The mushroom body D1 dopamine receptor controls innate courtship drive

J. Lim‡, 1, A. I. Fernandez1, S. J. Hinojos1, G. P. Aranda1, J. James1, C.-S. Seong1,1 and K.-A. Han*1

Neuromodulation Disorders Cluster at Border Biomedical Research Center, Department of Biological Sciences, University of Texas at El Paso, El Paso, TX, USA
†Present address: Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322, USA
‡Present address: Department of Hematology and Medical Oncology, Emory University School of Medicine, Winship Cancer Institute of Emory University, GA 30322, USA
1K.A.H. conceived the experiments; J.L., A.I.F., S.J.H., G.P.A., J.J. and C.S.S. performed and analyzed experiments; and K.A.H. wrote the manuscript.

*Corresponding author: K.-A. Han, Department of Biological Sciences, Biosciences Building, Room 3.152, University of Texas at El Paso, 500 West University, El Paso, TX 79968, USA. E-mail: khan@utep.edu

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, Pfau 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 (Alexseyenko 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 hours 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 (Eijima et al. 2007; Tompkins et al. 1993). 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).

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

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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 cV 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 dumb1 with inversion mutation in the gene coding for D1 receptor-dDA1, dumb2 with insertional mutation in D1-dDA1, dumb with deletion mutation in D5-DAMB, d2rr (106521) with insertional mutation in D2-dD2R, and doppec (hereafter der) with insertional mutation in DopECR. All mutants were placed in the CS background. We previously reported dumb, dumb, and UAS-dDA1dumb (Kim et al. 2007) and dumb(Cassar et al. 2015); and d2rr and der have been characterized previously (Ishimoto et al. 2013; Marella et al. 2012). dumb, dumb, and der are high-homomorphic 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); dumb is a null allele as it has no DAMB transcripts detectable (Cassar et al. 2015); and d2rr is a strong hypomorphic or null allele since the mutation completely abrogates the dopamine neuronal signal for proboscis extension (Marella et al. 2012). The Mio4437 line (dumb) carrying the Minos MiMIC insertion (Venken et al. 2010) in the first intron of the dDA1/dumb gene was backcrossed and placed in the CS background. The fly lines dumb4 (A3773), der (10847), c379-GAL4 (7362), and c305a-GAL4 (38829) were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA); d2rr (106521) from the Exelixis Collection (Harvard Medical School, Boston, MA, USA); NP1131-GAL4 from Dr. Dumb (Stony Brook 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 CO2 within 12–18 h after eclosion and housed individually in a food vial. 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 x 8 x 5 mm3) 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 courter (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 (CImax) and without (mock; CImock) conditioning, was calculated by [100× (CImock − CImax)] where CImock 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 NaH2PO4, 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 x20 or x40 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 SPSS 17.0. 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 responds to dopamine and ecdysone; (Srivastava et al. 2005)].

To identify the dopamine receptors important for generalized courtship suppression, we examined the dumb\(2^\), dumb, 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\(2^\), dumb 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\(2^\), dumb and dd2r males showed comparable Pls 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 naive 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 M104437 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 2: dDA1 is needed to court less appealing mates. (a) Naïve CS and dumb² 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 Ml{MIC} containing splice acceptor (SA) in dumb² and dumb⁴, respectively. The orange-colored boxes represent the open reading frame downstream of the UAS in dumb², which corresponds to the previously characterized dDA1 (Sugamori et al. 1995). The whole mount CS and dumb⁴ 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 dumb⁴ are shown (scale bar, 50 μm). Yellow arrowheads and arrows demarcate the $\alpha$, $\alpha'$, $\beta$, and $\gamma$ lobes in CS and the MB lobe areas in dumb⁴, respectively. (d) CI of the naïve dumb⁴ 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, $n = 32–34$. 

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transcript splicing, likely generating truncated mRNA. Supporting the notion, the MI04437 brain had barely detectable dDA1 immunoreactivity (Fig. 2c) thus was named as dumb allele since the dumb allele has been previously reported (Bang et al. 2011). Like dumb, dumb and dumb, dumb 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 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 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 male’s courtship. In general, CS males exhibit infrequent but measurable courtship activity toward other males. When tested with a male, dumb and dumb 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 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 males would show increased courtship latency. Should it be due to diminished courtship rigor, on the other hand, dumb males would exhibit limited courtship advancement, resulting in premature termination of the courtship ritual. Both CS and dumb 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 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 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 males.

We next examined whether dumb 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 advancement stages toward a decapitated female (Fig. 4c), indicating that the dumb’s low courtship activity was not due to premature termination of the courtship ritual. Similarly, the dumb’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 males observed when they were not courting a decapitated female. Also, there was no difference in locomotor activity of CS and dumb males in the courtship chamber (P > 0.05, Fig. 4d). These observations together show that dumb 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 dumb2 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 singing, 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 \( \alpha/\beta \) and \( \gamma \) 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 dumb2 via MB-GAL4 and UAS present in the dumb2 locus (UAS-dDA1\( ^{dumb2} \)) (Kim et al. 2007). Reinstated dDA1 expression in the MB \( \alpha/\beta \) and \( \gamma \) neurons through MB247-GAL4 or NP1131-GAL4;NP3061-GAL4 (Fig. 5) completely restored courtship drive in dumb2 males toward a decapitated female (Fig. 6). To identify whether dDA1 in the \( \alpha/\beta \) or \( \gamma \) neurons alone is sufficient, we used c739- and NP1131-GAL4 drivers that are expressed in the \( \alpha/\beta \) or \( \gamma \) neurons, respectively. dDA1 restored in \( \alpha/\beta \) or \( \gamma \) did not rescue the dumb2 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, dumb², 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 dumb². Yellow arrowheads demarcate the α, α’, β, and γ lobes. Scale bar, 25 μm.
D1 receptor in courtship drive

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 dumb2 males along with the dumb2 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.

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 mediates 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 dDAl 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 dDAl 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 (Griffith & Ejima 2009). The study by Griffith & Ejima (2009) Generalization of courtship learning in Drosophila is mediated by cis-vaccenyl acetate. Curr Biol 17, 599–605.

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