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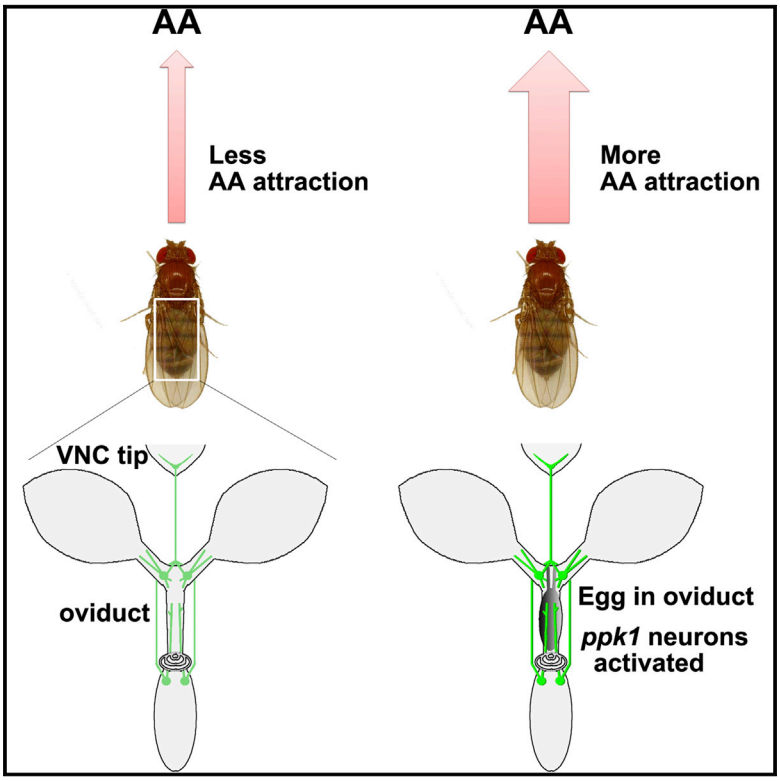
Mechanosensitive Neurons on the Internal Reproductive Tract Contribute to Egg-Laying-Induced Acetic Acid Attraction in *Drosophila*

Graphical Abstract



Highlights

Drosophila females activate acetic acid attraction prior to egg laying

Distention of the internal reproductive tract can trigger AA attraction

Tract distention is monitored by mechanosensitive tract *ppk1* neurons

Function of mechanosensitive tract *ppk1* neurons is essential for AA attraction

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In Brief

Pregnancy can change the behavior of females significantly. Gou et al. show that for female fruit flies, the need to lay eggs temporarily increases their attraction for acetic acid and that mechanical stretch of their internal reproductive tract—induced by egg delivery in the tract—is one source of such need. This study identifies the neural basis of an important reproductive need for female flies and provides a model for studying how such need modifies behavior and sensory processing of females.

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Mechanosensitive Neurons on the Internal Reproductive Tract Contribute to Egg-Laying-Induced Acetic Acid Attraction in *Drosophila*

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SUMMARY

Selecting a suitable site to deposit their eggs is an important reproductive need of *Drosophila* females. Although their choosiness toward egg-laying sites is well documented, the specific neural mechanism that activates females' search for attractive egg-laying sites is not known. Here, we show that distention and contraction of females' internal reproductive tract triggered by egg delivery through the tract plays a critical role in activating such search. We found that females start to exhibit acetic acid (AA) attraction prior to depositing each egg but no attraction when they are not laying eggs. Artificially distending the reproductive tract triggers AA attraction in non-egg-laying females, whereas silencing the mechanosensitive neurons we identified that can sense the contractile status of the tract eliminates such attraction. Our work uncovers the circuit basis of an important reproductive need of *Drosophila* females and provides a simple model for dissecting the neural mechanism that underlies a reproductive need-induced behavioral modification.

INTRODUCTION

The need to care for offspring can alter behaviors of animal mothers significantly. Many commonly described maternal behaviors such as feeding and aggression against intruders serve the purpose of nurturing and protecting the newborns. But in some species, pregnancy alone is sufficient to induce changes in sensory processing and behaviors (Rosenblatt and Lehrman, 1963). Pregnancy-induced hormonal changes are thought to play a role in activating "prenatal care behaviors" (Kristal, 2009), but the exact circuit mechanism by which the presence of a fetus in utero modifies the behaviors of expectant mothers remains not well understood.

The fruit fly *Drosophila melanogaster* has emerged as a suitable model to study the genetic and circuit basis of female reproductive behaviors. Similar to higher animals, virgin and mated/pregnant flies show significant differences in their behaviors and physiologies. For example, virgins are receptive to male courtship and lay very few eggs, whereas mated females are unreceptive to courtship, lay eggs frequently, and preferentially consume proteins over sugars (Carvalho et al., 2006; Kubli, 2003; Ribeiro and Dickson, 2010). Much progress has been made in recent years in elucidating the molecular and circuit basis by which the experience of mating modifies physiologies and behaviors of female flies (Bussell et al., 2014; Feng et al., 2014; Häsemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009; Zhou et al., 2014). In contrast, *whether* and *how* egg-laying need influences how female flies interpret the valence of external stimuli—so as to guide their decision of whether to move toward or away from specific stimuli—remains little explored, despite the fact that female flies are known to be highly selective about where to lay eggs (Azanchi et al., 2013; Dweck et al., 2013; Joseph et al., 2009; Joseph and Heberlein, 2012; Rockwell and Grossfield, 1978; Schwartz et al., 2012; Yang et al., 2008).

Here, we show that egg-laying need increases female flies' attraction for acetic acid (AA) significantly. Behavioral analysis reveals that signs of AA attraction emerge prior to physical egg deposition. Manipulating the internal egg-delivery process (that precedes physical egg deposition) reveals that artificial distention of the internal reproductive tract is sufficient to activate AA attraction and that mechanical stretch of the reproductive tract is sensed and relayed to the CNS by a set of *piezo*-expressing sensory neurons. Our results suggest a model in which *Drosophila* females modify their AA attraction by assessing, via mechanosensitive neurons on the tract, whether eggs are being pushed through their reproductive tract. We propose such activation of AA attraction in anticipation of impending physical egg laying may be considered a rudimentary form of maternal care and provide a suitable model to study the circuit mechanism by which reproductive needs modify female behaviors.

RESULTS

Egg Laying, but Not Mating, Correlates with Mated Females' Positional Preference for AA

It has been shown that, as a population, mated females show stronger positional preference for AA than virgins do, and they also prefer to lay eggs on an AA-containing versus an AA-free

substrate (Joseph et al., 2009). Because mating induces several behavioral and physiological changes, we first ascertained that mated females' increased AA preference is triggered by egg-laying need as opposed to other mating-induced changes. We custom-built an apparatus that contains several chambers, each of which can house one AA+ and one AA− substrate (Figure S1B). We loaded females into this apparatus (one per chamber), recorded their behaviors for 4 hr (Figure S1A; Movie S1), and tracked their positions using Ctrax (Branson et al., 2009). Similar to what was reported previously (Joseph et al., 2009), we found that mated, egg-laying females show a clear preference to lay eggs and to spend time on an AA site (Figure 1A; Table S1).

To disentangle the impact of mating versus egg laying on positional preference for AA, we compared AA preference of mated females that lay very few eggs versus virgins that lay many. (We have discovered that mated females reduce their egg-laying rate significantly if their diet is deprived of yeast paste, a protein source that boosts egg production. Conversely, virgins that have been continuously fed yeast paste after eclosion can sometimes lay many eggs.) We found that mated females that lay very few eggs show no positional preference for AA, but virgins that lay many do (Figure 1A). Importantly, neither yeast-fed males nor yeast-fed virgins that lay few eggs show any AA preference (Figure S1F). Together, these results suggest egg laying, not mating or yeast feeding, is responsible for mated females' positional preference for AA.

Egg-Laying Females Show "Active Attraction" for AA

We next determined whether egg-laying need triggers "active AA attraction" in addition to positional preference for AA. We noticed that females move constantly between AA+ and AA-free substrates but occasionally reverse their running direction in the middle of the chamber. We labeled a reversal where females would switch from moving away to moving toward AA substrate an "attractive return" and proposed that it indicates active AA attraction (Figure 1B, right panel). A recent report uses a similar criterion to define behavioral attraction (Gao et al., 2013). Similarly, we proposed a reversal from moving toward to moving away from AA substrate signals active AA aversion and labeled it an "aversive return" (Figure 1B, left panel). We can then determine net AA attraction by calculating an "AA attraction index" (Figure 1B). Indeed, regardless of their mating status, females that lay many eggs show a significantly stronger AA attraction than ones that lay very few eggs (Figure 1B; Figure S1G), suggesting increased egg laying is sufficient to induce active AA attraction.

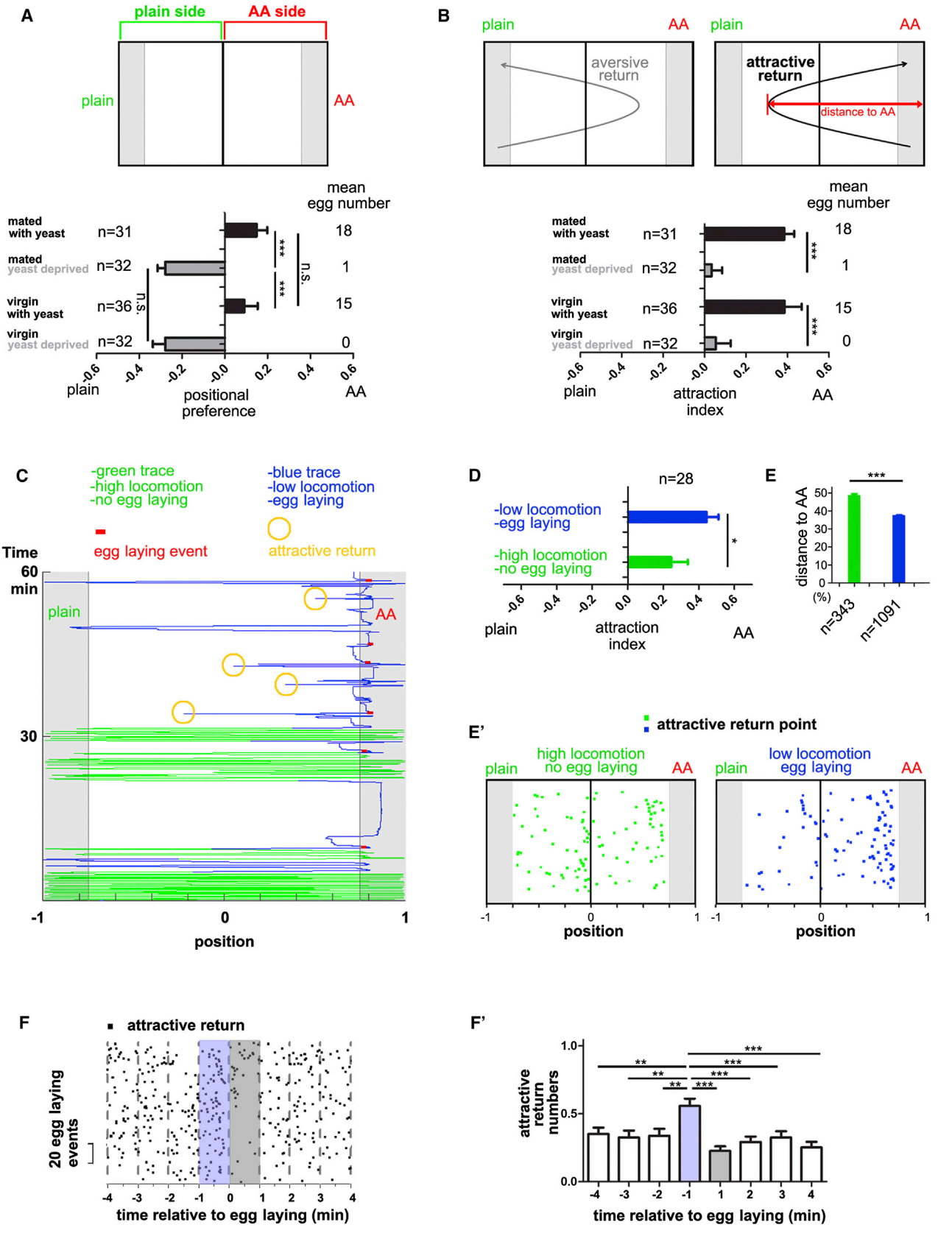
We have so far compared AA attraction of two types of females: one that lays many eggs and one that does not. But if egg-laying need is indeed the trigger for AA attraction, the same female should exhibit different levels of AA attraction depending on her egg-laying rate in a given time period. So we sought to correlate egg-laying rate and AA attraction in the same animals and found that temporal distribution of egg-laying events is nonuniform: there are clearly periods when a female lays eggs frequently and ones when she does not (Figure 1C, blue and green traces). Moreover, females consistently move faster during "no egg-laying" periods than "high egg-laying" periods (Figure 1C, blue and green traces), allowing us to use locomotion speed to separate their trajectories into high versus low egg-laying states (Figures 1C, S1C, and S1D). We found that individuals show stronger AA attraction when they are actively laying eggs than when they are not (Figure 1D). An examination of the "return point" (Figure 1B) further revealed that females in a high egg-laying state tend to execute their attractive returns at positions closer to AA (Figures 1E and 1E′), suggesting that when laying eggs actively, females are less tolerant about staying away from AA.

Finally, if egg-laying need is a trigger for AA attraction, then signs of attraction might emerge prior to each egg laying. We examined the trajectory in the 1 min window immediately before and after 151 egg-laying events and found that, indeed, attractive returns tend to occur before, but not after, egg laying (Figures 1F and 1F′). Moreover, when we segmented the trajectory before each egg laying into four consecutive 1 min periods, we found that the number of attractive returns increases as females are nearing to laying an egg (Figures 1F and 1F′). In contrast, aversive returns occur very rarely in these windows (Figure S1E). Taken together, our analysis shows that *Drosophila* females are more attracted to AA when they are actively laying eggs and that such attraction emerges prior to physical egg laying.

Persistent Presence of Egg(s) in the Internal Reproductive Tract Is Sufficient to Trigger AA Attraction Independent of Egg Laying

Next, we wanted to uncover the neural basis by which egg-laying need activates AA attraction prior to physical egg laying. We looked closer at the egg-delivery process that occurs in females' internal reproductive tract. While egg-laying becomes obvious to observers only when females begin to display the ovipositor motor program (Yang et al., 2008), it has begun once an egg starts to descend from the ovaries into the internal reproductive tract—an epithelial tube that connects the two ovaries to the uterus, where eggs are fertilized (Figure 2A). The reproductive tract has a small diameter and is encased by muscles that are innervated by sensory and motor neurons (Castellanos et al., 2013; Häsemeyer et al., 2009; Yang et al., 2009). Because egg delivery in the tract precedes physical egg deposition, we hypothesize that perhaps females start to increase AA attraction once they sense eggs are being pushed through the tract.

To test this idea, we first assessed AA attraction of females who have eggs "persistently trapped in the tract." It has been shown recently, and we confirmed, that some of the *ILP7-Gal4*-expressing neurons in the ventral nerve cord (VNC) are motor neurons that innervate the tract (Figure 2B) (Castellanos et al., 2013). Importantly, inhibiting *ILP7* neurons with *Kir2.1* (Baines et al., 2001) consistently causes one or more eggs to be "jammed" in the tract (Castellanos et al., 2013; Yang et al., 2008) (Figure 2C), providing us with the desired phenotype to test our hypothesis. Indeed, females with inhibited *ILP7* neurons showed clear AA attraction, despite that they cannot physically lay any eggs (because their tract is jammed) (Figure 2E). Importantly, females of the same genotype but deprived of yeast paste showed much reduced egg jamming (Figure 2D) as well as much reduced AA attraction (Figure 2E), suggesting that



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the persistent presence of eggs in the tract, not other changes induced by silencing *ILP7* neurons, is what triggers AA attraction. Thus, we propose that during regular egg laying, AA attraction is activated each time an egg is being squeezed through the tract.

A Group of *ppk1*-Expressing Sensory Neurons on the Reproductive Tract Can Sense Contraction of the Tract

How do females sense that an egg is being squeezed through their reproductive tract? We speculate some sensory neurons on the tract are mechanosensors. We have previously shown that the *ppk-Gal4*-labeled sensory neurons on the tract can be divided into at least two groups (Häsemeyer et al., 2009; Yang et al., 2009) (Figure 3A). The first group expresses the *fruitless* transcripts, senses sex peptide (SP), and has relatively short dendrites (Häsemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009). We suspect *ppk* neurons of the second group, two on lateral ducts and four to six at the base of the common duct (Figures 3B, S2H and S2I), are mechanosensors. They have large dendrites that "tile" the entire surface of tract (Figure 3B) and appear to express the mechanosensitive channel Piezo (Coste et al., 2010; Coste et al., 2012; Kim et al., 2012) (Figures 3C and S2E). However, there is no direct evidence showing that these "tract-tiling" *ppk1* neurons are indeed capable of sensing tract contraction/distention.

Next, we devised a three-step approach to test whether these tract-tiling *ppk1* neurons are mechanosensors (Figure 3F). First, we used the calcium sensor GCaMP3 (Tian et al., 2009) to monitor activities of axonal termini of tract *ppk1* neurons. (The GCaMP3 signal in *ppk* axons in the VNC is less likely to move out of the focal plane when tract contraction occurs.) To define the specific VNC region targeted by tract *ppk1* neurons, we made use of a *ppk1.0-Gal80* (Häsemeyer et al., 2009). This *ppk1.0-Gal80* suppresses *Gal4*-dependent expression in nearly all *ppk1-Gal4* neurons except ones that reside on the tract, revealing that axons of tract *ppk1* neurons consistently target to the posterior tip of the VNC (Figures 3D, 3E, S2A, and S2B). This projection is further confirmed when we used the "FLP-out" approach (Gordon and Scott, 2009) to sparsely label *ppk1-Gal4* neurons (Figures S2F and S2G). Second, we used a chemical-genetic approach (Lima and Miesenböck, 2005; Yao et al., 2012) to stimulate contraction of tract muscles. We expressed the ATP-gated P_2X_2 channel in *ILP7* neurons and found that stimulating them with ATP induces robust contraction of the tract (Figures 3G and 3H; Movie S2). Finally, we used the *LexA/LexAop2* and *Gal4/UAS* system to express *P_2X_2* and *GCaMP3* in *ILP7* and *ppk1* neurons, respectively (Figure 3F), allowing us to record *ppk1* neurons while activating tract muscle contraction.

CGaMP3 signal in the axonal termini of tract *ppk1* neurons increased significantly when we stimulated *ILP7* neurons (Figures 3I–3K; Movie S3). To ensure that such GCaMP3 increase was not due to "local interaction" between *ppk1* and *ILP7* neurons (their processes overlap extensively in the VNC; Figure S3A), we physically severed the connectives between the tract and the VNC. *ILP7* neurons in such "severed" preparation still responded to ATP robustly (Figure S3B–S3D), but their stimulation no longer induced a significant GCaMP3 increase in *ppk1* axons (Figure 3K), ruling out the possibility that *ILP7* neurons *locally* activate *ppk1* axons in the VNC. Thus, our results suggest that the tract-tiling *ppk1* neurons are mechanosensors that can detect contraction/distention of the tract.

ppk1-Expressing Tract Sensory Neurons Are Required for Egg-Laying-Induced AA Attraction

To determine if the mechanosensitive tract *ppk1* neurons play a role in promoting AA attraction, we next targeted them for inhibition. In our first "subtraction-based" approach, we used the *ppk1-Gal4* to express *Kir2.1* in the presence of *ppk1-Gal80*. This manipulation caused females to have eggs jammed in their tract (Figure 4A), but unlike *ILP7*-inhibited

Figure 1. Egg Laying, but Not Mating, Triggers Acetic Acid Attraction in Mated Females

(A) Mated and virgin flies that lay more eggs (with yeast, more than ten eggs in 4 hr) show positional preference for acetic acid (AA). Mated and virgin ones that lay few eggs (yeast deprived, one or no eggs in 4 hr) show positional avoidance of AA. The schematic shows the configuration of an egg-laying chamber. Shaded areas are "troughs" where we place substrates. Plain, 1% agarose; AA, 1% agarose with 3% (v/v) AA. Positional preference for AA is calculated as: $(T_{AA} - T_{plain})/(T_{AA} + T_{plain})$, where T_{AA} and T_{plain} are time spent on the AA (red bracket) half and the plan half of the chamber (green bracket), respectively. See Figure S1B for chamber picture. *p < 0.05, **p < 0.01, ***p < 0.0001. n.s., not significant, p > 0.05. Student's t test.

(B) Mated and virgin flies that lay many eggs show stronger "active" AA attraction than ones that lay few eggs. "Aversive return" and "attractive return" are as diagramed. The vertical bar in the right panel denotes the "return point" of an attractive return. AA attraction index is calculated as $(R_{attractive} - R_{aversive})/(R_{attractive} + R_{aversive})$, where $R_{attractive}$ and $R_{aversive}$ represent the number of attractive and aversive returns in a given trajectory, respectively. *p < 0.05, **p < 0.01, ***p < 0.0001. Student's t test.

(C) Example of a 1 hr, annotated trajectory of a single female. y axis, time; x axis, animal position. The green and blue traces show the segments of the trajectory where the animal is in a high versus low locomotion state. The two states can be separated using speed threshold of 0.7 mm/s (see Experimental Procedures and Figures S1C and S1D). Note that all the egg-laying events (short red ticks) occur in the low-locomotion state. Yellow circles, "attractive returns" in the trajectory.

(D) Individual females show stronger AA attraction when they are actively laying eggs than when they are not (blue versus green trace). *p < 0.05, Student's t test.

(E and E′) Return points of attractive returns are closer to AA when animals are in a high egg-laying state (343 returns) than in a low egg-laying state (1,091 returns). The return point of an attractive return is when the animal reverses its running direction (see also B). Distance between the return point and the AA substrate is calculated as % chamber length. ***p < 0.001, Student's t test. (E′) Representative return points of 100 attractive returns in low versus high egg-laying states.

(F) Raster of attractive returns surrounding 151 egg-laying events. There are 151 "invisible" horizontal lines, each of which represents an 8 min trajectory where a single egg-laying event occurs at time 0. Each dot along the horizontal line denotes one attractive return. Light purple and gray highlights the 1 min window before and after egg laying, respectively.

(F′) Average number of attractive returns during each minute of the 8 min timeline in (F). *p < 0.05, **p < 0.01, ***p < 0.0001. n.s., not significant, p > 0.05. Student's t test.

Note that all error bars indicated in this work represent SEM.

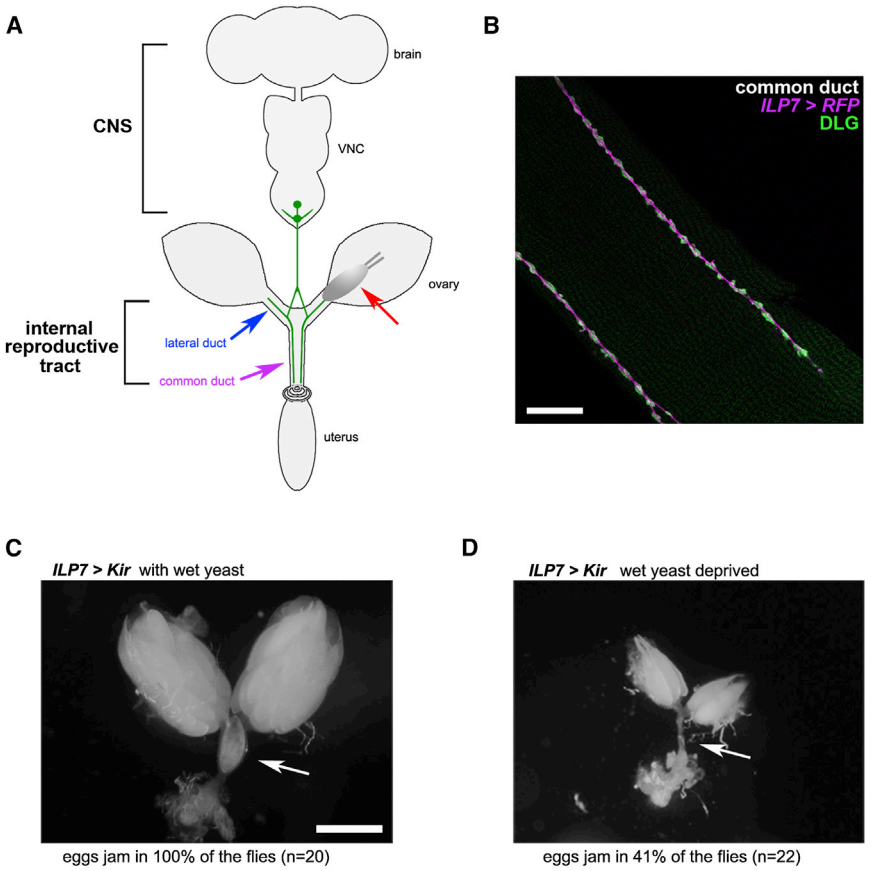

Figure 2. Persistent Presence of an Egg in the Reproductive Tract Is Sufficient to Trigger AA Attraction

(A) A diagram depicting the reproductive tract and its relative position to the CNS. Eggs (red arrow) are produced in the ovaries. To be deposited, they must be pushed out of the ovaries, squeezed through the lateral and common ducts, and fertilized in the uterus. In our article, internal reproductive tract = lateral ducts + common duct. One *ILP7*-expressing motor neuron whose axon descends from the VNC to innervate the tract is shown in green.

(B) *ILP7* axons (magenta) form DLG-positive synapses (green) with tract muscles. *ILP7* axons are labeled by *mCD8-RFP*. DLG, Disc Large, a neuromuscular junction marker. Scale bar is 25 μm.

(C and D) Inhibiting *ILP7* neurons consistently (20/20) causes egg jamming in the tract (white arrow) of yeast-fed mated females (C). In contrast, inhibiting *ILP7* neurons did not cause egg jamming consistently if females were yeast deprived (D). Note that ovaries from yeast-fed females are much larger (they contain many more eggs) than those from yeast-deprived ones. The two pictures were taken at the same magnification. Scale bar is 500 μm.

(E) Yeast-fed and yeast-deprived *ILP7*-inhibited mated females show strong and much-reduced AA attraction, respectively. One-way ANOVA with Bonferroni's post test. *$p < 0.05$. n.s., not significant, $p > 0.05$.

females, they showed no AA attraction (Figure 4A). In our second ''intersection-based'' approach, we used the *21-7-Gal4* that labels tract *ppk1* neurons, but not many of the none tract *ppk1* neurons (Song et al., 2007), to express an *UAS-FRT-stop-FRT-Kir2.1* (Yang et al., 2009) and introduced a *ppk1-LexA* and a source of *flp* into the same animals. Because *21-7-Gal4* and *ppk1-LexA* are coactive mostly only in the tract *ppk1* neurons, animals bearing all four transgenes (*ppk1-LexA*, *LexAop2-flp*, *21-7-Gal4*, and *UAS-FRT-stop-FRT-Kir2.1*) should mostly have only their tract *ppk1* neurons inhibited (Figures S2C and S2D). Again, these ''intersected'' animals show an ''egg-jamming'' phenotype but no AA attraction (Figure 4C). Lastly, restricting *Kir2.1* expression to only the adult stage still reduced AA attraction (Figure 4B), ruling out potential developmental problems due to chronic silencing of *ppk1* neurons as the cause for the lack of AA attraction we observed. Thus, active tract *ppk1* sensory neurons are required to activate AA attraction induced by egg presence in the tract.

To test whether the mechanosensitivity of the tract *ppk1* neurons is important for AA attraction, we reduced their *piezo* expression. We first used *21-7-Gal4* to express a *piezo-RNAi* (Kim et al., 2012). In addition, we also used two copies of *ppk1-Gal4*—in the presence of *ppk1-Gal80*—to express the *piezo-RNAi*. Neither set of animals showed AA attraction, suggesting that *piezo* expression in tract *ppk1* neurons is important for AA attraction (Figure 4C).

Finally, we asked whether artificial activation of tract *ppk1* neurons in the absence of active egg delivery is sufficient to trigger AA attraction. We found that expressing in tract *ppk1* neurons the sodium channel *NaChBac* (Luan et al., 2006), a commonly used effector for increasing membrane potential, failed to trigger AA attraction in yeast-deprived females (Figure 4D). Because *NaChBac* overexpression may not be effective in stimulating *ppk1* neurons, we also used the heat-gated *dTRPA1* to stimulate *ppk1* neurons. However, increasing temperature alone causes a significant alteration in AA attraction, making data interpretation difficult. Thus, we are unable to conclude whether stimulating *ppk1* neurons is sufficient to induce AA attraction.

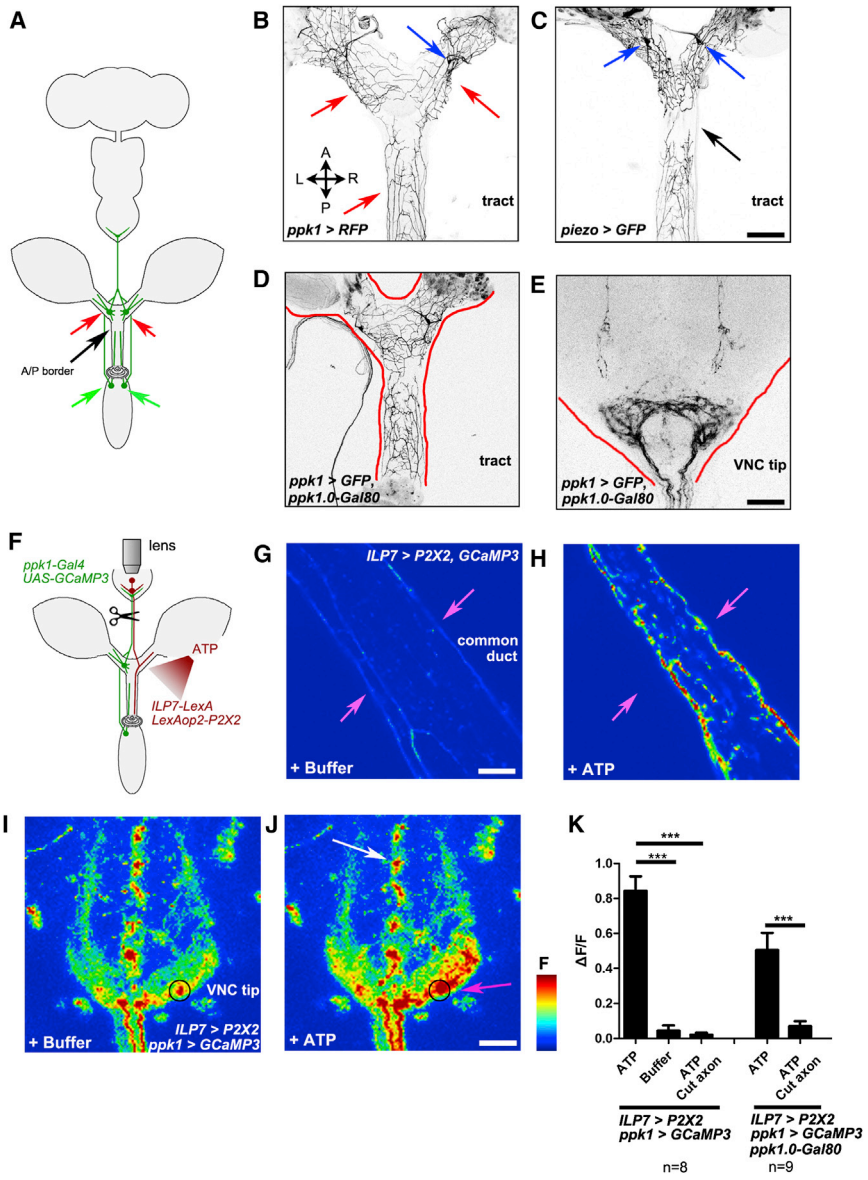

Figure 3. A Subgroup of *ppk1*-Expressing Sensory Neurons that Innervate the Reproductive Tract Can Sense Tract Contraction

(A) A diagram showing a subset of *ppk1* neurons that extends dendrites on the tract and project axons to the VNC. There is one *ppk1* neuron on each lateral duct (red arrows) and two to three on each side of the base of the common duct (green arrows). Black arrow points to the anterior/posterior (A/P) divide demarcated by the *ppk1* dendrites. Note that we use *ppk1* and *ppk1-Gal4* interchangeably.

(B) Dendrites (red arrows) of *ppk1* neurons partition the tract into distinct domains. Note that cell bodies of *ppk1* neurons on the lateral ducts are suspended outside of the tract and are easily torn off during dissection, but one cell body remains attached to the tract in this picture (blue arrow). Scale bar is 100 μm for (B)–(D).

(C) *piezo-Gal4* labels the same "tract-tiling" neurons as *ppk1-Gal4*. Blue arrows, somas of the sensory neurons on the lateral ducts; black arrow, A/P divide.

(D and E) Dendrites and axons of the tract *ppk1* neurons labeled by *ppk1-Gal4* driving *GFP* in the presence of *ppk1-Gal80*. (D) The "subtracted" animals still show labeling of tract-tiling sensory neurons. (E) The tract-tiling *ppk1* neurons target their axons to the posterior tip of the VNC. Scale bar for (E) is 25 μm. See Figure S2 for a comparison of the axonal projection labeled by *ppk1-Gal4* before and after *ppk1-Gal80*-mediated "subtraction."

(F) Our preparation. To activate *ILP7* neurons, we used *ILP7-LexA* to express *LexAop2-P2X2* and bath-applied ATP. To assess activity changes of *ppk1* neurons, we used *ppk1-Gal4* to express *UAS-GCaMP3* and imaged their axonal termini in the VNC. The connectives between VNC and reproductive tract in were usually carefully preserved, but we severed them when assessing whether the GCaMP3 increase in *ppk1* axons might be due to local activation of *ppk1* axons by *ILP7* neurons (see also Figure S3).

(G and H) Stimulating *ILP7* neurons induces a clear contraction of the reproductive tract. (G) Tract before stimulation. *ILP7* axons are visible because they coexpress *GCaMP3* and P_2X_2. (H) ATP causes a clear contraction of the tract and a significant GCaMP3 increase (n = 10 animals). Scale bar for (G) and (H) is 25 μm. See also Movie S2.

(I–K) Stimulating tract contraction (via stimulating *ILP7* neurons) induces a clear GCaMP3 increase in tract *ppk1* neurons with (9/20) and without (8/8) the presence of *ppk1-Gal80*. (I) GCaMP3 response of *ppk1* axons when perfused with buffer. (J) GCaMP3 response of the same axons when perfused with ATP. (K) Quantification of the ATP-induced GCaMP3 change. ***$p < 0.001$. Mann-Whitney test. Note that the GCaMP3 increase is more pronounced in the area where tract *ppk1* axons terminate (pink arrow) than where other axons terminate (white arrow). See also Movie S3.

DISCUSSION

In this report, we discovered that egg-laying need can activate AA attraction in *Drosophila* females and that mechanical stretch of the tract—induced by egg delivery through their internal reproductive tract—is one origin of the "egg-laying need." We demonstrate that egg delivery in the internal reproductive tract is an important physiological signal that modulates how *Drosophila* females interpret the valence of sensory stimuli. The flexibility in egg-laying need-induced modification of sensory processing contrasts significantly to that triggered by SP: once SP gains control of the female CNS, it keeps the mated females unreceptive to male courtship for days (Rezával et al., 2012; Ribeiro and Dickson, 2010; Yang et al., 2009). In contrast, mated females can readily turn on and off their AA attraction depending on their egg-laying need at a given moment. Perhaps this is because while there is no need for females to remate until the stored sperm is depleted, in between egg laying, they have to tend to other needs and are better off not "too attached" to an AA site.

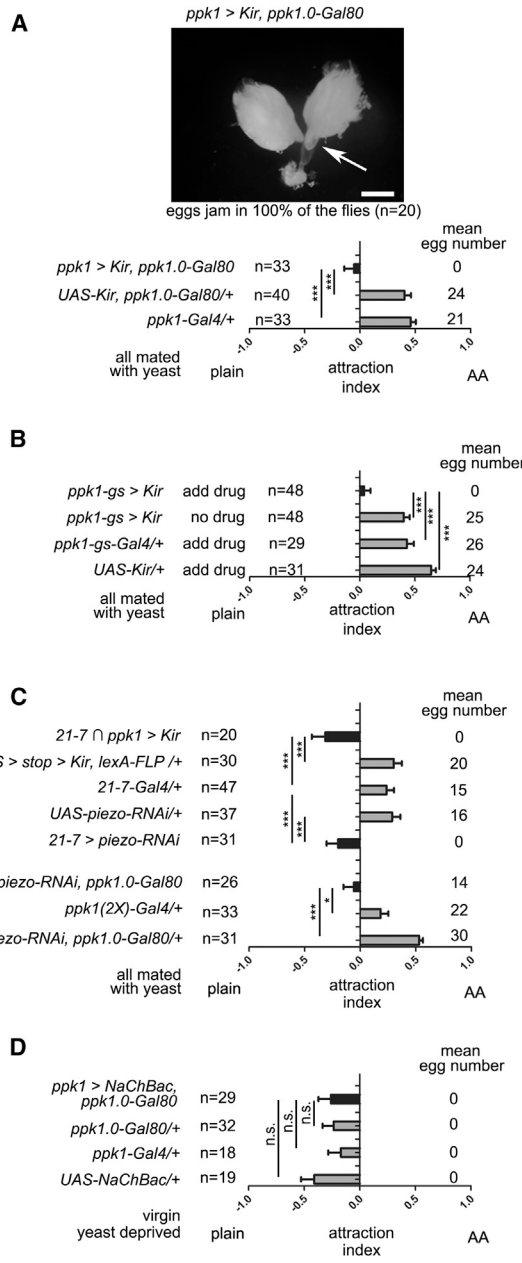

Figure 4. Functional Tract *ppk1* Neurons Are Required for Egg-Laying-Induced AA Attraction

(A) Inhibiting *ppk1* neurons in the presence of *ppk1-Gal80* causes egg jamming (white arrow) and reduces AA attraction. Scale bar is 500 μm. One-way ANOVA with Bonferroni's post test. *$p < 0.05$. n.s., not significant, $p > 0.05$.

(B) Inhibiting *ppk1* neurons specifically in adult stage reduces AA attraction still. One-way ANOVA with Bonferroni's post test. n.s., not significant. ***$p < 0.001$.

(C) Inhibiting tract *ppk1* neurons by the "intersection method" reduces AA attraction, as does reducing *piezo* expression in *21-7* neurons or tract *ppk1* neurons (2× *ppk1-Gal4* plus *ppk1-Gal80*).

(D) Expressing *NaChBac* in tract *ppk1* neurons fails to activate AA attraction.

If tract *ppk1* neurons play a critical role in AA attraction, why did our activation experiment fail? There are several possibilities. First, a temporally/spatially precise stimulation of *ppk1* neurons is needed. Second, there are additional mechanosensors on the tract that act together with *ppk1* neurons to promote AA attraction. Third, presence of eggs in the tract may signal via both a mechanical and a nonmechanical means. For example, passage of eggs through the tract may trigger release of hormones from the tract that then act together with *ppk1* neurons to modulate behavior. Nevertheless, it is interesting to note that *Drosophila* is not the only species that relies on reproductive tract-generated mechanical stimuli to activate "maternal behaviors." Mechanical stimulation of uterine wall has been shown to contribute to maternal behavior activation in sheep, dogs, and rats (Hayes and De Vries, 2007; Kendrick et al., 1991; Keverne et al., 1983; Lévy et al., 2010; Poindron et al., 1989; Yeo and Keverne, 1986), raising the possibility that this feature of behavior control may be evolutionarily conserved.

Which sensory system is responsible for egg-laying-induced AA attraction? Several reports suggest AA can promote attraction through the olfactory system (Ai et al., 2010; Root et al., 2011; Semmelhack and Wang, 2009), but Joseph et al. showed that egg-laying preference for AA depends on taste, but not olfaction (Joseph et al., 2009). Indeed, we have found that *Ir64a* and *Or83b*, two olfaction mutants with defective AA sensing (Ai et al., 2010; Semmelhack and Wang, 2009), both show strong egg-laying-induced AA attraction (Figures S4E and S4F). But if egg-laying-induced AA attraction is indeed taste driven, then it may be partly driven by taste memory. This is because "attractive returns" occur in the middle of the chamber where females' taste neurons are not in contact with AA. Mushroom body (MB) has been shown to regulate egg-laying-induced positional preference for AA (Joseph et al., 2009), but we did not observe a significant change in AA attraction when we ablated it (Figures S4A–S4D), suggesting the taste memory that guides attractive returns in our paradigm is likely stored elsewhere. Determining the identity of the AA-sensing taste neurons that promote AA attraction will be an important next step.

Lastly, we also do not know which brain center(s) responds to tract *ppk1* neurons to modify valence of AA signal. *Pars intercerebralis*, the neuroendocrine center, may be one potential (indirect) target, given that it was proposed recently to be a potential target of tract neurons that signal mating status change (Feng et al., 2014) and that hypothalamus, its vertebrate counterpart, is important for modulating reproductive behaviors also.

EXPERIMENTAL PROCEDURES

Stocks

The following stocks were used in this work: *ILP7-Gal4* (Yang et al., 2008), *piezo-Gal4* (Kim et al., 2012), *21-7-Gal4* (Song et al., 2007), *hs-FLP* (Gordon and Scott, 2009), *UAS-Kir2.1eGFP* (Baines et al., 2001), *ppk1-Gal4, UASmCD8-GFP, UAS-mCD8-RFP, UAS-NaChBac* (Bloomington Drosophila Stock Center), *UAS-FRT-CD2-stop-FRT-Kir2.1eGFP* (Yang et al., 2009), *UAS-P_2X_2* (Lima and Miesenböck, 2005), *UAS-GCaMP3* (Tian et al., 2009), *UAS-piezo-RNAi* (Kim et al., 2012), *ppk1-LexA, ppk1.0-Gal80* (Häsemeyer et al., 2009), *ILP7-LexA, LexAop2-FLP* (Pan et al., 2012), *LexAop2-P_2X_2, ppk1-GS-Gal4, LexAop2-GCaMP3*, and w^{1118}.

Transgenic Animals

ILP7-LexA, ppk1-LexA constructs were produced by cloning the 1 kb promoter upstream of *ILP7* and *ppk1* genes, respectively, into the recently

modified *LexA* construct (Pfeiffer et al., 2010). *LexAop2-GCaMP3* and *LexAop2-P2X2* were constructed by cloning the *GCaMP3* (Tian et al., 2009) and P_2X_2 (Lima and Miesenböck, 2005) genes into *LexAop2* vectors (Pfeiffer et al., 2010). These constructs were then injected into attP-carrying animals following standard protocol.

Immunohistochemistry

Tissues were processed following the same protocol as previously described (Yang et al., 2008). Images were acquired using a Zeiss LSM 700 laser scanning confocal microscope. The following primary antibodies were used: mouse anti-Dlg (1:10, DHSB), rat anti-mCD8 (1:100, Invitrogen), mouse anti-GFP (1:100, Sigma-Aldrich), and rabbit anti-GFP (1:1,000, Invitrogen). The following secondary antibodies were used: goat anti-mouse Alexa 488, goat anti-mouse Cy3, goat anti-rat Alexa 488, goat anti-rabbit Alexa 488, and goat anti-rabbit Cy3.

Hydroxyurea Treatment to Ablate the Mushroom Body

To ablate the mushroom body, 0- to 1-hr-old larvae were collected and fed hydroxyurea (HU) (H8627, Sigma) dissolved in inactive yeast paste (50 mg/ml) for 5 hr and then transferred into fresh HU-free food.

Egg-Laying Preference Assay

Females were collected into groups of 20–30 with 15–20 males at the first day of eclosion and kept in fly incubator (25°C and 65% humidity) for 4–5 days. The vials where females were kept were supplemented with active yeast paste unless we wanted to reduce their egg production. Prior to egg-laying/behavior assays, individual female flies were loaded into chambers in a custom-designed two-choice egg-laying apparatus (Figures S1A and S1B). The 3% AA (v/v) substrate and plain substrate were made from 1% agarose that has been preheated in the 55°C water bath. The substrates were allowed to set for 30 min before experiments commence.

Behavior Analysis

Flies were raised and placed into chambers as described above. A camera holder with four Microsoft LifeCam Cinema cameras was then attached to the top of the chamber-containing apparatus (Figure S1A). Each camera was positioned above two egg-laying chambers to record two flies. Videos were acquired by CamUniversal and then converted (to allow faster tracking) using Avidemux. Egg-laying events were annotated manually by visually inspecting the videos. Ctrax (Branson et al., 2009) was used to track the fly positions. Custom MATLAB code was used to detect parameters we used to calculate positional preference index and attraction index and to separate the trajectories into high and low egg-laying states based on the flies' locomotion speed (Figures S1C and S1D). The speed is calculated first by determining the path length every 2 s (at 7.5 frames/s). We imposed a 1 min threshold when separating the low versus high locomotion states. This is because flies sometimes increase their running speed suddenly and briefly—even though they have been consistently in a state of low locomotion—and they then immediately slow down and lay an egg (Figure 1C). Thus, if flies do not maintain their increased speed for at least 1 min, we consider such changes too transient to be labeled as "high locomotion state."

Tract Contraction Assay

To determine whether stimulating *ILP7* can cause tract contraction (Figures 3G and 3H), we isolated the tract from the rest of the body and pinned it onto a custom-made perfusion chamber that contains insect physiology buffer (Xiang et al., 2010). We then either perfused the chamber with buffer alone or with buffer with ATP (400 μM).

GCaMP3 Recording

Intact VNC (with their connection to the tract carefully preserved) was dissected and mounted in a custom-built perfusion chamber and imaged through a water-immersion 40× lens on our Zeiss LSM 700 confocal scope. To image neuronal activity, we recorded GCaMP3 fluorescence at one frame per second using the time-series function software. We normally waited for the baseline to stabilize before perfusing buffer alone for 3 min and then buffer with ATP to stimulate specific neurons and recorded changes in GCaMP3 signal. The acquired images were then analyzed using a custom MATLAB code. Change in fluorescence (ΔF/F) in a given ROI was averaged from signals from all focal planes.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.09.033.

AUTHOR CONTRIBUTIONS

C.-H.Y. conceived the project with help from B.G. and Y.L. B.G. and Y.L. performed the experiments and analyzed the results with help from A.G, U.S., and C.-H.Y. U.S. designed the behavior setup. B.G, Y.L., and C.-H.Y. wrote the paper.

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REFERENCES

Ai, M., Min, S., Grosjean, Y., Leblanc, C., Bell, R., Benton, R., and Suh, G.S. (2010). Acid sensing by the *Drosophila* olfactory system. Nature *468*, 691–695.

Azanchi, R., Kaun, K.R., and Heberlein, U. (2013). Competing dopamine neurons drive oviposition choice for ethanol in Drosophila. Proc. Natl. Acad. Sci. USA *110*, 21153–21158.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J. Neurosci. *21*, 1523–1531.

Branson, K., Robie, A.A., Bender, J., Perona, P., and Dickinson, M.H. (2009). High-throughput ethomics in large groups of Drosophila. Nat. Methods *6*, 451–457.

Bussell, J.J., Yapici, N., Zhang, S.X., Dickson, B.J., and Vosshall, L.B. (2014). Abdominal-B neurons control Drosophila virgin female receptivity. Curr. Biol. *24*, 1584–1595.

Carvalho, G.B., Kapahi, P., Anderson, D.J., and Benzer, S. (2006). Allocrine modulation of feeding behavior by the Sex Peptide of *Drosophila*. Curr. Biol. *16*, 692–696.

Castellanos, M.C., Tang, J.C., and Allan, D.W. (2013). Female-biased dimorphism underlies a female-specific role for post-embryonic Ilp7 neurons in *Drosophila* fertility. Development *140*, 3915–3926.

Coste, B., Mathur, J., Schmidt, M., Earley, T.J., Ranade, S., Petrus, M.J., Dubin, A.E., and Patapoutian, A. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. Science *330*, 55–60.

Coste, B., Xiao, B., Santos, J.S., Syeda, R., Grandl, J., Spencer, K.S., Kim, S.E., Schmidt, M., Mathur, J., Dubin, A.E., et al. (2012). Piezo proteins are pore-forming subunits of mechanically activated channels. Nature *483*, 176–181.

Dweck, H.K., Ebrahim, S.A., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., Hansson, B.S., and Stensmyr, M.C. (2013). Olfactory preference for egg laying on citrus substrates in *Drosophila*. Curr. Biol. *23*, 2472–2480.

Feng, K., Palfreyman, M.T., Häsemeyer, M., Talsma, A., and Dickson, B.J. (2014). Ascending SAG neurons control sexual receptivity of *Drosophila* females. Neuron *83*, 135–148.

Gao, X.J., Potter, C.J., Gohl, D.M., Silies, M., Katsov, A.Y., Clandinin, T.R., and Luo, L. (2013). Specific kinematics and motor-related neurons for aversive chemotaxis in *Drosophila*. Curr. Biol. *23*, 1163–1172.

Gordon, M.D., and Scott, K. (2009). Motor control in a *Drosophila* taste circuit. Neuron *61*, 373–384.

Häsemeyer, M., Yapici, N., Heberlein, U., and Dickson, B.J. (2009). Sensory neurons in the *Drosophila* genital tract regulate female reproductive behavior. Neuron *61*, 511–518.

Hayes, U.L., and De Vries, G.J. (2007). Role of pregnancy and parturition in induction of maternal behavior in prairie voles (*Microtus ochrogaster*). Horm. Behav. *51*, 265–272.

Joseph, R.M., and Heberlein, U. (2012). Tissue-specific activation of a single gustatory receptor produces opposing behavioral responses in Drosophila. Genetics *192*, 521–532.

Joseph, R.M., Devineni, A.V., King, I.F., and Heberlein, U. (2009). Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in Drosophila. Proc. Natl. Acad. Sci. USA *106*, 11352–11357.

Kendrick, K.M., Lévy, F., and Keverne, E.B. (1991). Importance of vaginocervical stimulation for the formation of maternal bonding in primiparous and multiparous parturient ewes. Physiol. Behav. *50*, 595–600.

Keverne, E.B., Levy, F., Poindron, P., and Lindsay, D.R. (1983). Vaginal stimulation: an important determinant of maternal bonding in sheep. Science *219*, 81–83.

Kim, S.E., Coste, B., Chadha, A., Cook, B., and Patapoutian, A. (2012). The role of *Drosophila* Piezo in mechanical nociception. Nature *483*, 209–212.

Kristal, M.B. (2009). The biopsychology of maternal behavior in nonhuman mammals. ILAR J. *50*, 51–63.

Kubli, E. (2003). Sex-peptides: seminal peptides of the *Drosophila* male. Cell. Mol. Life Sci. *60*, 1689–1704.

Lévy, F., Keller, M., Cornilleau, F., Moussu, C., and Ferreira, G. (2010). Vaginocervical stimulation of Ewes induces the rapid formation of a new bond with an alien young without interfering with a previous bond. Dev. Psychobiol. *52*, 537–544.

Lima, S.Q., and Miesenböck, G. (2005). Remote control of behavior through genetically targeted photostimulation of neurons. Cell *121*, 141–152.

Luan, H., Lemon, W.C., Peabody, N.C., Pohl, J.B., Zelensky, P.K., Wang, D., Nitabach, M.N., Holmes, T.C., and White, B.H. (2006). Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila. J. Neurosci. *26*, 573–584.

Pan, Y., Meissner, G.W., and Baker, B.S. (2012). Joint control of *Drosophila* male courtship behavior by motion cues and activation of male-specific P1 neurons. Proc. Natl. Acad. Sci. USA *109*, 10065–10070.

Pfeiffer, B.D., Ngo, T.-T.B., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., and Rubin, G.M. (2010). Refinement of tools for targeted gene expression in Drosophila. Genetics *186*, 735–755.

Poindron, P., Rempel, N., Troyer, A., and Krehbiel, D. (1989). Genital stimulation facilitates maternal behavior in estrous ewes. Horm. Behav. *23*, 305–316.

Rezával, C., Pavlou, H.J., Dornan, A.J., Chan, Y.-B., Kravitz, E.A., and Goodwin, S.F. (2012). Neural circuitry underlying *Drosophila* female postmating behavioral responses. Curr. Biol. *22*, 1155–1165.

Ribeiro, C., and Dickson, B.J. (2010). Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in *Drosophila*. Curr. Biol. *20*, 1000–1005.

Rockwell, R., and Grossfield, J. (1978). Drosophila: behavioral cues for oviposition. Am. Midl. Nat. *99*, 361–368.

Root, C.M., Ko, K.I., Jafari, A., and Wang, J.W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. Cell *145*, 133–144.

Rosenblatt, J.S., and Lehrman, D.S. (1963). Maternal behavior in the laboratory rat. In Maternal Behavior in Mammals, H. Rheingold, ed. (New York: Wiley), pp. 8–57.

Schwartz, N.U., Zhong, L., Bellemer, A., and Tracey, W.D. (2012). Egg laying decisions in *Drosophila* are consistent with foraging costs of larval progeny. PLoS ONE *7*, e37910.

Semmelhack, J.L., and Wang, J.W. (2009). Select *Drosophila* glomeruli mediate innate olfactory attraction and aversion. Nature *459*, 218–223.

Song, W., Onishi, M., Jan, L.Y., and Jan, Y.N. (2007). Peripheral multidendritic sensory neurons are necessary for rhythmic locomotion behavior in *Drosophila* larvae. Proc. Natl. Acad. Sci. USA *104*, 5199–5204.

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods *6*, 875–881.

Xiang, Y., Yuan, Q., Vogt, N., Looger, L.L., Jan, L.Y., and Jan, Y.N. (2010). Light-avoidance-mediating photoreceptors tile the *Drosophila* larval body wall. Nature *468*, 921–926.

Yang, C.H., Belawat, P., Hafen, E., Jan, L.Y., and Jan, Y.N. (2008). *Drosophila* egg-laying site selection as a system to study simple decision-making processes. Science *319*, 1679–1683.

Yang, C.H., Rumpf, S., Xiang, Y., Gordon, M.D., Song, W., Jan, L.Y., and Jan, Y.N. (2009). Control of the postmating behavioral switch in *Drosophila* females by internal sensory neurons. Neuron *61*, 519–526.

Yao, Z., Macara, A.M., Lelito, K.R., Minosyan, T.Y., and Shafer, O.T. (2012). Analysis of functional neuronal connectivity in the *Drosophila* brain. J. Neurophysiol. *108*, 684–696.

Yeo, J.A., and Keverne, E.B. (1986). The importance of vaginal-cervical stimulation for maternal behaviour in the rat. Physiol. Behav. *37*, 23–26.

Zhou, C., Pan, Y., Robinett, C.C., Meissner, G.W., and Baker, B.S. (2014). Central brain neurons expressing doublesex regulate female receptivity in *Drosophila*. Neuron *83*, 149–163.