B)**. Male female courtship index in 1st min, 2min, 3min, 4 min, 5min of exposure period and test period in both experimental group and control group. There is no significant difference between this groups. **(C)**. Courtship latency of exposure period and test period in both experimental group and control group. There is no significant difference between this groups. n= 20。 Mean ± s.e.m. Standard deviation is illustrated in FIGure.

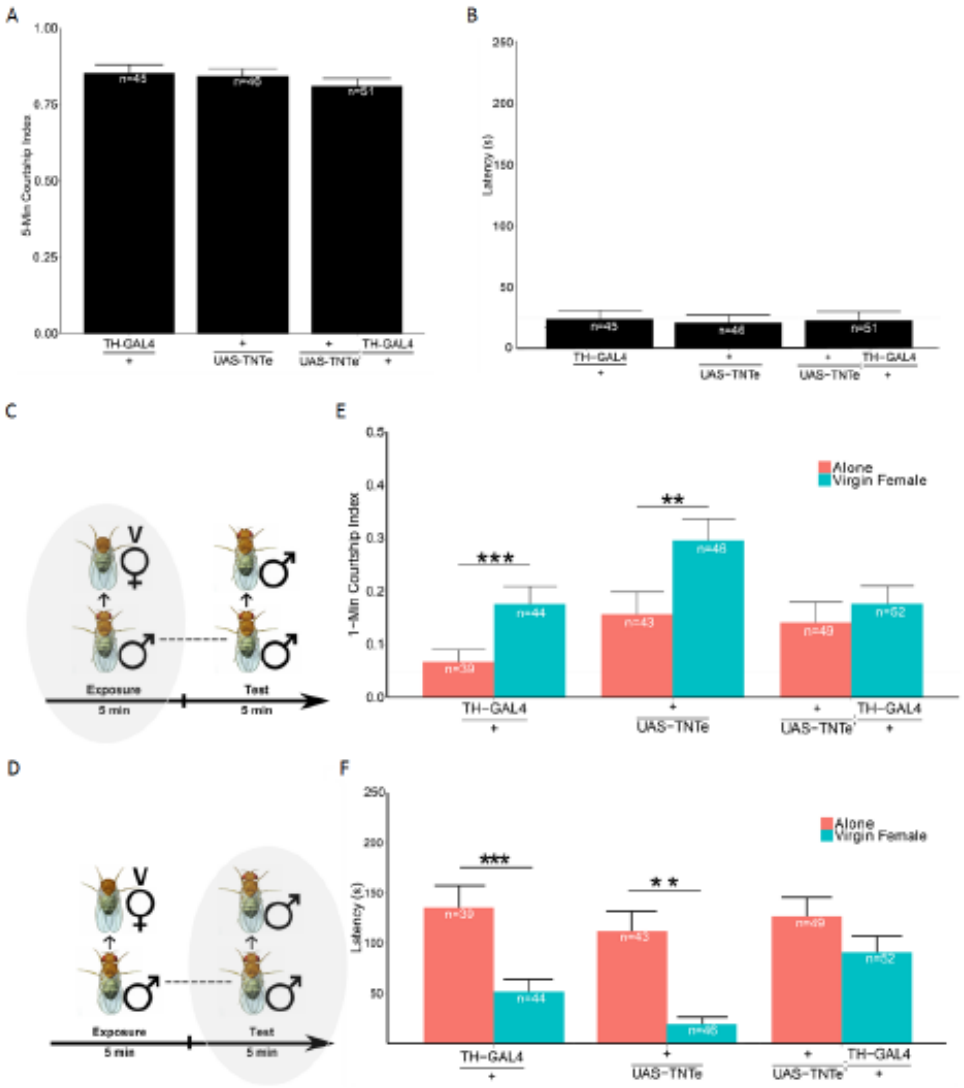

FIG 4 Blocking dopaminergic neurotransmission destroys sex-driven male-male courtship behavior, but it doesn't influence male-female courtship behavior during exposure period. (A),(B). During exposure period, male flies that blocks dopaminergic transmission (UAS- TNTe/+; TH-GAL4/+) display same intense male-female courtship behavior to CS female fly as control fly, TH-Gal4/+ and UAS-TNTe/+. **(A)**. Courtship index for male-female courtship behavior. **(B)**. Courtship latency for male-female courtship behavior. **(C)**. Experimental illustrator indicates that data of A and B come from male-female courtship behavior during exposure period. n=45~51. **(D)**. Experimental illustrator indicates that data of A and B come from male-male courtship behavior during test period. **(E),(F)**. During test period, male flies (UAS-TNTe/+; TH-GAL4/+) that blocking dopaminergic transmission displays no increased male-male courtship behavior comparing with control group. While control fly TH-Gal4/+ and UAS-TNTe/+ display increased sex-driven male- male courtship behavior. **(E)**. Courtship index of male-male courtship behavior. For UAS- TNTe/+genotype, p = 0.0003148, for TH-GAL4/+ genotype, p = 0.00342, **(F)**. Courtship latency of male-male courtship behavior. For UAS-TNTe/+

genotype, p = 3.637e-05, for TH-GAL4/+ genotype, p = 0.007924. n =39~52. Standard deviation is illustrated in FIGure. Mean ± s.e.m. * indicates P<0.05, ** indicates that P < 0.01.

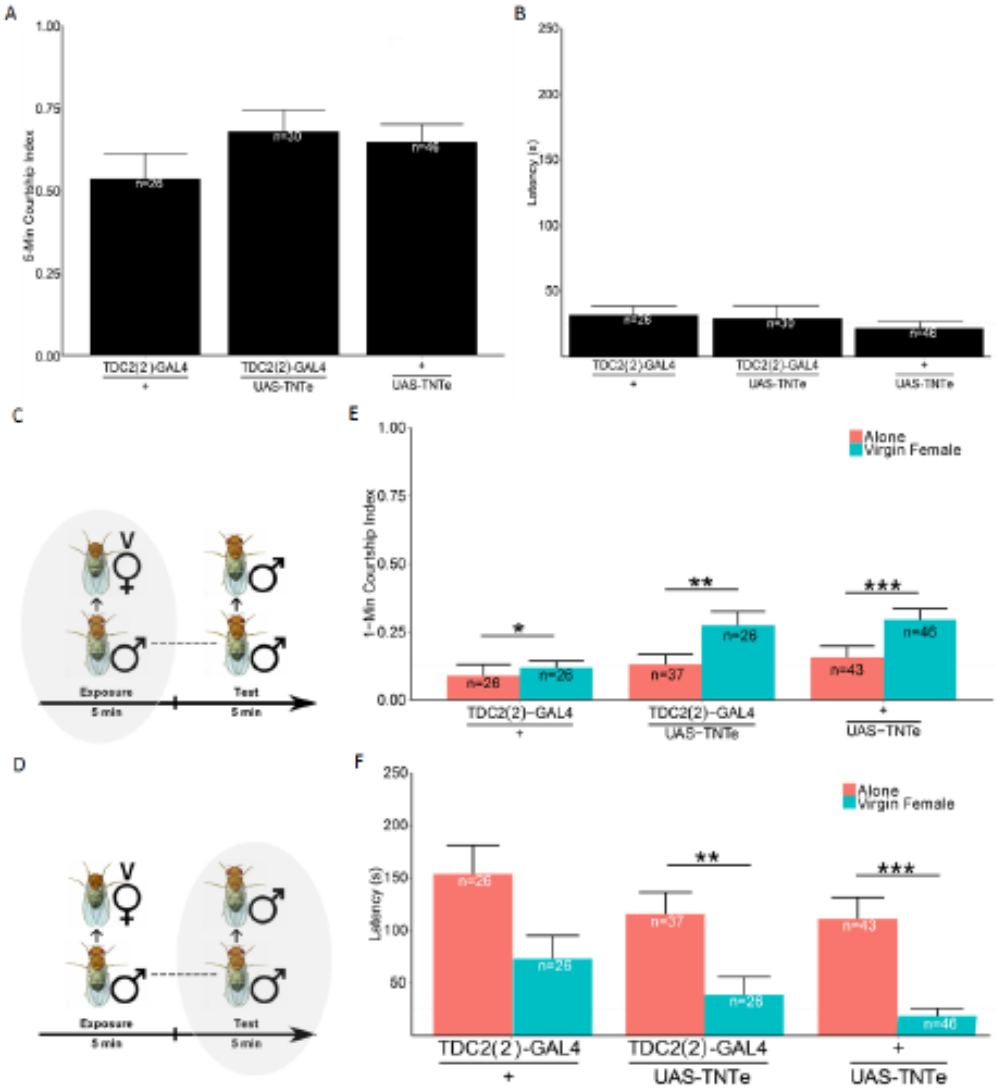

FIG 5 Blocking octopaminergic neurotransmission has no influence on sex-driven male-male courtship behavior, and it doesn't influence male-female courtship behavior during exposure period. (A), **(B)**. During exposure period, male flies that blocks dopaminergic transmission (UAS-TNTe/+; TDC2(2)-GAL4/+) display same intense male-female courtship behavior to CS female fly with control fly, TDC2(2)-Gal4/+ and UAS-TNTe/+. **(A)**. Courtship index for male-female courtship behavior. **(B)**. Courtship latency for male-female courtship behavior. **(C)**. Experimental illustrator indicates that data of A and B come from male-female courtship behavior during exposure period. n = 26~46 **(D)**. Experimental illustrator indicates that data of A and B come from

male-male courtship behavior during test period. **(E)**,**(F)**. During test period, male flies (UAS-TNTe/+; TDC2(2)-GAL4/+) that blocking dopaminergic transmission displays increased male-male courtship behavior comparing with control group. Also control fly TDC2(2)-Gal4/+ and UAS- TNTe/+ display increased sex-driven male-male courtship behavior. **(E)**. Courtship index of male- male courtship behavior. **(F)**. Courtship latency of male-male courtship behavior. n = 26~46. Standard deviation is illustrated in FIGure. Mean ± s.e.m. * indicates P<0.05, ** indicates that P < 0.01.

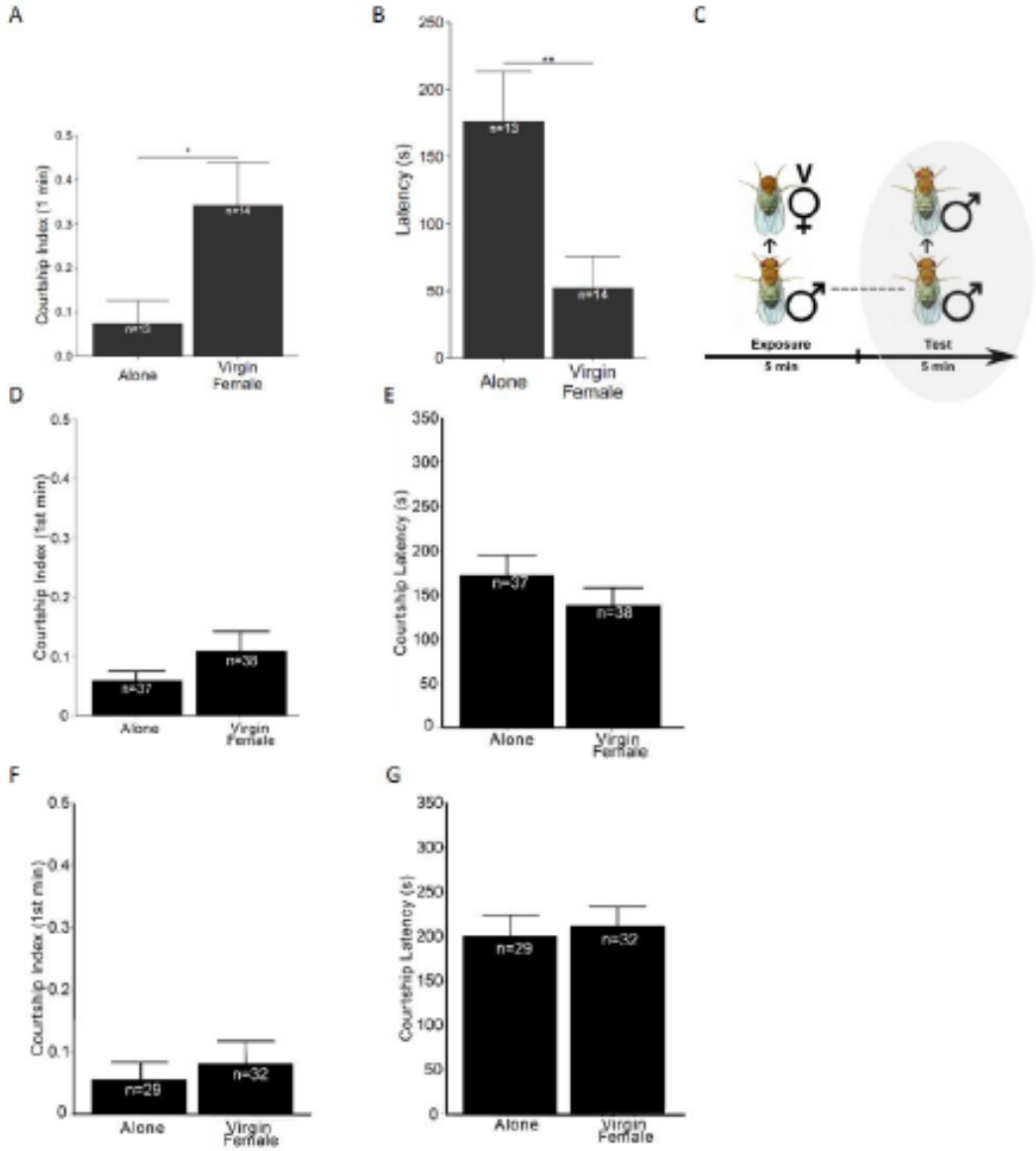

FIG 6 Vision deprivation but not olfaction deprivation will decrease sex-driven male-male courtship behavior. (A). **(B)**. After olfaction deprivation, the wild-type male fly could still display sex-driven male-male courtship behavior. **(A)**. Male-male courtship index (P=0.0009536, Wilcoxon rank sum test), **(B)**. Male-male courtship latency. (P=0.008138, Wilcoxon rank sum test). n =13~14. **(C)**. Experimental illustrator indicates

that data of (A), (B), (D), (E), (F), (G) come from male-male courtship behavior during exposure period in experimental group. n=45~51. $W^{1118}$and WCS are used respectively as the test fly with the vision deprivation in (D), (E) and (F), (G). They don't display sex-driven male-male courtship behavior. **(D)** and **(F)**, Courtship index. **(E)** and **(G)**, Courtship latency. n=34~37. Mean ± s.e.m. Standard deviation is illustrated in FIGure. * indicates $P < 0.05$, **indicates that $P < 0.01$.