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The mushroom body D1 dopamine receptor controls innate courtship drive

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Mating is critical for species survival and is profoundly regulated by neuromodulators and neurohormones to accommodate internal states and external factors. To identify the underlying neuromodulatory mechanisms, we investigated the roles of dopamine receptors in various aspects of courtship behavior in *Drosophila*. Here, we report that the D1 dopamine receptor dDA1 regulates courtship drive in naïve males. The wild-type naïve males actively courted females regardless their appearance or mating status. On the contrary, the dDA1 mutant (*dumb*) males exhibited substantially reduced courtship toward less appealing females including decapitated, leg-less and mated females. The *dumb* male's reduced courtship activity was due to delay in courtship initiation and prolonged intervals between courtship bouts. The dampened courtship drive of *dumb* males was rescued by reinstated dDA1 expression in the mushroom body α/β and γ neurons but not α/β or γ neurons alone, which is distinct from the previously characterized dDA1 functions in experience-dependent courtship or other learning and memory processes. We also found that the dopamine receptors dDA1, DAMB and dD2R are dispensable for associative memory formation and short-term memory of conditioned courtship, thus courtship motivation and associative courtship learning and memory are regulated by distinct neuromodulatory mechanisms. Taken together, our study narrows the gap in the knowledge of the mechanism that dopamine regulates male courtship behavior.

Keywords: Conditioning, courtship, D1 receptor, dopamine, Drosophila, learning, mating decision, memory, motivation, mushroom body

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Mating is important for fitness and survival in sexually reproducing species. Multidimensional internal and external drives regulate mating behavior through neuroendocrine and neuromodulatory systems. Dopamine, in particular, facilitates mating behavior across species including reptiles, rodents and humans (Gobrogge & Wang 2016, Hull 2011, Pfaus 2009, Woolley *et al.* 2004), however, the underlying neural and cellular mechanisms remain elusive. In the fruit fly *Drosophila melanogaster*, dopamine enhances mating as well. Blockade of dopamine neurotransmission significantly reduces a male's courtship toward a female (Alekseyenko *et al.* 2010; Chen *et al.* 2013) while reinstated vascular monoamine transporter (VMAT) expression in dopamine neurons alleviates defective courtship of the male lacking VMAT (Chen *et al.* 2013). In the past several years, advances in genetic and imaging tools have allowed remarkable progress in identifying the modulatory neural circuits for experience- and age-dependent changes in mating drive (Kuo *et al.* 2015; Zhang *et al.* 2016). For example, recently mated males show reduced courtship drive, which is mediated by the dopamine neurons in the superior medial protocerebrum through the D5 dopamine receptor DAMB/DopR2 in the P1 neurons (Zhang *et al.* 2016). Aged males on the other hand have declined sexual drive, which is due to a decreased dopamine level in the PPL2ab neurons (Kuo *et al.* 2015) although the receptor mediating this activity is unknown.

Dopamine is also involved in courtship learning and memory in *Drosophila* (Keleman *et al.* 2012; Montague & Baker 2016). A naïve male fly courts a virgin or mated female vigorously. In return, a virgin female, which is fully receptive, copulates with a courting male. A mated female, in contrast, is reluctant to remate and rejects an approaching male by kicking and/or running away. A rejected male subsequently does not court other females including virgin females for several hoursr or many days (McBride *et al.* 1999; Siegel & Hall 1979). Aversive volatile pheromone *cis*-vaccenyl acetate (cVA) transferred to a mated female during copulation plays a major role in generalized courtship suppression, while physical insult and fruitless attempts to mate facilitate conditioning (Ejima *et al.* 2007; Tompkins *et al.* 1983). Two studies have independently shown that the PAM dopamine neurons projecting to the mushroom body (MB) γ lobe mediate courtship learning and memory (Keleman *et al.* 2012, Montague & Baker 2016).

Keleman *et al.* (2012) have also found that the D1 dopamine receptor dDA1/DopR1, but not DAMB and the D2 receptor dD2R, enhances the male's response to cVA in a mated female for learning and memory of courtship suppression.

The findings described above elucidate the discrete dopamine neural circuits for several aspects of mating behavior. Nonetheless, a couple of key issues needs clarification. Keleman *et al.* study (Keleman *et al.* 2012) has used a mated female as a trainer as well as a tester in courtship conditioning. This raises a possibility that courtship learning and memory reported in the study may involve a nonassociative learning or memory component. It remains to be clarified whether dDA1 or other dopamine receptors are involved in associative courtship memory. Also, the dopamine receptors that mediate mating drive in naïve males are unknown. We have investigated these knowledge gaps in this report.

Materials and methods

Drosophila stocks and culture

The wild-type strain *Canton-S* (*CS*) was used as a control in all experiments. The dopamine receptor mutants used in this study include $dumb^1$ with inversion mutation in the gene coding for D1 receptor-dDA1, $dumb^2$ with insertional mutation in D1-dDA1, *damb* with deletion mutation in D5-DAMB, *dd2r* (*f06521*) with insertional mutation in D2-dD2R, and $dopecr^{c02142}$ (hereafter *der*) with insertional mutation in DopEcR. All mutants were placed in the *CS* background. We previously reported $dumb^1$, $dumb^2$ and $UAS\text{-}dDA1^{dumb2}$ (Kim *et al.* 2007) and *damb* (Cassar *et al.* 2015); and *dd2r* and *der* have been characterized previously (Ishimoto *et al.* 2013; Marella *et al.* 2012). $dumb^1$, $dumb^2$ and *der* are strong hypomorphic alleles as their phenotypes are comparable to those of their transheterozygotes with respective deficiency yet they have low levels of transcripts (Ishimoto *et al.* 2013; Kim *et al.* 2007). *damb* is a null allele as it has no DAMB transcripts detectable (Cassar *et al.* 2015). *dd2r* is a strong hypomorphic or null allele since the mutation completely abrogates the dopamine neuronal signal for proboscis extension (Marella *et al.* 2012). The *MI04437* line ($dumb^4$) carrying the *Minos Mi{MIC}* insertion (Venken *et al.* 2011) in the first intron of the *dDA1/dumb* gene was backcrossed and placed in the *CS* background. The fly lines $dumb^4$ (43773), *der* (10847), *c739-GAL4* (7362), and *c305a-GAL4* (30829) were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA); *dd2r* (f06521) from the Exelixis Collection (Harvard Medical School, Boston, MA, USA); *NP1131-GAL4* from Dr. Dubnau (Stony Book University School of Medicine, Stony Brook, NY, USA); *MB247-GAL4* from Dr. Waddell (University of Oxford, Oxford, UK). Flies were reared on a standard cornmeal/agar medium at 25°C with 50% relative humidity under the 12 h light/12 h dark cycle. Male flies were collected under CO_2 within 12–18 h after eclosion and housed individually in a food vial for 3–4 days. Virgin *CS* females were collected within 12 h after eclosion and housed together in a food vial. Virginity was verified by confirming the absence of progeny in a food vial. Mated *CS* females were prepared by housing a single virgin female with three to four mature *CS* males for 18–24 h in a small vial containing food and then used for conditioning. Virgin *CS* females were decapitated and only moving flies were used within 1 h. The 4- to 5-day-old males and females were used in all experiments.

Courtship assays

All courtship assays were performed in a courtship chamber ($8 \times 8 \times 5\,mm^3$) as previously described (Ejima & Griffith 2011). A wet filter paper was placed at the bottom of each chamber in order to maintain humidity. For a basal courtship assay, a tester male was transferred by aspiration to a chamber and acclimated for 10 min, and then a single courtee (i.e. intact or decapitated virgin *CS* female, a leg-amputated virgin *CS* female, a mated *CS* female or a male of the same genotype) was transferred to the chamber by aspiration. The chamber was videotaped for 1 h to score individual courtship steps (orientation and following, tapping, singing, licking, attempted copulation and copulation) (Yamamoto & Koganezawa 2013). The percentage of time that a male spent courting during the first 10 min of pairing or before copulation [courtship index (CI)] was used to represent courtship activity. Locomotor activity was measured by counting the number of times that a male crosses a midline drawn across the courtship chamber as previously described (Joiner & Griffith 2000).

For courtship conditioning, a tester male was introduced to a chamber containing a recently mated female for 1 h (conditioning phase). To control for experimental manipulation, a tester male was placed in a chamber alone for 1 h (mock exposure). In the test phase of conditioned courtship, a tester female is typically decapitated to avoid a potential effect of female's courtship solicitation and thereby to focus on male's behavior (Ejima & Griffith 2011). Thus, in an acquisition test, the conditioned or mock-exposed male was transferred to a chamber containing a decapitated virgin female right after conditioning (acquisition). In a memory test, the conditioned or mock-exposed male was housed in a food vial for 1 h and then tested with a decapitated virgin female. The male's courtship behavior was videotaped and scored during 10 min of pairing. The performance index (PI), a percent reduction in courtship activity with (CI_{test}) and without (mock; CI_{mock}) conditioning, was calculated by $[100 \times 1 - (CI_{test}/mCI_{mock})]$ where mCI_{mock} is the mean CI of the mock-exposed males, and used to represent acquisition and short-term memory. The PI value of 100 indicates perfect memory and 0 no memory. All experiments were performed in an environmental chamber maintained at 25°C with 60–70% humidity. The genotypes of tester males were blinded to the experimenters who set up courtship assays and scored courtship activity.

Immunohistochemical analysis

Immunostaining of dDA1 was performed as previously described (Kim *et al.* 2003; Kim *et al.* 2007). Briefly, the brains were dissected in phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde in PBS for 20 min. The brains were washed once with PBS and three times with PBHT (20 mM NaH_2PO_4, 0.5 M NaCl, 0.2% Triton X-100, pH 7.4) for 10 min each. The brains were then treated with 1% Triton X-100 in PBHT for 1 h, blocked with 5% normal goat serum for 2 h and incubated with the mouse polyclonal anti-dDA1 antibody (1:1000) overnight. After three washes with PBHT, the brains were incubated with the goat anti-mouse IgG antibody conjugated with Alexa 488 (1:1000, Invitrogen, Carlsbad, CA, USA) for 2 h followed by PBHT and 0.12 M Tris–HCl (pH 7.2) washes. The brains were mounted in the Vectashield medium (Vector Lab, Burlingame, CA, USA). All procedures were performed at room temperature. Images were scanned using the ×20 or ×40 oil-immersion objective in the LSM700 confocal microscope (Zeiss, Thornwood, NY, USA).

Data analysis

Statistical analyses were performed using Minitab 16 (Minitab, State College, PA, USA) and JMP 13 (SAS, Cary, NC, USA). All data are reported as mean + standard error of mean. Normality was determined by the Anderson Darling goodness-of-fit test. The normally distributed data of two groups were analyzed by the two-tailed Student's *t*-test. The data with three or more groups were analyzed by general linear model with *post hoc* Tukey–Kramer HSD. Nonnormally distributed data sets were analyzed by the Mann–Whitney *U* test for two groups and the Kruskal–Wallis test followed by *post hoc* Mann–Whitney *U* or Dunn with Control for Joint Ranks test.

Results

Dopamine receptors are dispensable for associative courtship memory acquisition and short-term memory

Drosophila has four dopamine receptors: dDA1 [D1 type; (Sugamori *et al.* 1995)], DAMB [D5 type; (Han *et al.* 1996)],

dD2R [D2 type; (Hearn *et al.* 2002)] and DopEcR [D1 type that responses to dopamine and ecdysone; (Srivastava *et al.* 2005)]. To identify the dopamine receptors important for generalized courtship suppression, we examined the *dumb*^{2}, *damb*, *dd2r* and *der* mutant flies deficient in dDA1, DAMB, dD2R and DopEcR, respectively, in conditioned courtship. When tested with a decapitated virgin female right after training, the wild-type *CS* as well as all dopamine receptor mutant males showed significantly reduced courtship activity (i.e. low CI) compared to the mock-exposed males ($P < 0.0001$; Fig. 1a). Also, the PI, which represents the percent reduction in courtship activity with and without conditioning, of the *dumb*^{2}, *damb* or *der* males was similar to that of *CS* males ($P > 0.05$, Fig. 1b). The PI of the *dd2r* males was slightly higher than that of *CS* males, which was marginally significant ($P = 0.0491$, Fig. 1b). Thus, we conclude that the dopamine receptor mutants had normal acquisition of associative courtship memory.

We next investigated whether dopamine receptors are important for short-term memory of courtship suppression. When tested at 1 h after training, *dumb*^{2}, *damb* and *dd2r* males showed comparable PIs to *CS* ($P > 0.05$, Fig. 1c). The *der* males, on the other hand, had significantly reduced PI ($P < 0.05$). It has previously shown that DopEcR in response to ecdysone, but not to dopamine, mediates short-term memory but not acquisition of the memory (Ishimoto *et al.* 2013), and our data are consistent with this finding. This indicates that the parameters used in our study are appropriate to detect differences in courtship memory. Taken together, these observations show that individual dopamine receptors dDA1, DAMB and dD2R are dispensable for acquisition and short-term memory of associative conditioned courtship.

Dumb males exhibit reduced courtship activity toward less appealing mates

The courtship conditioning experiments allowed somewhat unexpected discovery. The mock-exposed *dumb*^{2} males that were used as a control for the memory test showed highly reduced courtship activity with a decapitated female unlike *CS* or other dopamine receptor mutant males (Fig. 1a). We pursued this further by testing courtship activity of naïve *CS*, *dumb* and other dopamine receptor mutant males toward an intact or decapitated virgin female without mock exposure. *Damb, dd2r* and *der* males displayed courtship activity toward an intact or decapitated virgin female comparable to that of *CS* males similar to their mock-exposed counterparts (data not shown, Fig. 1a). When paired with an intact virgin female, *dumb*^{2} males exhibited slightly enhanced courtship activity, whereas the *dumb*^{1} and *dumb*^{1}/*dumb*^{2} transheterozygote males exhibited courtship activity similar to that of *CS* ($P > 0.05$, Fig. 2a). This indicates that dDA1 is not essential for courtship drive toward an intact virgin female. When paired with a decapitated virgin female, however, all *dumb* mutants (*dumb*^{1}, *dumb*^{2} and *dumb*^{1}/*dumb*^{2}) displayed substantially reduced courtship compared to *CS* ($P < 0.0005$, Fig. 2a). Upon comparison of courtship toward an intact vs. decapitated virgin female, *CS* males exhibited less courtship with a decapitated female than with an intact female, nevertheless the extent of courtship reduction was substantially greater in all *dumb* mutants compared to *CS* ($P < 0.0005$, Fig. 2b). To substantiate this finding further, we tested an additional *dumb* allele. The *MI04437* line has the transposon *Mi{MIC}* (Venken *et al.* 2011) inserted in the first intron of the *dDA1/dumb* gene (Fig. 2c). The inserted *Mi{MIC}* has the splice acceptor in the right direction for dDA1

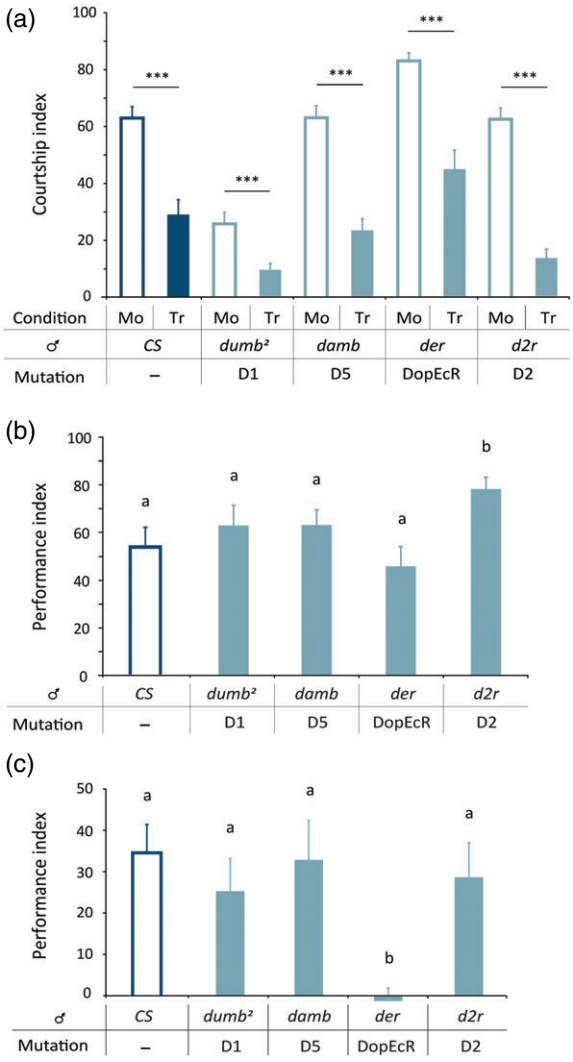

Figure 1: Dopamine receptors are dispensable for acquisition and short-term memory in associative courtship conditioning. The wild-type *CS* and dopamine receptor mutants were trained with a mated female or mock-exposed and then tested with a decapitated virgin female right after or 1 h training. (a) The CI of the mock-exposed (Mo) and trained (Tr) dopamine receptor mutants right after training. Mann–Whitney *U* test: $***P < 0.0001$; $**P < 0.01$; $n = 32–48$. (b) PI right after training. Kruskal–Wallis test, $P = 0.0193$; the letters on the bars denote significant difference from the control *CS* by Dunn method for Joint Ranking; $P = 0.049$ for b. (c) PI at 1 h after training. Kruskal–Wallis test, $P > 0.05$; b, $P = 0.0189$, Dunn for Joint Ranking with *CS*; $n = 22–73$.

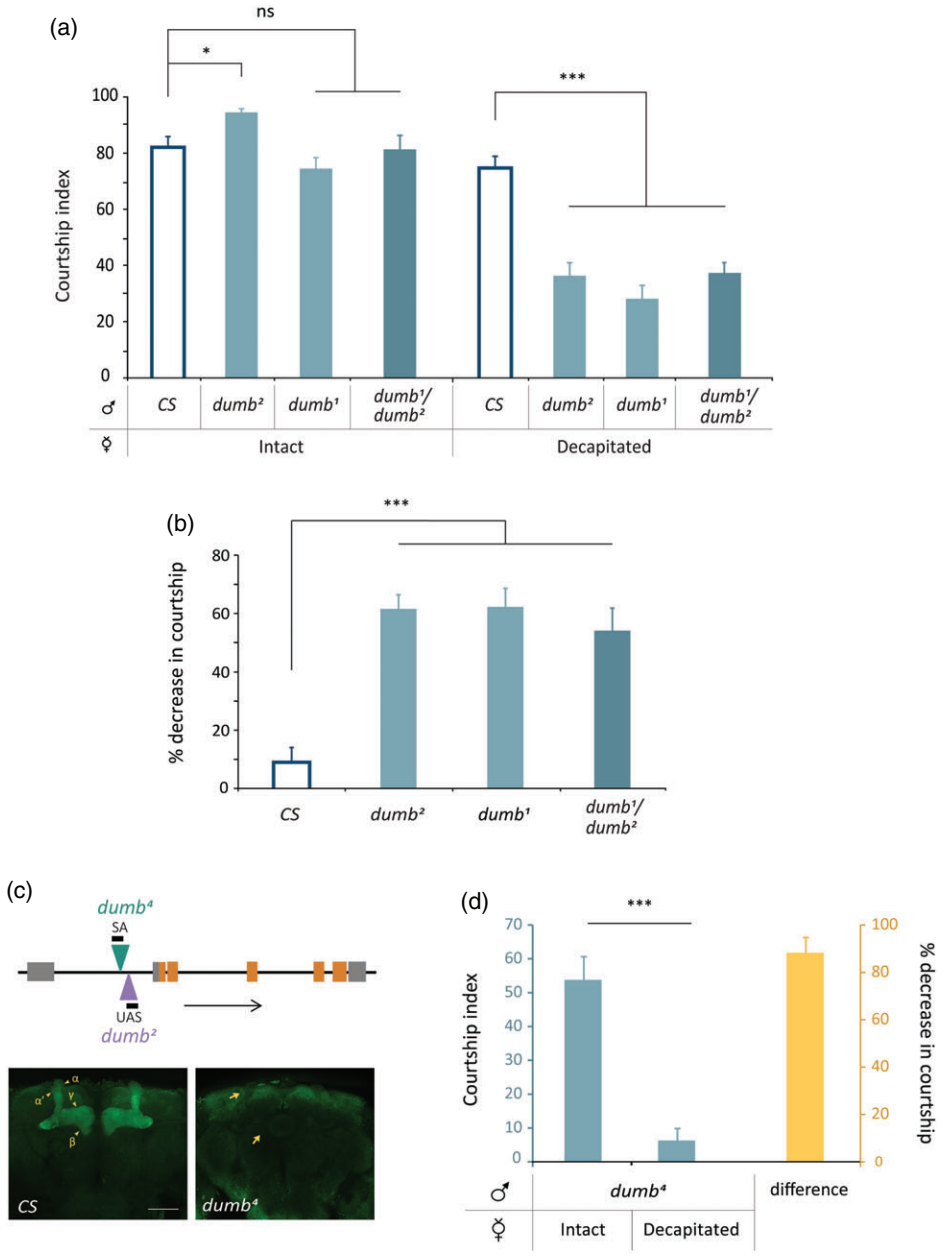

Figure 2: dDA1 is needed to court less appealing mates. (a) Naïve *CS* and *dumb*^{2} males were paired with either an intact or decapitated virgin female, and the percent time that a male courting a female (CI) was measured. With an intact female: Kruskal–Wallis, $P < 0.0001$; b, $P = 0.0130$ by Dunn for Joint Ranking with the control *CS*; ns, not significant; $n = 20–28$. With a decapitated female: Kruskal–Wallis, $P < 0.0001$; ***$P < 0.0005$ by Dunn for Joint Ranking with the control *CS*; $n = 27–28$. (b) The percent reduction of CI with a decapitated virgin female calculated from the mean CI with an intact virgin female. Kruskal–Wallis, $P < 0.0001$; ***$P < 0.0005$ by Dunn for Joint Ranking with the control *CS*; $n = 27–28$. (c) Transposon locations in *dumb* alleles (top) and dDA1 immunoreactivity (bottom). Boxes indicate exons and triangles denote the transposons *piggyBac{WH}* containing UAS and *Mi{MIC}* containing splice acceptor (SA) in *dumb*^{2} and *dumb*^{4}, respectively. The orange-colored boxes represent the open reading frame downstream of the UAS in *dumb*^{2}, which corresponds to the previously characterized dDA1 (Sugamori *et al.* 1995). The whole mount *CS* and *dumb*^{4} brains were stained with anti-dDA1 antibody and the Alexa 488-labeled secondary antibody. The stacked optical sections of the MB lobe areas in *CS* and *dumb*^{4} are shown (scale bar, 50 μm). Yellow arrowheads and arrows demarcate the α, α′, β, and γ lobes in *CS* and the MB lobe areas in *dumb*^{4}, respectively. (d) CI of the naïve *dumb*^{4} males paired with either an intact or decapitated virgin *CS* female (left two columns) and the percent reduction of CI with a decapitated virgin female calculated from the mean CI with an intact virgin female (right column). ***$P < 0.0001$ by Mann–Whitney *U* test, $n = 32–34$.

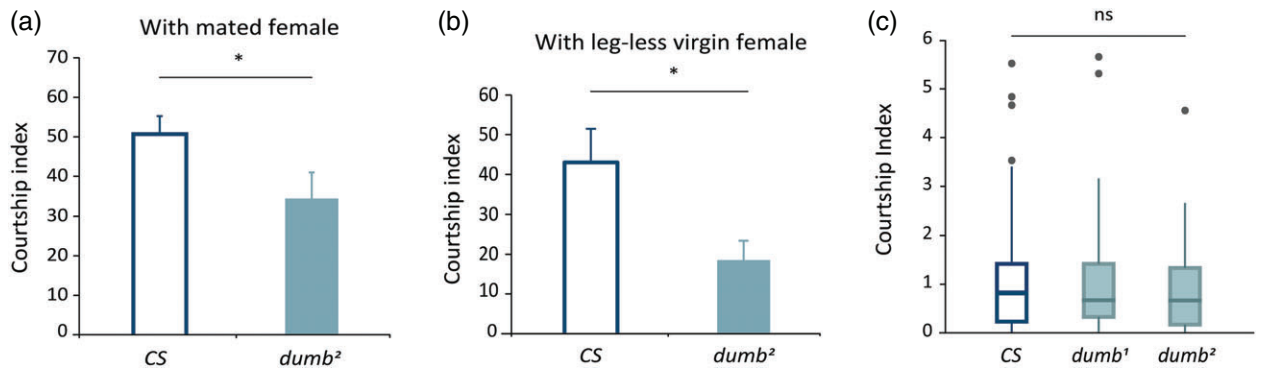

Figure 3: dDA1 is required to court suboptimal females. (a, b) CI of *CS* and *dumb*^{2} males with a mated female (a) or a leg-less virgin female (b). *$P < 0.05$ by Mann–Whitney *U* test, $n = 20–67$. (c) *CS*, *dumb*^{1} and *dumb*^{2} males exhibit comparable courtship activity with a male of the same genotype. The boxplot delineates minimum, the first quartile, median, third quartile and maximum as long with outliers in each genotype. ns, $P > 0.05$ by Kruskal–Wallis test, $n = 49–75$.

transcript splicing, likely generating truncated mRNA. Supporting the notion, the *MI04437* brain had barely detectable dDA1 immunoreactivity (Fig. 2c) thus was named as *dumb*^{4} allele since the *dumb*^{3} allele has been previously reported (Bang *et al.* 2011). Like *dumb*^{1}, *dumb*^{2} and *dumb*^{1}/*dumb*^{2}, *dumb*^{4} males showed substantially reduced courtship toward decapitated virgin females (Fig. 2d). These observations together suggest that dDA1 function is imperative to court less appealing mates.

Decapitated virgin females have appetitive pheromones such as intact virgin females but also have altered physical appearance and show limited movement. To examine a factor(s) responsible for the *dumb* male's reduced courtship, we used a mated female that freely moves around or an intact virgin female with amputated legs as a courtee. As with a decapitated female, *dumb*^{2} males exhibited significantly reduced courtship with both courtees ($P < 0.05$, Fig. 3a and b), suggesting that altered physical appearance, mated female characteristics (e.g. an aversive pheromone, a protruded ovipositor and rejection behavior) or limited movement be sufficient to discourage *dumb*^{2} male's courtship. In general, *CS* males exhibit infrequent but measurable courtship activity toward other males. When tested with a male, *dumb*^{1} and *dumb*^{2} males showed the courtship activity comparable to *CS* ($P < 0.05$, Fig. 3c), indicating that they can effectively distinguish males. Thus, *dumb* males do not have grossly altered visual or pheromone perception.

Diminished courtship drive accounts for dampened courtship activity of dumb males

The courtship activity quantified as CI represents the percentage of time that a male spent on all courtship activity. The reduced courtship activity of *dumb*^{2} males could be due to dampened courtship drive or rigor, both of which would contribute to low CI. Should it be due to dampened courtship drive, *dumb*^{2} males would show increased courtship latency. Should it be due to diminished courtship rigor, on the other hand, *dumb*^{2} males would exhibit limited courtship advancement, resulting in premature termination of the courtship ritual. Both *CS* and *dumb*^{2} males exhibited rapid courtship initiation with an intact virgin female, which did not differ between two genotypes ($P > 0.05$, Fig. 4a). The courtship initiation toward a decapitated female was delayed in both genotypes; however, the delay was significantly longer in *dumb*^{2} compared to *CS* males ($P < 0.005$, Fig. 4a). A *Drosophila* male performs multiple courtship bouts before he succeeds in copulation. Each courtship bout consists of the stereotyped ritual in the order of following and orientation, tapping, singing (wing vibration), licking and attempted copulation. When courtship ends in the middle of the ritual, a male begins from the first step (Han & Kim 2010). Like courtship initiation, *dumb*^{2} males had increased interval between courtship bouts compared to CS ($P < 0.005$, Fig. 4b). Thus, both delay in courtship initiation for the first bout and prolonged interval between bouts account for dampened courtship activity of *dumb*^{2} males.

We next examined whether *dumb*^{2} males have reduced courtship rigor. This was achieved by measuring duration of courtship bouts that ended at each courtship advancement (i.e. wing vibration, licking or copulation attempt), and calculating the percentage of the CI ended at each courtship step from the total CI. If all bouts ended at attempted copulation, the percent courtship advancement of attempted copulation would be 100 while the percentages of wing vibration and licking would be 0. We did not observe any difference between *CS* and *dumb*^{2} males in all courtship advancement stages toward a decapitated female ($P > 0.05$, Fig. 4c), indicating that the *dumb*^{2}'s low courtship activity was not due to premature termination of the courtship ritual. Similarly, the *dumb*^{2}'s low courtship activity was not due to aberrant behavior since there was no visible difference in resting and grooming behavior of *CS* and *dumb*^{2} males observed when they were not courting a decapitated female. Also, there was no difference in locomotor activity of *CS* and *dumb*^{2} males in the courtship chamber ($P > 0.05$, Fig. 4d). These observations together show that *dumb*^{2} males have diminished courtship drive, but not courtship rigor, toward a decapitated virgin female.

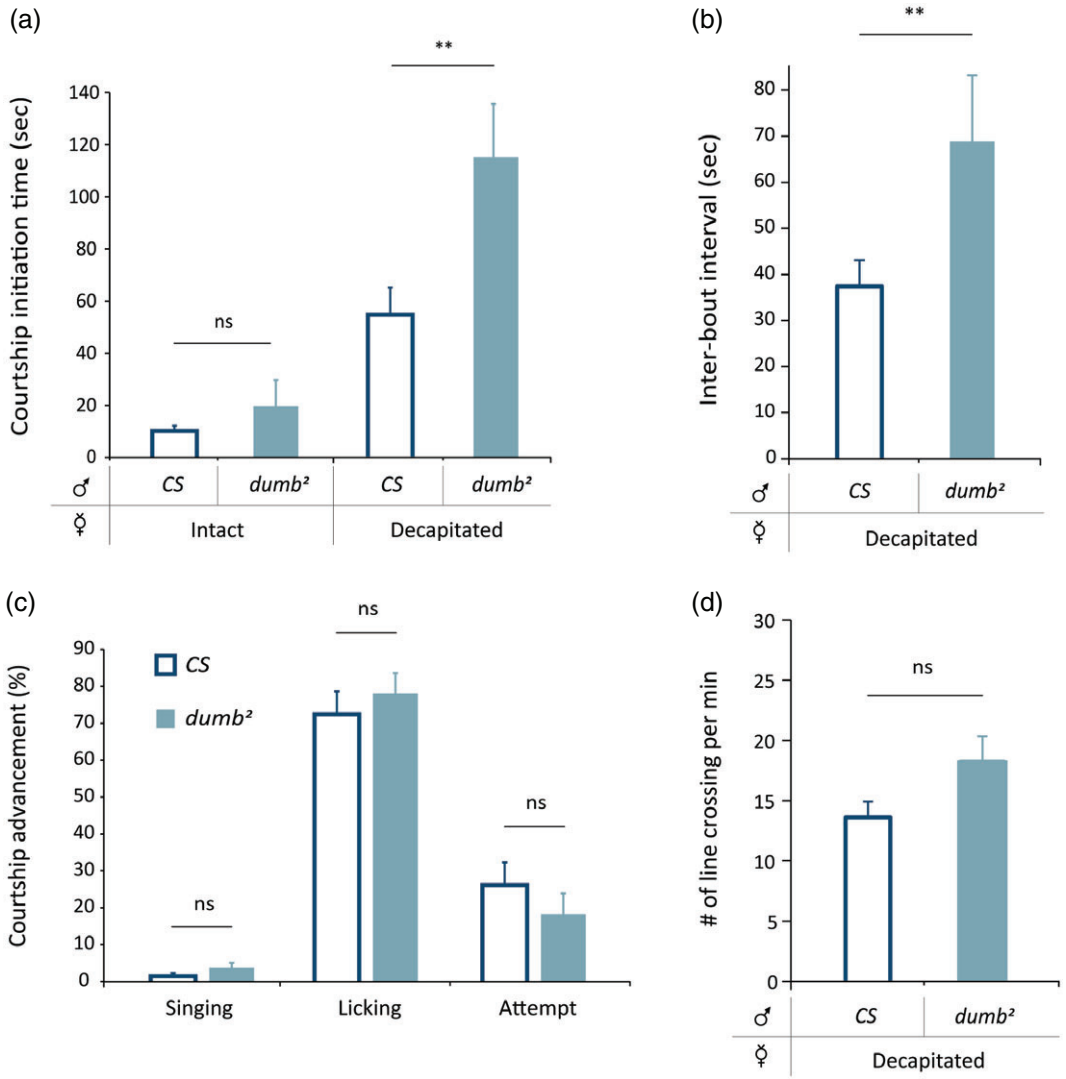

Figure 4: dDA1 is important for courtship drive but not for courtship rigor. (a) Courtship latency. The time that *CS* or *dumb*^{2} males began courting an intact or decapitated virgin female was measured. Mann–Whitney *U* test: ns, $P > 0.05$; $^{**}P < 0.005$; $n = 30–42$. (b) Interval between courtship bouts. Mann–Whitney *U* test: $^{**}P < 0.005$; $n = 30–42$. (c) Courtship rigor. The percentage of CI ended at each courtship step such as singling, licking or copulation attempt with the decapitated virgin female was calculated from the total CI to represent percent courtship advancement. Mann–Whitney *U* test: ns, $P > 0.05$, $n = 30–42$. (d) Locomotor activity. The number of times that a male crosses a midline drawn across the courtship chamber per min is shown. ns, $P > 0.05$ by two-tailed Student's *t*-test, $n = 32–42$.

dDA1 in the MB α/β and γ neurons mediates courtship drive

The study by Sakai & Kitamoto 2006 shows that blockade of the MB synaptic transmission delays courtship initiation and reduces courtship activity toward a virgin female, indicating an indispensable role of the MB for courtship motivation. Notably, these behavioral manifestations are similar to the phenotypes of *dumb* mutant males, implicating that the MB may be the site of dDA1's function in courtship drive. Supporting this notion, dDA1 is highly enriched in the MB lobes (Kim *et al.* 2003). We tested this notion by restoring dDA1 in the MB of *dumb*^{2} via MB-GAL4 and UAS present in the *dumb*^{2} locus (*UAS-dDA1*^{dumb2}) (Kim *et al.* 2007). Reinstated dDA1 expression in the MB *α/β* and *γ* neurons through *MB247-GAL4* or *NP1131-GAL4;NP3061-GAL4* (Fig. 5) completely restored courtship drive in *dumb*^{2} males toward a decapitated female (Fig. 6). To identify whether dDA1 in the *α/β* or *γ* neurons alone is sufficient, we used *c739-* and *NP1131-GAL4* drivers that are expressed in the *α/β* or *γ* neurons, respectively. dDA1 restored in *α/β* or *γ* did not rescue the *dumb*^{2} male's courtship drive (Figs. 5 and 6). dDA1 in the pigment dispersing factor (PDF) neurons is shown to be

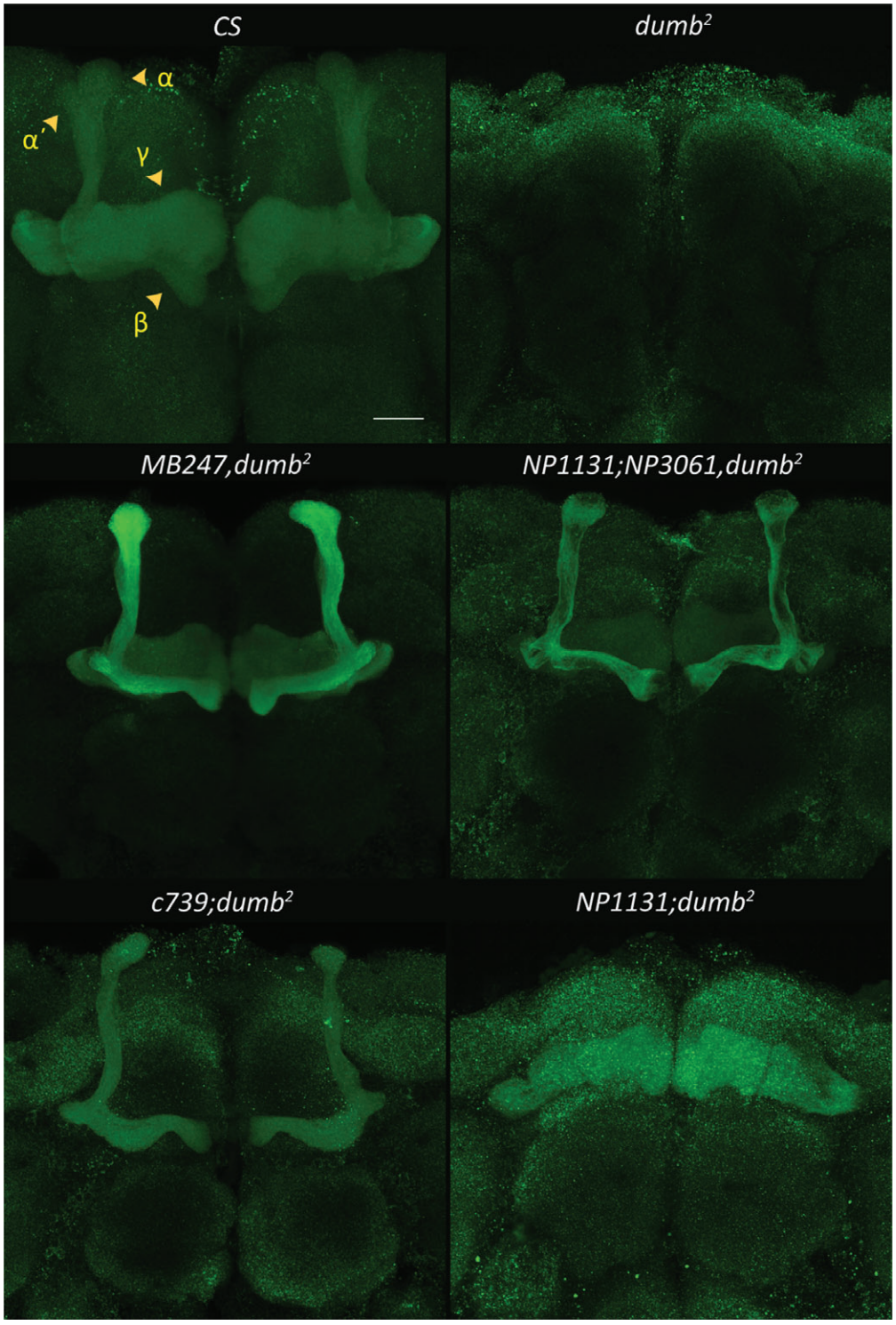

Figure 5: Restored dDA1 expression in the MB. dDA1 immunoreactivity was visualized by the Alexa 488-labeled secondary antibody. Shown are the stacked optical sections of the MB lobe areas in *CS*, *dumb*^{2}, *dumb*^{2} with reinstated dDA1 expression driven by *MB247-GAL4* in the α/β and γ lobes, *NP1131-GAL4;NP3061-GAL4* in the α/β and γ lobes, *c739-GAL4* in the α/β lobe, and *NP1131-GAL4* in the γ lobe. dDA1 expression is visible in all MB lobes in *CS* but undetectable in *dumb*^{2}. Yellow arrowheads demarcate the α, α', β, and γ lobes. Scale bar, 25 μm.

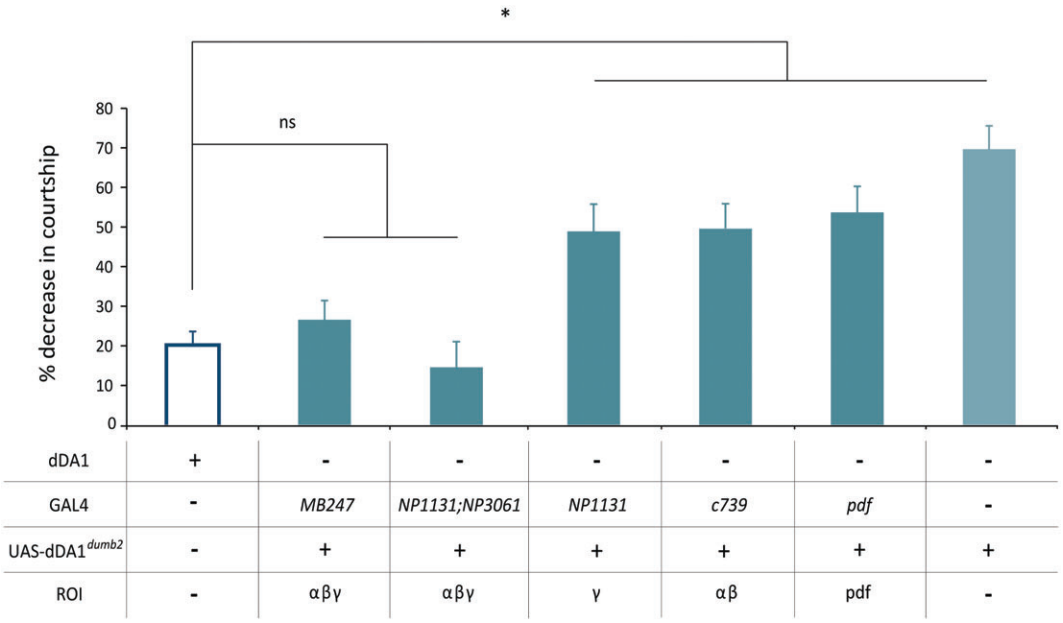

Figure 6: dDA1 in the MB α/β and γ lobes regulates courtship drive. The percent reduction of the CI with the decapitated female calculated from the mean CI with the intact female was measured in *CS* and *dumb*^{2} males along with the *dumb*^{2} males with restored dDA1 expression in the α/β and γ lobes (*MB247-GAL4* and *NP1131-GAL4;NP3061-GAL4*), γ lobe (*NP1131-GAL4*), α/β lobe (*c739-GAL4*), or PDF neurons (*PDF-GAL4*). Kruskal–Wallis test, $P < 0.0001$; ns, $P > 0.05$; $^*P < 0.05$ by Dunn for Joint Ranking with the control *CS*; $n = 31–42$.

important for locomotor arousal (Lebestky *et al.* 2009); however, dDA1 reinstated in the PDF neurons had no effect either (Fig. 6). These observations indicate the critical role of dDA1 in the MB α/β and γ neurons, but not in α/β or γ neurons alone, in courtship drive.

Discussion

The capacity to pursue and copulate with a potential mate correlates with reproductive success of an individual and species. In a natural competitive environment, a male fly would mate with a female that he first encounters, and failure to grasp the chance could diminish his reproductive success. Dopamine is shown to facilitate mating behavior and our study identifies dDA1 as the key receptor mediating innate mating drive of a naïve male. This corroborates the findings of the pharmacological studies on the roles of D1 receptor for male sexual motivation of songbirds and rodents (Riters *et al.* 2014, Stolzenberg & Numan 2011). Interestingly, the male fly deficient in dDA1 function courts well with an intact virgin female. This is in contrast to the previous finding that the male fly with defective dopamine neurotransmission exhibits reduced courtship toward an intact virgin female (Alekseyenko *et al.* 2010; Chen *et al.* 2013). This discrepancy could be due to redundant or compensatory function of other dopamine receptors. Such redundant or compensatory function could be sufficient for courting a highly receptive female that likely demands less courtship drive, but not for courting a decapitated female needing stronger motivation. The study of double or triple mutant combination should help clarify it. Alternatively, courtship drive for an intact virgin female may involve additional neuromodulator or neurotransmitter released from dopamine neurons (see below for further elaboration). Such neuromodulator or neurotransmitter would confer courtship drive toward an intact female in the absence of dDA1 function.

We have identified the MB α/β and γ neurons as the functional sites where dDA1 regulates courtship drive. The activity of dopamine neurons projecting to the γ lobe is crucial for aversive and appetitive olfactory memory formation and reinforcing or deprivation state (hunger, thirst or unsuccessful courtship) dependent motivation control, which is mediated by dDA1 in the γ neurons (Keleman *et al.* 2012, Kim *et al.* 2007, Krashes *et al.* 2009, Lin *et al.* 2014, Qin *et al.* 2012). Our finding is that dDA1 function in the γ neurons alone is not sufficient for innate courtship drive is intriguing. It is possible that the mechanisms by which dDA1 mediate innate courtship drive and experience-dependent motivation and plasticity could be distinct. Supporting this notion, dopamine regulates innate drive for sugar and this activity relies on dD2R in the subesophageal ganglion (Marella *et al.* 2012), whereas sugar reinforcement in appetitive conditioning is processed by dDA1 in the γ neurons (Kim *et al.* 2007, Liu *et al.* 2012). Perception of a potential mate involves multimodal sensory information (i.e. visual, olfactory, gustatory and auditory) processing (Clowney *et al.* 2015; Kohatsu & Yamamoto 2015). The MB receive multiple sensory information and moreover, olfactory and visual neural pathways

directly converge onto the MB α/β and γ (Aso *et al.* 2014, Vogt *et al.* 2016, Yagi *et al.* 2016). Profoundly increased courtship latency of *dumb* males implicates the active role of dDA1 in the α/β and γ neurons in evaluating a potential mate's information for mating decision, which is distinct from the previously characterized dDA1 functions in experience-dependent courtship or other learning and memory processes.

Our study indicates that individual dopamine receptors are dispensable for acquisition and short-term memory of associative conditioned courtship. This is in contrast to the study showing the role of dDA1 in courtship memory (Keleman *et al.* 2012). Courtship conditioning involves multiple types of behavioral plasticity including nonassociative and associative learning and memory (Griffith & Ejima 2009). The study by Keleman *et al.* has employed a mated female for training and testing whereas our study used a mated female for training and a virgin female for testing. A tester female lacking the unconditioned stimulus cVA seems better suited to address associative learning and memory. It is conceivable that dDA1 in the γ lobe could be involved in experience-dependent courtship motivation or nonassociative memory but not for associative courtship memory. The MB's role in courtship memory has been well established (Joiner & Griffith 1999, Keleman *et al.* 2007, McBride *et al.* 1999) and also in the α/β lobe, the α1-like octopamine receptor OAMB mediates acquisition of courtship memory (Zhou *et al.* 2012). It seems that other neuromodulator receptors including OAMB in the MB play major roles in associative courtship memory. Notably, our study shows that the neuromodulatory mechanisms for courtship drive and acquisition of associative courtship memory are distinct. This reinforces the notion that innate drive and experience-dependent courtship suppression are independently processed in the MB.

Montague & Baker 2016 have reported that blockade of synaptic output of the dopamine neurons projecting to the MB impairs courtship memory. In the mammalian brain, dopamine neurons have co-transmitters such as glutamate and GABA (Koos *et al.* 2011; Root *et al.* 2014; Tritsch *et al.* 2012). It is conceivable that the paradoxical findings made by manipulations of dopamine neuronal activity and dopamine receptors could be due to potential co-transmitters. Supporting this notion, metabotropic glutamate receptor antagonist or GABA treatments rescue defective courtship behavior including courtship memory of *dfmr1* (Fragile X gene) mutant males (Chang *et al.* 2008; McBride *et al.* 2005). It remains to be determined whether dopamine neurons projecting to the MB also release glutamate and/or GABA.

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