Contribution of visual and circadian neural circuits to memory for prolonged mating induced by rivals

Woo Jae Kim, Lily Yeh Jan & Yuh Nung Jan

Rival exposure causes *Drosophila melanogaster* males to prolong mating. Longer mating duration (LMD) may enhance reproductive success, but its underlying mechanism is currently unknown. We found that LMD is context dependent and can be induced solely via visual stimuli. In addition, we found that LMD involves neural circuits that are important for visual memory, including central neurons in the ellipsoid body, but not the mushroom bodies or the fan-shaped bodies, and may rely on the rival exposure memory lasting for several hours. LMD is affected by a subset of learning and memory mutants. LMD depends on the circadian clock genes *timeless* and *period*, but not *Clock* or *cycle*, and persists in many arrhythmic conditions. Moreover, LMD critically depends on a subset of pigment dispersing factor neurons rather than the entire circadian neural circuit. Our study thus delineates parts of the molecular and cellular basis for LMD, a plastic social behavior elicited by visual cues.

Competition between males contributes to sexual selection, which Charles Darwin defined as the “struggle between the individuals of one sex, generally the males, for the possession of the other sex”1. In *Drosophila*, males vigorously compete with one another, and this aggressive behavior may help with the acquisition or defense of food resources as well as gaining access to mates2.

In addition to aggression, male-male competition may take the form of sperm competition3, a process for the males’ sperm to compete in fertilizing the ova4. One of the tactics for this process is prolonged copulation. In *Drosophila*, whereas there appears to be little correlation between the mating duration and fertility based on counting progeny numbers3, lengthening a male’s mating duration increases its paternity share among the progeny, indicative of greater reproductive success4.

Mating duration can be influenced by many factors, including environment5, age6, body size7, infection status of the male8 and whether the female is a virgin9. The mating duration is plastic and depends on the male’s social experience; *D. melanogaster* males respond to the presence of other males by prolonging the mating duration10. As little is known about the molecular and cellular basis of rival-induced LMD, we took advantage of the fact that mating duration traits are under male control11 and are therefore more amenable to genetic analysis than other social behaviors12, and characterized the underlying circuity.

**RESULTS**

**LMD in *D. melanogaster* is a plastic behavior**

The mating duration for the Dahomey strain is longer for males housed together with rivals for 5 d before mating10. Using the same experimental protocol, we found that the mating duration of group-reared wild-type Canton-S males also lasted longer (by more than 5 min) than that of singly reared males (Fig. 1a,b), although they had comparable timing for mating initiation (Fig. 1c) and mating success ratio over the course of 60 min (~85%). Similar LMD was observed in wild-type Berlin and Oregon R strains (Fig. 1a) and in another species of fruit flies, *D. simulans* (Supplementary Fig. 1a). LMD did not seem to depend on the number of rivals present during group rearing (Fig. 1d), as has been reported for the Dahomey strain13, nor did LMD vary with the number of males present during the mating duration assay (Supplementary Fig. 1g,h), in contrast with what has been reported for the Dahomey strain10. The white mutant (w1118) males showed no LMD (Supplementary Fig. 1b–f).

LMD was evident for Canton-S males with either Canton-S or w1118 female partners (Fig. 1e), consistent with findings that the mating duration is largely under male control in *D. melanogaster* and *D. simulans*11. When we introduced three Canton-S virgin females, housed them together with a single Canton-S male and replaced the females with virgin females daily over the course of 5 d, we found that the mating duration for these Canton-S males with prior exposure to females was shorter than that of singly or group-reared males (Fig. 1f), probably reflecting the fact that the males’ resource for mating is not unlimited13. By quantifying the mating duration every other day, we found that the group-reared males began to show LMD by the fifth day of cohabitation (Fig. 1g), whereas the mating duration of singly reared males became progressively shorter after 5 d of isolation (Fig. 1g).

To test for the reversibility of LMD, we shifted singly reared males to group rearing and moved group-reared males to subsequent housing in isolation. The mating duration was shorter for males that were group-reared for the first 5 d and then shifted to singly rearing for 5 d than for males that were group-reared for 10 d (Fig. 1h). In contrast, the mating duration was longer for males singly reared for the first 5 d and then group-reared for 5 d than for males that were singly reared for 10 d (Fig. 1h). Exposure of males that had been...
singly reared for 5 d to rivals for 24 h was sufficient to induce LMD (Fig. 1i). Thus, LMD is plastic; it can change over time in a way that depends on the male's experience with his rivals.

Visual stimuli are required for inducing LMD

LMD appears to require visual inputs, as Canton-S males that were singly or group-reared for 5 d in the dark did not show LMD (Fig. 2a). LMD was absent in blind males with photoreceptors ablative via GMR-hid (Fig. 2a and Supplementary Fig. 2c) or mutants with impaired vision, such as ninaB4 (which do not synthesize the rhodopsin chromophore14), ninaC3 (with degeneration of all rhabdomeres), ninaE7 (which lack opsin in R1–6 photoreceptors) and rh51 mutants (which lack opsin in R8 photoreceptors15) (Fig. 2b). To explore the possible involvement of other sensory stimuli, we tested Or83b1/Or83b2 mutants that showed no behavioral or electrophysiological responses to many odorants and found that group-reared males displayed LMD as compared with singly reared males (Fig. 2b) or males that were housed with four Canton-S females for 1 d (Supplementary Fig. 2d). In the w1118 genetic background with impaired vision, however, neither Or83b1/Or83b2 mutants nor the heterozygous controls displayed LMD (Supplementary Fig. 2g), indicating that Or83b-dependent olfactory inputs are dispensable, whereas the visual inputs are essential for LMD. Moreover, presentation of male odors to singly reared males could not induce LMD (Supplementary Fig. 2f). We also tested the iav1 auditory mutants18 and found that they showed LMD (Fig. 2b). Thus, visual stimuli, but not other sensory stimuli, are required for LMD, although we cannot rule out the possible involvement of nonvisual sensory stimuli.

We next investigated the nature of the visual inputs that elicit LMD. Drosophila can detect color and motion17,18. We presented different color stimuli to singly reared males and could not induce LMD (Supplementary Fig. 2a,b). Notably, rearing a Drosophila melanogaster Canton-S male together with three Drosophila simulans or Drosophila virilis males induced LMD of the Canton-S male (Fig. 2c). In contrast with the male's indifference to w1118 male rivals, LMD was induced by w1118 male rivals bearing the mini-white transgene that bestowed the red eye color (Fig. 2c), as well as by male rivals with orange eyes (Supplementary Fig. 2c). Given that stationary color dots did not induce LMD, but moving males with red compound eyes did, we hypothesized that both color and motion are important for inducing LMD. As a test, we placed a small mirror at the bottom of the vial that housed a single male. The mating duration was longer for singly reared males with a mirror that reflected the male’s image than for control singly reared males with the mirror turned upside-down so that the male could not see his reflection (Fig. 2d,e). Although the singly reared male visited the area occupied by the mirror or the upside-down mirror with similar frequency (8.1 visits per h with a mirror, 7.5 visits per h with a reversed mirror), it lingered for an average of 60 s over the reversed, upside-down mirror (Supplementary Fig. 2h). We then tested the effect of viewing moving females with red eyes and found that LMD was displayed by singly reared males that were separated by a transparent film from three other male or female flies (Fig. 2f). Thus, the visual stimulus that induces LMD in nature likely corresponds to flies with red compound eyes in motion.
Figure 2 LMD is induced by visual stimuli. (a) Mating duration assays of group-reared (G; white bars) or singly reared (S; black) Canton-S males in constant darkness for 5 d and of GMR-Hid flies (blind). From left to right, n = 29, 29, 15 and 19 (t = 1.247 and 0.4406). (b) Mating duration assays of various vision, olfactory and auditory mutants. The genotypes are indicated below the bars. From left to right, n = 17, 20, 29, 23, 27, 19, 26, 44, 25, 20 and 23 (t = 0.2103, 0.1664, 0.7524, 1.672, 3.436 and 4.724). (c) Mating duration assays of group-reared males (white bars), singly reared males (black) or males reared with three D. simulans or D. virilis (red), w* strains (pink), or w1118 males (gray). The genotype of the w* strain was w*, tub-GAL80ts. Eye color of w*, tub-GAL80ts strain is bright red. From left to right, n = 22, 22, 27, 27, 30 and 24 (F = 20.83). (d) Mating duration assays of group-reared males with a mirror or reversed mirror (G w/mirror and G w/mirror rev), or singly reared males with a mirror or reversed mirror (S w/mirror and S w/mirror rev) in place for 5 d. Reversed mirror is a mirror placed upside-down at the bottom of the vial, so that the male cannot see his reflection in the mirror. The color of the backside of the mirror was dark green. From left to right, n = 15, 25, 24 and 23 (F = 4.891). (e) The presentation of a mirror to a male fly. A small round mirror was placed at the bottom of the food vial to generate the male’s reflection. (f) Mating duration assays of group-reared males (white bars), singly reared males (black), and males reared with either three other males (blue) or three females (red) separated by transparent film. From left to right, n = 28, 22, 18 and 20 (F = 19.19). **p < 0.01, Student’s t test. *p < 0.05, Dunn’s multiple comparison test.

Timeless and per period are involved in LMD

Transcriptional feedback loops are critical for circadian clocks. In Drosophila, the Clock (Clk) and cyc (cyc) genes activate the period (per), timeless (tim), vrille (vri), PAR domain protein 1 (Pdp1) and clockwork orange (cwo) genes, which in turn inhibit CLK-activated transcription or regulate Clk transcription. Clock genes also regulate non-circadian phenomena, such as the male courtship song frequency, developmental time, sleep length, delayed cocaine sensitization and the time course of giant fiber habituation. Moreover, tim and per regulate the mating duration of Drosophila melanogaster.

LMD was exhibited by Clk and cyc mutant males, but not tim and per mutant males (Fig. 3a,b) or males with mutation of cryptochrome (cry), which regulates the function of TIM, or mutation of doubletime (dbt), which encodes the kinase that phosphorylates PER (Fig. 3c). Moreover, LMD persisted in males with mutation of cwo, which encodes a putative bHLH transcription factor that acts preferentially at night to help terminate CLK-CYC–mediated transcription of target genes (Fig. 3c). Thus, PER and TIM, but not CLK and CYC, are required for LMD, even though these core clock gene products act together to regulate circadian rhythm.

Figure 3 LMD is affected in tim and per mutants, but not in Clk or cyc mutants. (a) Mating duration assays of various tim and per mutants. The genotypes are indicated below the bars. From left to right, n = 42, 32, 18, 15, 21, 18, 21, 24, 21, 27, 24, 28 and 28 (t = 0.3659, 0.9971, 1.869, 1.534, 0.02206, 0.7068 and 0.3400). (b) Mating duration assays of various Clk and cyc mutants. The genotypes are indicated below the bars. From left to right, n = 16, 20, 28, 24, 24, 26, 27 and 26 (t = 5.475, 3.742, 4.758 and 5.754). (c) Mating duration assays of various cry, dbt and cwo mutant animals. The genotypes are indicated below the bars. From left to right, n = 21, 20, 21, 20, 22, 16, 20, 24, 28 and 27 (t = 0.3774, 0.09724, 1.989, 0.9722 and 3.081). (d) Mating duration assays of group- or singly reared Canton-S males at 22 °C, 25 °C and 29 °C. From left to right, n = 83, 65, 46, 46, 22 and 20 (t = 8.108, 9.143 and 3.060). (e) Mating duration assays of group- or singly reared Canton-S males in standard 12-h dark:12-h light condition (D:L), in constant dark condition (D:D) and in constant light condition (L:L) at 25 °C. From left to right, n = 78, 62, 30, 30, 23 and 18 (t = 7.706, 1.247 and 4.554). (f) Mating duration assays of group- or singly reared Canton-S males at various time points of the 24-h circadian cycle (Zeitgeber time (ZT) = 0 at the onset of light for 12-h dark:12-h light condition). Mating duration assays were performed at Z0, T6, T12 or T18. From left to right, n = 24, 24, 16, 21, 18, 16, 30 and 17 (t = 6.350, 4.951, 6.599 and 4.140). *p < 0.05, **p < 0.01 and ***p < 0.001, Student’s t test.
Circadian clocks may be synchronized by light:dark cycles or by temperature fluctuations. We tested Canton-S males reared at different temperature for 5 d, and found that they all exhibited LMD (Fig. 3d). LMD was also intact for males that were constantly exposed to light (Fig. 3e), a condition that is known to induce arrhythmic activities, and those exposed at different times of the day (Fig. 3f). Although there may be a tendency for the mating duration to be shorter in the evening, the variability of this measurement, in contrast with the consistent display of LMD, precluded any quantitative comparisons of mating durations under different conditions. Moreover, expression of PER or TIM in per and tim mutants with the pan-neuronal GAL4 driver (elav-GAL4) was able to rescue LMD (Fig. 4a,b), even though the transgene mRNA expression is not rhythmic, indicating that LMD depends on TIM and PER function rather than their cyclic expression during circadian rhythm.

To identify the neurons that require the PER activity for LMD, we used different GAL4 drivers to express PER in various subsets of circadian pacemaker neurons in per mutants. Circadian pacemaker neurons are divided into six groups: three dorsal clusters (DN1-3) and three lateral clusters (LNp, 1-LNv, and s-LNv) (Fig. 4). LMD was restored in per mutants expressing the UAS-per transgene via GAL4 drivers in broad circadian cells, pigment dispersing factor (PDF)-expressing neurons and lateral clock neurons including PDF-expressing cells (Fig. 4c and Supplementary Fig. 3f), and have adult-specific expression of UAS-per via tub-GAL80P was sufficient for LMD (Fig. 4b). However, LMD was not rescued by expressing the UAS-per transgene in all of the neuronal progenitors except for the PDF-expressing neurons via a pan-neuronal GAL4 driver combined with pdf-GAL80 (Fig. 4b). Moreover, LMD was abolished in pdf mutant flies lacking the neuropeptide PDF (Supplementary Fig. 3e). Thus, PER and PDF function in PDF neurons of the adult male are crucial for generating LMD.

To address the question of whether LMD requires the function of per and tim, but not CLK and cyc, in PDF neurons, we used a dominant-negative CLK transgene (UAS-CLKDN) that includes the protein interaction domains, but not the basic DNA-binding domain.

Disrupting the CLK function in PDF neurons in an adult-specific manner using tub-GAL80 had no effect on LMD (Fig. 4d), suggesting that the CLK function in PDF neurons is not required to generate LMD, even though disrupting CLK function in the PDF neurons abolishes circadian rhythm. Taken together, these findings indicate that circadian rhythm is not crucial for LMD.

**LMD involves a subset of circadian neural circuits**

To further characterize the neural circuits that are important for LMD, we electrically silenced defined groups of cells in a temperature-dependent manner by expressing Kir2.1 potassium channels using various GAL4 drivers together with tub-GAL80P, which allows Kir2.1 expression to be elevated by temperature shifts to silence the GAL4-expressing cells. Flies were reared at 29 °C for the first 2 d to strongly induce Kir2.1 expression and then shifted to 25 °C for the next 3 d for mild induction of Kir2.1 before testing (Fig. 5a).

LMD was not altered by expressing Kir2.1 in some of the peripheral sensory neurons (ppk-GAL4 and 21-7-GAL4), the wing boundary tissues that might be involved in male courtship songs (vg-GAL4), the octopamine/tyramine neurons (tdc-GAL4) or the dopaminergic neurons (Ddc-GAL4) involved in aggression (Fig. 5b). In contrast, expression of Kir2.1 in the visual system (GMR-GAL4 and ey-GAL4) abolished LMD (Fig. 5b and Supplementary Fig. 3a). Inactivation of subsets of neurons for sensing olfactory and gustatory stimuli did not abolish LMD either (Fig. 5b). Thus, neurons involved with visual information processing are important for the generation of LMD, consistent with our visual mutant findings (Fig. 2).

Given that LMD depends on a subset of the clock gene products, including PER function in PDF neurons, we next investigated the role of electrical activity in circadian pacemaker neurons. LMD was abolished following inactivation of all clock neurons by expressing Kir2.1 with tim-GAL4 or per-GAL4 (Fig. 5c). Inactivation of PDF-expressing cells, which include 1-LNv, and s-LNv, abolished LMD (Fig. 5c).
Inactivation of CRY-positive cells, which include most of the lateral neurons and a small subset of dorsal neurons, also abolished LMD (Fig. 5c). Inactivation of lateral neurons via the Mai179-GAL4 driver abolished LMD (Fig. 5c). Inactivation of a subset of LNv and LNp neurons that express neuropeptide P (NPF), a homolog of mammalian neuropeptide Y, via npf-GAL4 also abolished LMD (Fig. 5c). These findings implicate the electrical activity of lateral clock neurons in the generation of LMD.

It is well known that s-LNv neurons, which project dorsally to dorsal neurons, are the primary circadian pacemaker cells. Moreover, the DN3 and DNp cells send projections toward the s-LNv cell bodies. To test whether dorsal neurons are involved with LMD, we used Clk-GAL4 drivers that specifically target a subset of dorsal neurons. Notably, inactivation of a subset of dorsal neurons using Clk4.1M-GAL4 or Clk4.5F-GAL4 drivers had no effect on LMD (Fig. 5c). Thus, although the electrical activity required for LMD generation involves lateral neurons, it does not require this subset of dorsal neurons that are important for circadian rhythm generation. For all of the experiments involving UAS-Kir2.1, normal LMD was displayed by control flies carrying GAL4 or UAS-Kir2.1 reared at the non-permissive temperature and by flies carrying GAL4, tub-GAL80δ and UAS-Kir2.1 that were reared at the permissive temperature (Supplementary Fig. 4a–h).

Given that either electrical silencing or excessive excitation of clock neurons can abolish the rhythmic behavior, we sought to activate these same neurons by overexpressing the bacterial voltage-gated sodium channel NachBac using the pdf-GAL4 driver. We found no effect on LMD (Supplementary Fig. 4j), although UAS-NaChBac expression in LNp pacemaker neurons abolishes the cyclic accumulation of PDF and induces complex behavioral rhythms with multiple superimposed periods. However, overexpression of the temperature-sensitive dominant-negative dynamin mutant shibire (UAS-shi) in PDF neurons eliminated LMD (Supplementary Fig. 4i). Thus, electrical silencing or inhibition of dynamin-dependent endocytosis of PDF neurons may affect LMD, but activating those neurons has no effect on LMD. Our findings implicate a subset of the lateral neurons in LMD generation, which likely involves processes that are distinct from the regulation of circadian rhythm (Fig. 4e and Supplementary Fig. 3f).

LMD requires neurons in the ellipsoid body

In Drosophila, there are several phases of memory: short-term memory, which lasts for less than an hour, mid-term memory, which lasts for 1–3 h, and anesthesia-resistant memory and long-term memory, which are two forms of long-term memory that are distinguishable by training procedures. To test how long the rival exposure memory for LMD lasts, we shifted group-reared flies to single housing at various time points before the mating duration assay. LMD persisted at the same level for up to 6 h of single housing, but began to fade after 6–12 h of housing in isolation (Fig. 6a). Thus, it appears that the rival exposure memory for LMD lasts longer than short-term or mid-term memory. Furthermore, because every mating duration assay was performed after mild CO2 anesthesia, LMD likely relies on a form of rival exposure memory that is resistant to anesthesia.

Next, we tested the dnc, rut and amn mutants, which suffer from defective learning and memory. LMD was impaired in rut and amn mutants, but not in dnc mutants (Fig. 6b). To confirm that the LMD defects of amn mutants are indeed a result of amn mutation, we rescued the LMD defect of males bearing the amn mutation on the X chromosome by introducing a third chromosome that carries a short duplication of the X chromosome, including the amn gene (Supplementary Fig. 3c). Genetic intervention has provided strong evidence that the mushroom bodies act as the seat of memory for odors. In contrast, the visual pattern memory of D. melanogaster involves the central complex, which includes the ellipsoid body and fan-shape bodies. To identify brain regions that are likely to be involved in memory processing in LMD, we drove expression of UAS-Kir2.1 in the ellipsoid body, fan-shaped bodies or mushroom bodies using several GAL4 lines (Fig. 6). LMD was not altered by UAS-Kir2.1 expression in the mushroom bodies via ok107-GAL4 (Fig. 6c), but was abolished by Kir2.1 expression in the ellipsoid body via EB-GAL4 and c547-GAL4 (Fig. 6c). We also tested GAL4 drivers for expression of Kir2.1 in fan-shaped bodies via GAL414–94 and found no effect on LMD (Fig. 6c). Given that the mushroom body inputs in Drosophila are predominantly olfactory, the dependence of LMD on the ellipsoid body rather than mushroom bodies is consistent with our observation that LMD requires visual stimuli.

Having found that LMD depends on the rut gene and central neurons in the ellipsoid body, we used different GAL4 drivers to express the rut gene product in various subsets of neurons in rut mutants. Expression of the UAS-rut transgene with GAL4 drivers for expression in lateral clock neurons, mushroom bodies or fan-shaped bodies did not restore LMD to rut mutants (Fig. 6d).
Figure 6 LMD requires visual memory. (a) Flies were group- (white) or singly reared (black) for five days. Group-reared flies were shifted to single rearing condition for 2 h (yellow), 6 h (red), 12 h (sky blue), or 24 h (blue) before mating duration assay. From left to right, n = 46, 23, 31, 27, 22 and 24. F = 30.54. (b) Mating duration assays of memory mutants. The genotypes are indicated below the bars. From left to right, n = 21, 22, 14, 15, 12 and 14 (t = 5.2061, 0.5560 and 0.2420). (c) Mating duration assays of GAL4 drivers that label the central complex using tub-GAL80; UAS-Kir2.1. The experimental procedure was the same as described in Supplementary Figure 5a. GAL4 control experiments are shown in Supplementary Figure 4g. From left to right, n = 21, 18, 21, 20, 30, 28, 32 and 28 (t = 1.2925, 0.9622, 4.8442 and 4.3464). (d) Mating duration assays of rut2080 mutant with UAS-rut transgene expression driven by GAL4 drivers as indicated below the bars. GAL4 control experiments are shown in Supplementary Figure 3d. From left to right, n = 24, 24, 32, 31, 32, 35, 17, 17, 16 and 17 (t = 0.0664, 0.1289, 2.8196, 0.7981 and 0.8027). (e) GAL4 expression profiles in the brain. Flies expressing each of the GAL4 drivers were crossed with UAS-mCD8GFP flies and immunostained with antibodies to GFP (green) and nc82 (magenta) (c547-GAL4, ok107-GAL4 and GAL414–94). Top panels show 200x images of the boxed regions. White arrows indicate the brain regions labeled by GAL4 drivers (EB, ellipsoid body; MB, mushroom bodies; FB, fan-shape bodies). Scale bars represent 100 µm. (f) Mating duration assays for GAL4 drivers that label the central complex using tub-GAL80; UAS-Kir2.1. GAL4 drivers that affect LMD are marked in red and those with no effect on LMD are in blue. The names of the GAL4 drivers are indicated below the bars. GAL4 control experiments are shown in Supplementary Figure 5b. From left to right, n = 50, 33, 17, 15, 16, 14, 31, 31, 15, 13, 13, 16, 16, 16, 27, 32, 32 and 29 (t = 6.8137, 0.1023, 0.2649, 4.7739, 3.5733, 0.0471, 4.4566, 1.0110 and 4.6859). **P < 0.01 and ***P < 0.001, Student’s t test. *P < 0.05, Dunn’s multiple comparison test.

However, UAS-rut expression in the ellipsoid body driven by c547-GAL4 was able to rescue the LMD defect of rut2080 mutants (Fig. 6d) and rutMB2769 mutants (Supplementary Fig. 3b). Taken together with the previous report that UAS-rut expression in the ellipsoid body driven by c547-GAL4 could successfully restore the visual memory defect of rut2080 mutants35, our observations suggest that the expression of rut in the ellipsoid body is likely to be important for processing the visual memory required for LMD.

The central complex is composed of four interconnected neuropils: the protocerebral bridge, the fan-shaped bodies, the ellipsoid body and the noduli. By using several GAL4 lines with known expression patterns in the central complex37 to drive the expression of Kir2.1, we found that the LMD was eliminated by Kir2.1 expression via the c42-GAL4 line, which labels R2 and R4m neurons in the ellipsoid body, the c161-GAL4 and 0787-GAL4 lines, which label a small field in the ellipsoid body, and the c819-GAL4 line, which labels R2 and R4m neurons in a pattern similar to that of c547-GAL4 (Fig. 6f). Given that the c507-GAL4 line, which labels R3 and R4d neurons, had no effect on LMD, LMD likely requires the activity of R2 neurons in the ellipsoid body. The GAL4 lines that label other central complex areas had no effect on LMD (Fig. 6f).

Our findings implicate a subset of neurons in the ellipsoid body in the generation of LMD (Supplementary Fig. 5c).

To further narrow down the relevant subset of neurons, we used various specific promoters to drive GAL80, which blocks the transcription of GAL4, in combination with the GAL4 driver, so that only those neurons expressing GAL4, but not GAL80, would express Kir2.1. We used GMR-GAL80 to suppress expression of UAS-Kir2.1 in the compound eye, pdf-GAL80 to suppress expression in lateral clock neurons, or cry-GAL80 to suppress expression in lateral clock neurons and a subset of the dorsal clusters of clock neurons. LMD was not affected by combining UAS-Kir2.1 with GMR-GAL80 and either ey-GAL4 or ap-GAL4 (Fig. 7a), indicating that the compound eye is crucial for LMD. In contrast, LMD was eliminated by the expression of UAS-Kir2.1 driven by pdf-GAL4 or cry-GAL4, even in the presence of GMR-GAL80 (Fig. 7a), indicating that the clock neurons labeled by these GAL4 lines are important for LMD. Moreover, LMD was eliminated by Kir2.1 expression via GAL4 drivers that label lateral clock neurons, such as cry-GAL4, npf-GAL4 and Mai179-GAL4, but not when these GAL4 drivers were combined with pdf-GAL80 (Fig. 7b), indicating that PDF neurons are important for generating LMD. Similar results were obtained when we used cry-GAL80, which labels a broader subset of clock
neurons including PDF neurons (Fig. 7c). When combined with GMR-GAL80, pdf-GAL80 or cry-GAL80, only c547-GAL4 was able to drive Kir2.1 expression and eliminate LMD (Fig. 7), indicating that neuronal activity of the ellipsoid body is critical for generating LMD. Taken together, these findings implicate the compound eye, PDF neurons and neurons in the ellipsoid body in the generation of LMD. Although the compound eye processes visual inputs, the ellipsoid body is likely to be involved in visual memory. Given that PDF neurons may be connected with the ellipsoid body through l-LNv38 (Supplementary Fig. 5d), it would be interesting determining how PDF neurons might be involved in conveying the information regarding rival exposure to other central neurons.

DISCUSSION

Our findings provide evidence that males retain the memory of rival exposure, based primarily on visual stimuli, for several hours and lengthen mating duration accordingly. Indeed, LMD could be induced by allowing a male to view flies of either sex through a transparent partition, flies of different species or images of themselves in a mirror (Fig. 2), indicating that LMD could be generated by visual stimuli without chemical communication. We found that the circadian clock genes tim and per, but not clk or cye, are important for LMD (Fig. 3). Not only could the LMD defects of per and tim mutants be rescued by the expression of PER or TIM via the nonrhythmic GAL4 driver (Fig. 4a,b), expression of PER in PDF neurons was sufficient to restore LMD to per mutants (Fig. 4c). Moreover, LMD requires electrical activity in lateral neurons, but not some of the dorsal neurons that are important for circadian rhythm (Fig. 5). It therefore seems unlikely that circadian rhythm regulation is crucial for LMD. We found that LMD involves the memory of rival exposure that lasts for several hours and is resistant to anesthesia (Fig. 6a) and that it requires the rut function in the ellipsoid body (Fig. 6c,d). Finally, we found that LMD generation depends on the activity of the compound eye, the PDF neurons and a subset of neurons in the ellipsoid body (Fig. 7).

Recent studies of social experience–mediated and context-dependent sexual behaviors of the fruit fly imply chemical communication of males via pheromones as being important. We found that vision in a social setting is also important for generating LMD. Although a recent report found that males use multiple redundant cues to detect mating rivals, we found that LMD can be elicited by visual cues because rearing flies in constant darkness eliminated LMD (Fig. 2a), blind mutants and males with defective vision showed no LMD (Fig. 2a,b), and LMD can be generated simply by placing a mirror to allow a singly reared male fruit fly to see his reflection for 5 d (Fig. 2d). The visual stimulus for LMD likely derives from the red compound eye in motion because LMD can be induced by males of different species or females visible through a transparent film (Fig. 2c,f), but not by mutant males without red pigment in their compound eyes (Fig. 2c).

LMD provides a new method for studying visual memory. To date, learning and memory studies in flies have focused primarily on the memory circuits in mushroom bodies, but LMD requires a subset of neurons in the ellipsoid body rather than mushroom bodies (Fig. 6c–f). The ellipsoid body is the central brain region required for visual learning and memory, whereas mushroom bodies are not required for memory formation in visual learning in a flight simulator. Given that the mating duration assay is simpler than the flight simulator for the investigation of visual memory, it can be useful for large-scale genetic screens to identify mutants with altered visual memory.

METHODS

Methods and any associated references are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank S. Zhu for the unpublished fly line GAL41–94. We also thank A. Keene, J. Blau, M. Heisenberg, C. Helfrich-Förster, L. Griffith, R. Allada, M. Noll, J.L. Price, A. Sehgal, M. Young, J.D. Armstrong and M. Sato for kindly providing valuable flies. We are grateful to A. Keene for valuable discussion of this project and J. Berg for assisting with the writing of the manuscript. The work was supported by US National Institutes of Health grant 2R37NS040929 to Y.N.J. L.Y.J. and Y.N.J. are investigators of the Howard Hughes Medical Institute.

AUTHOR CONTRIBUTIONS

W.J.K. designed and performed the experiments. W.J.K., Y.N.J. and L.Y.J. wrote the manuscript. Y.N.J. and L.Y.J. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nn.3104. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Darwin, C. The Descent of Man, and Selection in Relation to Sex (John Murray, 1871).
2. Kim, Y.-K. Sexual selection and aggressive behavior in Drosophila. In Handbook of Behavior Genetics (ed. Kim, Y.-K.) 317–330 (Springer New York, 2009).
3. Ridley, M. Seminal work. Nature 397, 576–577 (1999).
ONLINE METHODS

Fly rearing and strains. *D. melanogaster* were raised on cornmeal-yeast medium at similar densities to yield adults with similar body sizes. Flies were kept in 12-h light:12-h dark cycles at 25 °C (ZT0 is the beginning of the light phase; ZT12 is the beginning of the dark phase) except for some experimental manipulation (constant dark, constant light and experiments with the flies carrying *tub-GAL80*).

Wild-type flies were Canton-S. To reduce the variation from genetic background, we backcrossed all flies for at least three generations to Canton-S strain. All of the mutants and transgenic lines that we used have been described previously.

**per**, **per**, **cycl**4, **Clk**4, **Clk**4, **tim-GAL4**; **GMR-GAL80**, *pdf-GAL80*, *cry-GAL80* and, and **UAS-CkDN** were obtained from A.C. Keene and J. Blau (New York University). Wild-type Berlin, **rbt** and **ninaE** were obtained from M. Heisenberg (Universität Würzburg)15. **Mat1**-**GALA4** was obtained from C. Helfrich-Förster (Universität Würzburg)27. **pdf**-**GAL4** was obtained from L.C. Griffith (Brandeis University)45. **Clk**4.5**F-GAL4** and **Clk**4.1**M-GAL4** were obtained from Ravi Allada (Northwestern University, USA)29. **ninaB** were obtained from M. Noll (University of Zürich)46, **dbt** and **dbt** were obtained from J.L. Price (University of Missouri)37. **per**3, **tim**2 and **tim** were obtained from M.W. Young (Rockefeller University). **c547-GAL4**, **c507-GAL4** and **121Y-GAL4** were obtained from J.D. Armstrong (University of Edinburgh). **bsh-GAL4**, **def-GAL4** and **bsh-GAL4** were obtained from M. Sato (Kanazawa University)48. **GMR-Hid**, **Ork83p**, **Ork73p**, **ninaC**4, **cyc5**7, **dnc**1, **ruf2080**, **ann1**, **cw51562**3, **cry**48**B0849**, **UAS-tubGAL80**6, **UAS-Kor2.1**, **UAS-shi**6, **UAS-NachBac**6, **dbt**4 and **UAS-rut**4 were obtained from J.D. Armstrong (University of Edinburgh). **c161-GAL4**, **c205-GAL4**, **c819-GAL4**, **c07Y-GAL4**, **c078Y-GAL4** and **w1118**; **Dp(1;3)DC368**, **PBac[DC368]** were obtained from the Bloomington Stock Center. **D. simulans** and **D. viridis** were obtained from the Kyoto Stock Center (Drosophila Genetic Resource Center). Small mirrors were obtained from Factory Direct Craft (3/4-inch round glass mosaic tile mirrors).

Mating duration assays. Males of the appropriate strain were collected individually and placed into vials with food. In the case of group-reared condition, four males from the same strain were placed into vials except for some experimental manipulation (for example, Fig. 2d). The day of eclosion was designated as day 1 of adult life. Canton-S females were collected from bottles and placed into vials for 5 d. Each vial contained 10 s, and total mating duration was calculated for each couple.

Immunostaining. To examine the expression pattern of GAL4 in adult brains, we crossed GAL4 transgenic lines with **UAS-mCD8-GFP** flies. Brains of adult flies were dissected 5 d after eclosion. Dissected brains were subjected to immunostaining as described previously40. Briefly, dissected adult brains were fixed in 4% formaldehyde (vol/vol) for 30 min at 20–22 °C. After fixation, brains were washed with 1% PBT (phosphate-buffered saline + Triton X-100, vol/vol) three times (30 min each) and blocked in 5% normal donkey serum (vol/vol) for 30 min. The brains were then incubated with primary antibodies in 1% PBT at 4 °C overnight followed by fluorophore-conjugated secondary antibodies for 2 h at 20–22 °C. Brains were mounted with anti-fade mounting solution (Invitrogen, catalog #S2828) on slides for imaging.

Statistical analysis. We performed three independent tests for all of the experiments. More than 12 males (group- or singly reared) were used for each independent experiment. Besides assessment of significance from statistical test (Student’s t test), we included the data set, which showed statistical significance for at least two of these three independent tests. Our experience suggests that relative mating duration value differences between group- and singly reared flies are always consistent; however, both absolute value and magnitude of the difference in each strain can vary. Thus, we always include internal controls for each treatment and believe that each test should be considered independently, as suggested by previous studies28. Statistical comparisons were made between groups that were or were not exposed to rivals in each experiment. As the mating duration of males showed normal distribution (Kolmogorov-Smirnov tests, *P* > 0.05), we used two-sided Student’s t tests.

When we compared the difference of mating duration in experiments without internal control built in, (for example, Fig. 2c), we always performed control experiments of wild type for each independent experiment for internal comparison. In this case, we analyzed data using ANOVA for statistically significant differences (at a 95.0% confidence interval) between the means of mating duration for all conditions. If a significant difference between the means was found by Kruskal-Wallis test, then the Dunn’s multiple comparison test was used to compare the mean mating duration of each condition to determine which conditions were significantly different from condition of interest.

44. Keene, A.C. et al. Clock and cycle limit starvation-induced sleep loss in *Drosophila*. *Curr. Biol.* 20, 1209–1215 (2010).
45. Parisky, K.M. et al. PDF cells are a GABA-responsive wake-promoting component of the *Drosophila* sleep circuit. *Neuron* 60, 672–682 (2008).
46. Krstic, D., Bolt, W. & Noll, M. Sensory integration regulating male courtship behavior in *Drosophila*. *PLoS ONE* 4, e4457 (2009).
47. Preuss, F. et al. *Drosophila* doubletime mutations which either shorten or lengthen the period of circadian rhythms decrease the protein kinase activity of casein kinase I. *Mol. Cell. Biol.* 24, 886–898 (2004).
48. Hasegawa, E. et al. Concentric zones, cell migration and neuronal circuits in the *Drosophila* visual center. *Development* 138, 983–993 (2011).
49. Yang, C.H. et al. Control of the postmating behavioral switch in *Drosophila* females by internal sensory neurons. *Neuron* 61, 519–526 (2009).
50. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22, 451–461 (1999).