Brief Communication

Fan-shaped body neurons are involved in *period*-dependent regulation of long-term courtship memory in *Drosophila*

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In addition to its established function in the regulation of circadian rhythms, the *Drosophila* gene *period* (*per*) also plays an important role in processing long-term memory (LTM). Here, we used courtship conditioning as a learning paradigm and revealed that (1) overexpression and knocking down of *per* in subsets of brain neurons enhance and suppress LTM, respectively, and (2) suppression of synaptic transmission during memory retrieval in the same neuronal subsets leads to defective LTM. Further analysis strongly suggests that the brain region critical for *per*-dependent LTM regulation is the fan-shaped body, which is involved in sleep-induced enhancement of courtship LTM.

[Supplemental material is available for this article.]

The *Drosophila period* (*per*) gene was identified as the first clock gene that plays a critical role in regulation of circadian rhythm (Konopka and Benzer 1971). Subsequent studies of *per* and other clock genes in *Drosophila* as well as in mammals led to the evolutionarily conserved molecular model of circadian timekeeping mechanisms, which revolutionized our understanding of the biological clock at the molecular level (King and Takahashi 2000; Hall 2003). We previously reported a surprising finding that, in addition to its well-known roles in circadian rhythm regulation, *per* is also involved in formation of long-term memory (LTM) induced by courtship conditioning (Sakai et al. 2004). Courtship conditioning is an established learning paradigm in *Drosophila*, in which male flies that have courted unreceptive, nonvirgin females subsequently suppress their courtship behavior even toward receptive virgin females (Siegel and Hall 1979; Mehren et al. 2004; Sakai et al. 2004; Griffith and Ejima 2009; Ishimoto et al. 2009). One-hour conditioning generates "courtship memory" detectable for several hours as an experience-dependent reduction in courtship activity (Siegel and Hall 1979), whereas 7-h conditioning results in LTM lasting at least 5 d (Sakai et al. 2004; Ishimoto et al. 2009). *per*-null mutant males show normal short-lasting courtship memory (Jackson et al. 1983; Gailey et al. 1991; Sakai et al. 2004). Interestingly, however, we found that they are defective in formation of courtship LTM (Sakai et al. 2004). In contrast to *per*, mutations in other clock genes do not affect courtship LTM (Sakai et al. 2004). Our results indicate that *per* plays a role in courtship LTM independently of its function as the core oscillator of circadian clock. The adverse effect of *per* mutations on courtship LTM was recently confirmed by Donlea et al. (2009). Furthermore, Chen et al. (2012) have shown that *per*, but not other clock genes, is required for LTM induced by *Drosophila* olfactory associative learning, suggesting the general role of *per* in control of different types of LTM.

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To better understand the mechanisms underlying the novel function of *per* in memory processing, it is essential to identify the neuronal substrates that are responsible for *per*-dependent regulation of LTM. To address this issue, we used the enhancing effect of *per* overexpression on courtship LTM. Our previous study has demonstrated that 5-h conditioning is insufficient for the formation of courtship LTM in wild-type males, but the same conditioning protocol becomes sufficient when *per* is temporarily but ubiquitously expressed using the *hsp70* promoter prior to 5-h conditioning (Sakai et al. 2004). In the present study, wild-type *per* was overexpressed using the GAL4/UAS system to identify particular neuronal subsets important for *per*-dependent regulation of LTM (see Supplemental Material for detailed experimental protocols).

Particular attention was paid to the fan-shaped body (FB) of the central complex for the following reasons: First, *per* is likely expressed in FB neurons because the genomic *per* regulatory region drives reporter gene expression in this neuronal subset (Kaneko and Hall 2000). Second, the FB is known to process different types of memory. For example, visual pattern memory requires expression of functional *rutabaga* adenylyl cyclase—a proposed molecular integrator critical for memory formation—in the FB neurons (Liu et al. 2006; Pan et al. 2009). In addition, CaMKII activity in the FB neurons is necessary for courtship memory (Joiner and Griffith 1999). Third, Donlea et al. (2011) have reported that consolidation of courtship LTM is facilitated when the FB neurons are artificially activated immediately after conditioning using the temperature-gated cation channel TRPA1 (Donlea et al. 2011).

To examine whether *per* overexpression in the FB neurons leads to LTM enhancement, we used an enhancer trap line, OK348, which drives GAL4 expression mainly in a subset of the FB neurons (Fig. 3A, below; Connolly et al. 1996). Indeed, when *per* was overexpressed with OK348, 5-h conditioning became sufficient for the formation of courtship LTM (Fig. 1A, OK348/2-4), as was observed when *per* was ubiquitously expressed using the *hsp70* promoter (Sakai et al. 2004). Similarly to wild-type males, control males showed courtship LTM after 7-h conditioning but not after 5-h conditioning (Fig. 1B, +/2-4 and OK348/+),

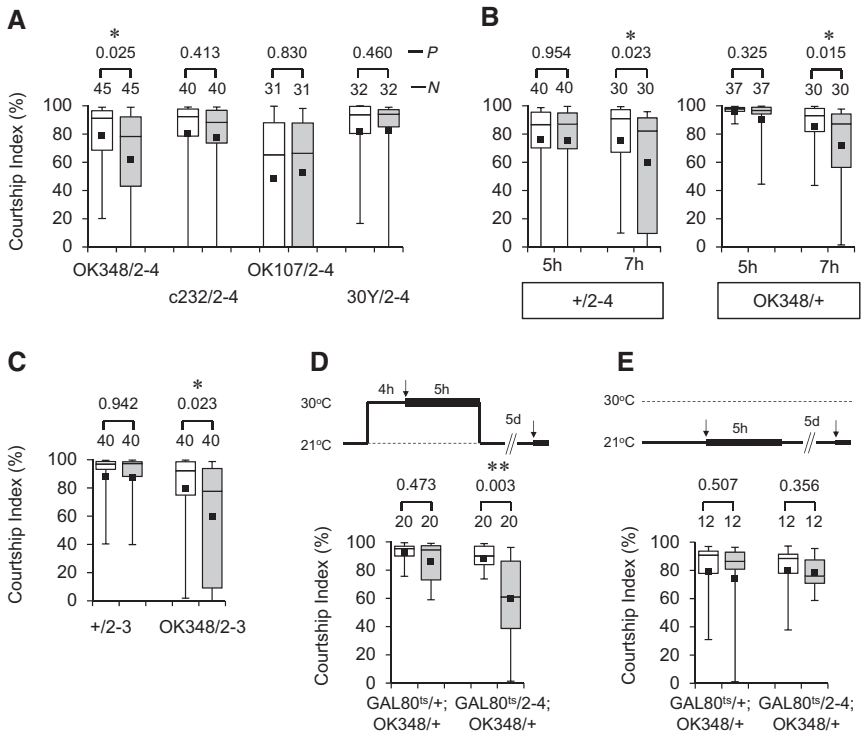

Figure 1. *per* overexpression in OK348-positive neurons enhances courtship LTM. In each box plot, the box encompasses the interquartile range. A line is drawn at median and vertical bars corresponding to the 10th and 90th percentiles. Each square within a box is the mean. White and gray boxes show the CIs of naive and conditioned males, respectively. In the labels for the *x*-axis, "2–4" and "2–3" represent UAS-*per*2-4 and UAS-*per*2-3, respectively. (*A*) 5-d memory after 5-h conditioning in OK348/UAS-*per*2-4, c232/UAS-*per*2-4, OK107/UAS-*per*2-4, and 30Y/UAS-*per*2-4 males. (*P*) Probability; (*N*) sample size; (*) $P < 0.05$. (*B*) 5-d memory after 5-h and 7-h conditioning in +/UAS-*per*2-4 and OK348/+ males; (*) $P < 0.05$. (*C*) 5-d memory after 5-h conditioning in +/UAS-*per*2-3 and OK348/UAS-*per*2-3 males; (*) $P < 0.05$. (*D*) 5-d memory in *tub*-GAL80^{ts}/+; OK348/+ and *tub*-GAL80^{ts}/UAS-*per*2-4; OK348/+ males. Males were kept at 21°C. Four hours before conditioning, males were transferred to an environment of 30°C (restrictive temperature), and subsequently conditioned for 5 h at this temperature. Experimental paradigms are indicated *above* the graphs. The first and second arrows indicate the beginning of the 5-h conditioning and the 10-min test, respectively; (**) $P < 0.01$. (*E*) 5-d memory after 5-h conditioning in *tub*-GAL80^{ts}/+; OK348/+ and *tub*-GAL80^{ts}/UAS-*per*2-4; OK348/+ males. All experiments were carried out at the permissive temperature (21°C).

indicating that the memory enhancement is due to overexpression of *per* in OK348-positive neurons. The LTM enhancement was also observed in combination of OK348 with a second UAS-*per* transformant (Fig. 1C, OK348/2-3).

The mushroom body (MB) is a brain structure centrally important for olfactory memory (Davis 2005; Margulies et al. 2005) and courtship memory (Joiner and Griffith 1999; McBride et al. 1999; Ishimoto et al. 2009). However, the MB does not seem to be a critical brain region for *per*-dependent courtship LTM, because *per* is not likely expressed in neurons comprising the MB (Kaneko and Hall 2000). Consistently, LTM enhancement was not observed when *per* expression was directed to the MB using OK107 or 30Y (Fig. 1A, OK107/2-4 and 30Y/2-4). Likewise, overexpression of *per* in the ellipsoid body of the central complex using c232 did not lead to enhancement of courtship LTM (Fig. 1A, c232/2-4).

Next, we used the TARGET system (McGuire et al. 2003) to determine whether transient expression of *per* in adult OK348-positive neurons is sufficient for the enhancement of courtship LTM. As shown in Figure 1D, the memory enhancement was observed when *per* expression was temporarily induced in the OK348-positive neurons prior to and during 5-h conditioning (Fig. 1D, GAL80^{ts}/2-4; OK348/+ [30°C]). In contrast, control males did not display LTM enhancement (Fig. 1D, GAL80^{ts}/+; OK348/+ [30°C]; Fig. 1E, 21°C). These results indicate that overexpressed *per* in the OK348-positive neurons facilitates physiological processes that are critical for formation or consolidation of courtship LTM.

We previously demonstrated that the activity of *per*-positive neurons is required for courtship LTM: disruption of synaptic transmission from *per*-GAL4-positive cells specifically blocks retrieval of LTM (Sakai et al. 2004). We, therefore, examined whether courtship LTM is also affected by conditional blockage of synaptic transmission from OK348-positive neurons. The temperature-sensitive dynamin mutation *shibire*^{ts1} (*shi*^{ts1}) (Kitamoto 2001) was used in combination with OK348 to disrupt synaptic transmission from the OK348-positive neurons in a temperature-dependent manner. First, all experimental procedures were carried out at a constant temperature of either 25°C (permissive) or 30°C (restrictive). We found that LTM is disrupted only when the restrictive temperature was used in the presence of both OK348 and UAS-*shi*^{ts1} (Fig. 2A). GAL4 and UAS control males showed normal courtship LTM at both 25°C and 30°C (Fig. 2B,C). To further examine the specific role of synaptic transmission from OK348-positive neurons, courtship LTM was analyzed in OK348/UAS-*shi*^{ts1} flies after temperature was shifted from permissive to restrictive during three distinct experimental phases—the period for courtship conditioning (memory formation), the test period (memory retrieval), and the period in between (memory storage). When temperature was increased to 30°C during the test period (Fig. 2D, 25-25-30), courtship LTM was not observed. However, as long as the temperature was permissive during the test period, normal courtship LTM was detected irrespective of the temperature during the conditioning period and the period in between (Fig. 2D, 30-25-25 and 25-30-25). These results indicate that synaptic transmission from the OK348-positive neurons is required during retrieval, rather than formation or storage, of courtship LTM.

Although OK348-driven GFP expression was primarily observed in the FB of the central complex (Fig. 3A, triangle) as previously reported (Connolly et al. 1996), additional expression was detected in clusters of the cells in the lateral region of the brain (Fig. 3A, arrows). GFP expression was also detected in a similar lateral region when the GFP reporter was driven by OK107 and 30Y (Fig. 3C,D, arrows). *per* is expressed in pacemaker neurons called lateral neurons (LNs), which include PDF neuropeptide-expressing neurons (Hall 2003). To examine whether the GAL4-positive neurons in the lateral region are circadian pacemaker neurons, we carried out a colocalization analysis using an anti-PDF antibody. As observed in *pdf*-GAL4 (Fig. 3E,G), PDF immunoreactivity was detected in some OK348- or 30Y-positive neurons in the lateral region (Fig. 3G). However, there was no coexpression of GAL4 and PDF in OK107-positive neurons (Fig. 3G).

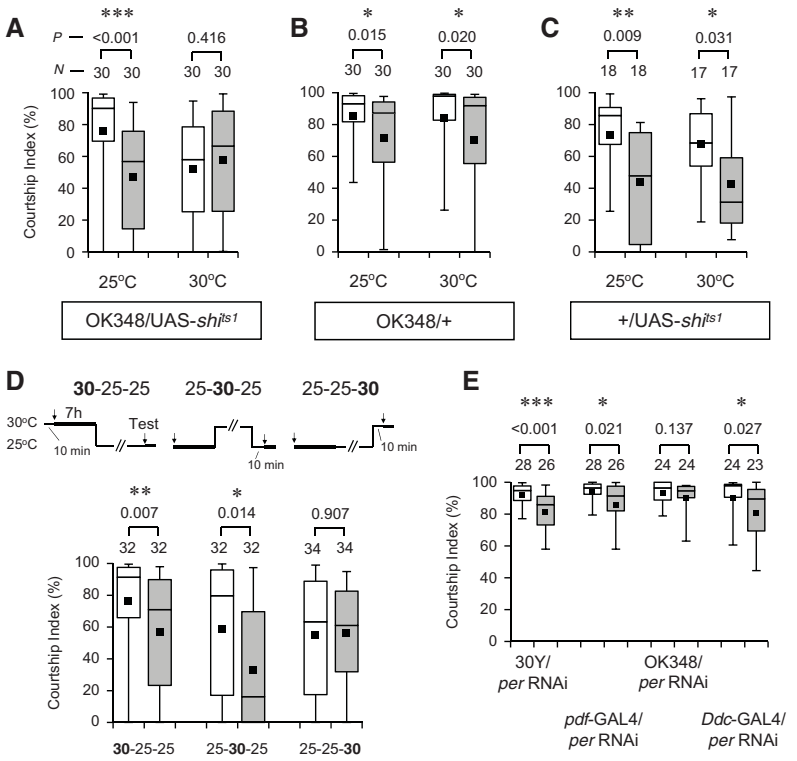

Figure 2. Effect of disruption of synaptic transmission in OK348-positive neurons on courtship LTM. (*A–C*) 5-d memory after 7-h conditioning. All procedures in the experiments were carried out at the permissive (25°C) or restrictive (30°C) temperature. OK348/UAS-*shi*^{ts1} (*A*), OK348/+ (*B*), and +/UAS-*shi*^{ts1} (*C*) males were used. (*P*) Probability; (*N*) sample size; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. (*D*) 5-d memory after 7-h conditioning in OK348/UAS-*shi*^{ts1} males. Experimental paradigms of temperature-shift are indicated *above* the graphs. The first and second arrows indicate the beginning of 7-h conditioning and 10-min test, respectively; (*) $P < 0.05$; (**) $P < 0.01$. (*E*) 5-d memory after 7-h conditioning in F_1 males between UAS-*per* RNAi and 30Y, *pdf*-GAL4, OK348, or *Ddc*-GAL4; (*) $P < 0.05$; (***) $P < 0.001$.

Because OK348-positive neurons include PDF neurons (Fig. 3G), it is possible that *per* expression in these PDF neurons, but not FB neurons, is critical for courtship LTM. To investigate this possibility, *per* expression was suppressed in PDF neurons using *per*-RNAi with *pdf*-GAL4, and its effect on courtship LTM was examined. We found that 30Y/UAS-*per* RNAi and *pdf*-GAL4/UAS-*per* RNAi males showed normal courtship LTM after the standard 7-h conditioning (Fig. 2E). In contrast, OK348/UAS-*per* RNAi males showed defective courtship LTM (Fig. 2E). These results strongly indicate that OK348-positive neurons other than PDF neurons are required for *per*-dependent courtship LTM. Thus, it is most likely that, among OK348-positive neurons, FB neurons are responsible for *per*-dependent regulation of courtship LTM.

Recently, Chen et al. (2012) have reported that *per* expression in two dorsal–anterior–lateral (DAL) neurons is required for LTM formation induced by olfactory conditioning. To determine whether formation of courtship LTM also requires *per* in the DAL neurons, we suppressed *per* expression in the DAL neurons and examined the effect of this suppression on courtship LTM. *Ddc*-GAL4, which directs GAL4 expression in the DAL neurons (Fig. 3F; Chen et al. 2012), was used in combination with UAS-*per* RNAi to suppress *per* expression in the DAL neurons. As shown in Figure 2E, *Ddc*-GAL4/UAS-*per* RNAi males were found to display normal courtship LTM after 7-h conditioning. This result indicates that, unlike olfactory LTM, courtship LTM does not require *per* expression in the DAL neurons.

Here, we have demonstrated that courtship LTM is suppressed when *per* expression in OK348-positive neurons is inhibited (Fig. 2E) or when synaptic transmission from OK348-positive neurons is blocked during the memory retrieval phase (Fig. 2D). These results suggest the possibility that courtship LTM is stored in these neurons and that their activation leads to manifestation of memory. Although *per* is a central component of the intricate gene regulatory network for circadian rhythm (Hall 2003), it could also control expression of the genes critical for formation or consolidation of LTM. Donlea et al. (2011) have recently shown that conditional activation of the FB neurons induces sleep and that this treatment also leads to enhancement of LTM—the effect we observed in this study when *per* was overexpressed in OK348-positive neurons

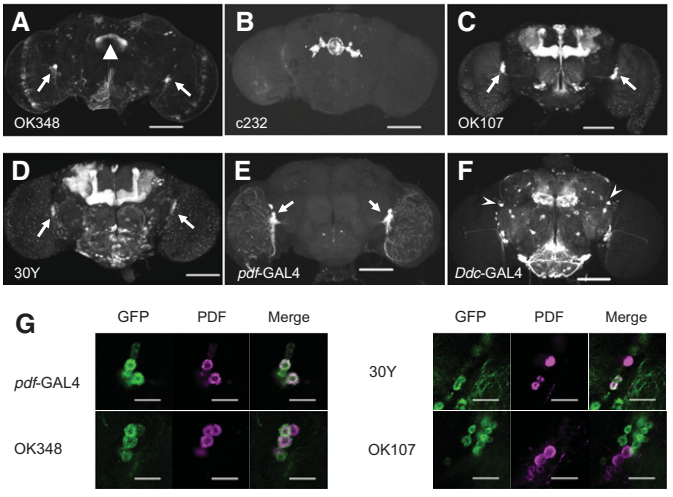

Figure 3. Confocal images of GAL4-positive neurons in the adult brain. (*A–F*) Frontal views of the adult brain. F_1 males generated between UAS-*mCD8::GFP* and OK348 (*A*), c232 (*B*), OK107 (*C*), 30Y (*D*), *pdf*-GAL4 (*E*), or *Ddc*-GAL4 (*F*) were used. GFP fluorescence was observed under a confocal microscope (Carl Zeiss LSM710). Z sections were collected at 1-μm intervals and processed to construct projections through an extended depth of focus. Scale bars, 100 μm. (Triangle) Fan-shaped body (*A*); (arrows) neurons in the lateral region (*A*,*C–E*); (arrowheads) DAL neurons (*F*). (*G*) GAL4-driven GFP in clusters of the cells in the lateral region of the brain (green) and PDF immunolabeling (magenta). F_1 males generated between UAS-*mCD8::GFP* and *pdf*-GAL4, OK348, 30Y, or OK107 were used. Scale bars, 20 μm.

(Fig. 1). Thus, the intriguing possibility arises that *per* may mediate the enhancement of courtship LTM following FB activation and sleep induction. Future studies are expected to clarify the possible mechanistic link between nonclock functions of *per* in the FB neurons and regulation of sleep as well as LTM.

Acknowledgments

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References

Chen CC, Wu JK, Lin HW, Pai TP, Fu TF, Wu CL, Tully T, Chiang AS. 2012. Visualizing long-term memory formation in two neurons of the *Drosophila* brain. *Science* **335:** 678–685.

Connolly JB, Roberts IJ, Armstrong JD, Kaiser K, Forte M, Tully T, O'Kane CJ. 1996. Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. *Science* **274:** 2104–2107.

Davis RL. 2005. Olfactory memory formation in *Drosophila*: From molecular to systems neuroscience. *Annu Rev Neurosci* **28:** 275–302.

Donlea JM, Ramanan N, Shaw PJ. 2009. Use-dependent plasticity in clock neurons regulates sleep need in *Drosophila*. *Science* **324:** 105–108.

Donlea JM, Thimgan MS, Suzuki Y, Gottschalk L, Shaw PJ. 2011. Inducing sleep by remote control facilitates memory consolidation in *Drosophila*. *Science* **332:** 1571–1576.

Gailey DA, Villella A, Tully T. 1991. Reassessment of the effect of biological rhythm mutations on learning in *Drosophila melanogaster. J Comp Physiol A* **169:** 685–697.

Griffith LC, Ejima A. 2009. Courtship learning in *Drosophila melanogaster*: Diverse plasticity of a reproductive behavior. *Learn Mem* **16:** 743–750.

Hall JC. 2003. Genetics and molecular biology of rhythms in *Drosophila* and other insects. *Adv Genet* **48:** 1–280.

Ishimoto H, Sakai T, Kitamoto T. 2009. Ecdysone signaling regulates courtship long-term memory formation in adult *Drosophila melanogaster*. *Proc Natl Acad Sci* **106:** 6381–6386.

Jackson FR, Gailey DA, Siegel RW. 1983. Biological rhythm mutations affect an experience-dependent modification of male courtship behavior in *Drosophila melanogaster*. *J Comp Physiol A* **151:** 545–552.

Joiner MA, Griffith LC. 1999. Mapping of the anatomical circuit of CaM kinase-dependent courtship conditioning in *Drosophila*. *Learn Mem* **6:** 177–192.

Kaneko M, Hall JC. 2000. Neuroanatomy of cells expressing clock genes in *Drosophila*: Transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J Comp Neurol* **422:** 66–94.

King DP, Takahashi JS. 2000. Molecular genetics of circadian rhythms in mammals. *Annu Rev Neurosci* **23:** 713–742.

Kitamoto T. 2001. Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive *shibire* allele in defined neurons. *J Neurobiol* **47:** 81–92.

Konopka RJ, Benzer S. 1971. Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci* **68:** 2112–2116.

Liu G, Seiler H, Wen A, Zars T, Ito K, Wolf R, Heisenberg M, Liu L. 2006. Distinct memory traces for two visual features in the *Drosophila* brain. *Nature* **439:** 551–556.

Margulies C, Tully T, Dubnau J. 2005. Deconstructing memory in *Drosophila*. *Curr Biol* **15:** R700–R713.

McBride SM, Giuliani G, Choi C, Krause P, Correale D, Watson K, Baker G, Siwicki KK. 1999. Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in *Drosophila melanogaster*. *Neuron* **24:** 967–977.

McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL. 2003. Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* **302:** 1765–1768.

Mehren JE, Ejima A, Griffith LC. 2004. Unconventional sex: Fresh approaches to courtship learning. *Curr Opin Neurobiol* **14:** 745–750.

Pan Y, Zhou Y, Guo C, Gong H, Gong Z, Liu L. 2009. Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. *Learn Mem* **16:** 289–295.

Sakai T, Tamura T, Kitamoto T, Kidokoro Y. 2004. A clock gene, *period*, plays a key role in long-term memory formation in *Drosophila*. *Proc Natl Acad Sci* **101:** 16058–16063.

Siegel RW, Hall JC. 1979. Conditioned responses in courtship behavior of normal and mutant *Drosophila*. *Proc Natl Acad Sci* **76:** 3430–3434.

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