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Long-term neuropeptide modulation of female sexual drive via the TRP channel in *Drosophila melanogaster*

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Connectomics research has made it more feasible to explore how neural circuits can generate multiple outputs. Female sexual drive provides a good model for understanding reversible, long-term functional changes in motivational circuits. After emerging, female flies avoid male courtship, but they become sexually receptive over 2 d. Mating causes females to reject further mating for several days. Here, we report that pC1 neurons, which process male courtship and regulate copulation behavior, exhibit increased CREB (cAMP response element binding protein) activity during sexual maturation and decreased CREB activity after mating. This increased CREB activity requires the neuropeptide Dh44 (Diuretic hormone 44) and its receptors. A subset of the pC1 neurons secretes Dh44, which stimulates CREB activity and increases expression of the TRP channel Pyrexia (Pyx) in more pC1 neurons. This, in turn, increases pC1 excitability and sexual drive. Mating suppresses *pyx* expression and pC1 excitability. Dh44 is orthologous to the conserved corticotrophin-releasing hormone family, suggesting similar roles in other species.

sexual drive | pC1 | Diuretic hormone 44 | Pyrexia | Sex Peptide

Significance

Animals experience the mating-related "ticking of a biological clock" as their mating motivation or sexual drive changes over their lifespan. In the fruit fly, a female develops sexual drive over a couple of days after emergence, which is almost completely suppressed later by mating. This study utilized female mating behavior as a model to investigate the reversible and long-term shifts of electric activity in the brain circuit involved in generating behavioral motivation. It uncovered a neuropeptide pathway, orthologous to the vertebrate corticotrophin-releasing hormone (CRH) family of neuropeptides, that enables such long-term shifts in the excitability of a group of brain neurons processing olfactory and auditory courtship cues, and sexual drive by regulating the production of an ion channel.

Sex behavior is crucial for the survival of many species. In most of these species, a male typically begins with a courtship display and his female target decides whether to accept his advances. To make this decision, the female must integrate sensory cues from the male, such as pheromones and vocal signals, with her own sexual motivation. Female sexual motivation changes in response to various social and environmental cues, including the presence of other females (1), perceived male quality (2, 3), male group quality (4), time of day (5), and food availability (6). Sexual motivation can also change on a longer time-scale depending on sexual maturity and past mating experience (7–9).

The fruit fly *Drosophila melanogaster* has a simple nervous system that enables it to perform complex behaviors. This model insect, for which many advanced molecular genetic tools have been developed, has been used extensively to study the development and function of the nervous system. Studies of the regulation of *Drosophila* sexual behavior have provided important insights into how genes influence the development and function of neurons that program behaviors. Genes such as *doublesex* (*dsx*) and *fruitless* (*fru*) determine the development and differentiation of the nervous system and generate sex-specific behaviors through alternative splicing that produces sexually dimorphic transcription factors (10–13). The male-specific *fru* protein (Fru^M) is sufficient to produce male-specific circuits and behaviors even in females (14, 15). Dsx is also crucial for sexually dimorphic brain connectivity and behaviors (16–20). The female-specific *dsx* protein (Dsx^F) is involved in various aspects of female reproductive behavior. For example, Dsx^F-producing neurons, such as the SPSNs (21–23), the SP abdominal ganglion (SAG) neurons (24), the pC1 neurons (25), the pCd neurons (25), and the vpoDNs (26), are critical for female mating behavior.

The pC1 neurons exhibit strong Ca^{2+} transients in response to the aphrodisiac pheromone 11-*cis*-vaccenyl acetate (cVA) or male courtship songs (25). They are connected to vpoDNs and control vaginal plate opening (VPO) behavior, allowing females to engage in vaginal coitus (26). The pC1 neurons also indirectly connect to oviDNs, which control oviposition. Highlighting the inverse relationship between mating and oviposition, activation of pC1 neurons in females suppresses oviposition via oviDNs (27). There are approximately fourteen pC1 neurons in each brain hemisphere, and an analysis of high-resolution electron microscopy (EM) connectome data (e.g., Hemibrain) grouped them into five subtypes (i.e., pC1a through pC1e) (27, 28). Recent genetic studies have suggested differential roles for each pC1 subtype. pC1d and pC1e, for example, were found to regulate aggression (28–30). The specific pC1 subset involved in mating behavior, however, remains unclear.

Drosophila females reach sexual maturity 2 to 3 d after they become adults (i.e., after eclosion). Freshly eclosed immature females show very low sexual drive and actively avoid

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courtship from males. Their gradual increase in sexual drive during sexual maturation correlates with oocyte development (8), and is regulated by gonadotropins such as juvenile hormone (JH) (31). The production of JH is driven by endocrine factors that stimulate the physiological and behavioral events leading to eclosion (32–34). JH titers peak on the day of eclosion, decreasing gradually thereafter. One recent study revealed that the SAG neurons, which make synaptic connections with pC1 neurons (see below), are involved in the gradual cessation of JH production after eclosion (34). The same study also showed that SAG neurons progressively increase neural activity over the 3 d following eclosion. Despite these findings, it is still unknown whether SAG neurons play a role in the generation of sexual drive during sexual maturation.

Past mating experience and pregnancy also have a significant impact on a female's mating drive. The behavioral and physiological changes that comprise the post-mating response (PMR) are triggered by a seminal protein called Sex Peptide (SP) (35, 36). Mated females who have not encountered SP show the same high level of sexual drive as virgin females (37, 38), whereas virgin females injected with synthetic SP exhibit decreased sexual drive and actively avoid male courtship (35, 39). This suggests that SP signaling affects unknown neural circuits that generate the sexual drive. During mating, the male injects SP along with his sperm into the female uterus (40). Within the uterus, SP activates the SP receptor (SPR) in SPSNs that project neural processes into the tip of the abdominal ganglion (AbG). In the AbG, the SP signal is transmitted to a small group of female-specific neurons known as the SAG neurons. SAG neurons provide excitatory inputs to pC1 neurons (24, 27). SPR is an inhibitory GPCR that, when activated, silences the neurons that express it (21, 22, 41). As a result, SP silences SPSNs, then SAG neurons, and then pC1 neurons in that order, suppressing mating receptivity and increasing oviposition in mated females.

Recent advances in the study of all the connections in the *Drosophila* brain or the *Drosophila* connectome have revealed neural circuit networks that control various functions, such as odor-evoked behaviors (42), learning and memory (43, 44), and innate behaviors (28, 45, 46). Connectome databases have also significantly improved our understanding of how these neural circuits influence female mating behavior. For instance, the Hemibrain EM database showed direct and strong connections between SAG neurons and pC1 neurons that were later validated by electrophysiological recordings (27). Further research using EM connectome data documented the circuit architecture of the complex interactions between pC1 neurons, vpoENs, vpoINs, and vpoDNs. This offered insight into how male courtship songs elicit female responses, such as the opening of the vaginal plates for sexual intercourse (26). Despite this progress, our understanding of how sexual drive or motivation is programmed within mating decision circuits remains limited.

The physiological properties of decision-making circuits are often altered by the action of neuromodulators, such as amines or neuropeptides (47). It is likely that the female mating decision circuits also exhibit significant modulation via the action of neuromodulators and their metabotropic receptors (i.e., GPCRs). Studies in both insects and mammals have already linked several neuromodulators to sexual behaviors. For instance, in female rodents, the neuropeptide kisspeptin and its GPCR receptor Kiss1R play a role in triggering pheromone-driven mate preference and copulatory behavior (48). In female flies, dopamine (49, 50), octopamine (51), SIFamide (52), Mip (53), and Drosulfakinin (54) are involved in mating behaviors. However, the specific brain neuromodulatory systems responsible for inducing long-term changes in female sexual drive during sexual maturation and after mating remain unknown.

In this study, we found that two GPCRs for the neuropeptide Diuretic hormone 44 (Dh44) are crucial for cAMP (i.e., cyclic adenosine monophosphate) response element binding protein (CREB) activity (its fly homolog, *CrebB*) in pC1 neurons, which increases during sexual maturation and decreases after mating. Dh44 is orthologous to the vertebrate corticotrophin-releasing hormone (CRH) family that includes CRH, urotensin I, sauvagine, and urocortin I, II, and III (55). Like its vertebrate counterparts, Dh44 is involved in multiple biological processes, such as diuresis (56), feeding (57), circadian activity (58), and sperm storage (59). Our findings suggest that a pC1 neuron subset receives excitatory input from SAG neurons, secretes Dh44, and activates CREB activity in a larger group of pC1 neurons that express Dh44Rs. The increased CREB activity in pC1 neurons increases their expression of *pyrexia* (*pyx*), a gene encoding a transient receptor potential (Trp) cation channel. This leads to increased sexual drive. After mating, SP silences the pC1 neurons, stopping their secretion of Dh44 and reducing their CREB activity. This down-regulates their expression of *pyx,* remodels their synapses, and reduces their excitability, leading to decreased sexual drive.

Results

During Sexual Maturation, pC1 Neurons Display Increased Excitability and Increased CREB Activity. *D. melanogaster* females are sexually immature when they emerge and show low sexual drive and mating receptivity. It takes 2 d for a female to develop full sexual drive (*SI Appendix*, Fig. S1*A*). To investigate whether pC1 neuron excitability also changes with sexual maturation, we examined the Ca^{2+} responses of pC1 neurons expressing the Ca^{2+} reporter GCaMP6m when we exposed them to courtship cues, such as the sex pheromone cVA or the courtship pulse song, before and after sexual maturation (Fig. 1*A* and *SI Appendix*, Fig. S1*B*). In 3-d-old mature virgin females, pC1 neurons produced robust Ca^{2+} transients in response to cVA or pulse song (Fig. 1*A* and *SI Appendix*, Fig. S1*C*). In contrast, the pC1 neurons of 1-d-old immature virgin females, which typically exhibit a mating rate of less than 10% when paired with wild-type *Canton-S* (*CS*) males, showed a much weaker Ca^{2+} response to cVA or pulse song (Fig. 1*A*).

To determine whether increased pC1 neuron excitability increases sexual drive, we used the temperature-gated cation channel dTrpA1 to activate pC1 neurons during mating assays in sexually immature 1-d-old females. Our results confirmed that pC1 neuron activation increased mating receptivity to a level comparable to that observed in sexually mature older females (Fig. 1*B*).

Our observation that the excitability of pC1 neurons increases as females undergo sexual maturation suggests pC1 neurons receive modulatory inputs that change with age. One previous study reported an association between female mating receptivity and cAMP, which functions downstream of neuromodulators and their GPCRs (60). To monitor cAMP activity specifically in pC1 neurons, we assembled three transgenes (i.e., *pC1-GAL4, UAS-FLP*, and *CRE-F-luc*) that allow pC1 neurons to produce a luciferase reporter in response to elevated intracellular cAMP levels (61, 62) (*SI Appendix*, Fig. S1*D*). Remarkably, we found a clear correlation between pC1 luciferase reporter activity (hereafter referred to as pC1-CREB activity) and the time course of mating receptivity changes during sexual maturation. Both pC1-CREB and mating receptivity gradually increased after eclosion, peaked within 2 d, and remained high thereafter (Fig. 1*C* and *SI Appendix*, Fig. S1*E*).

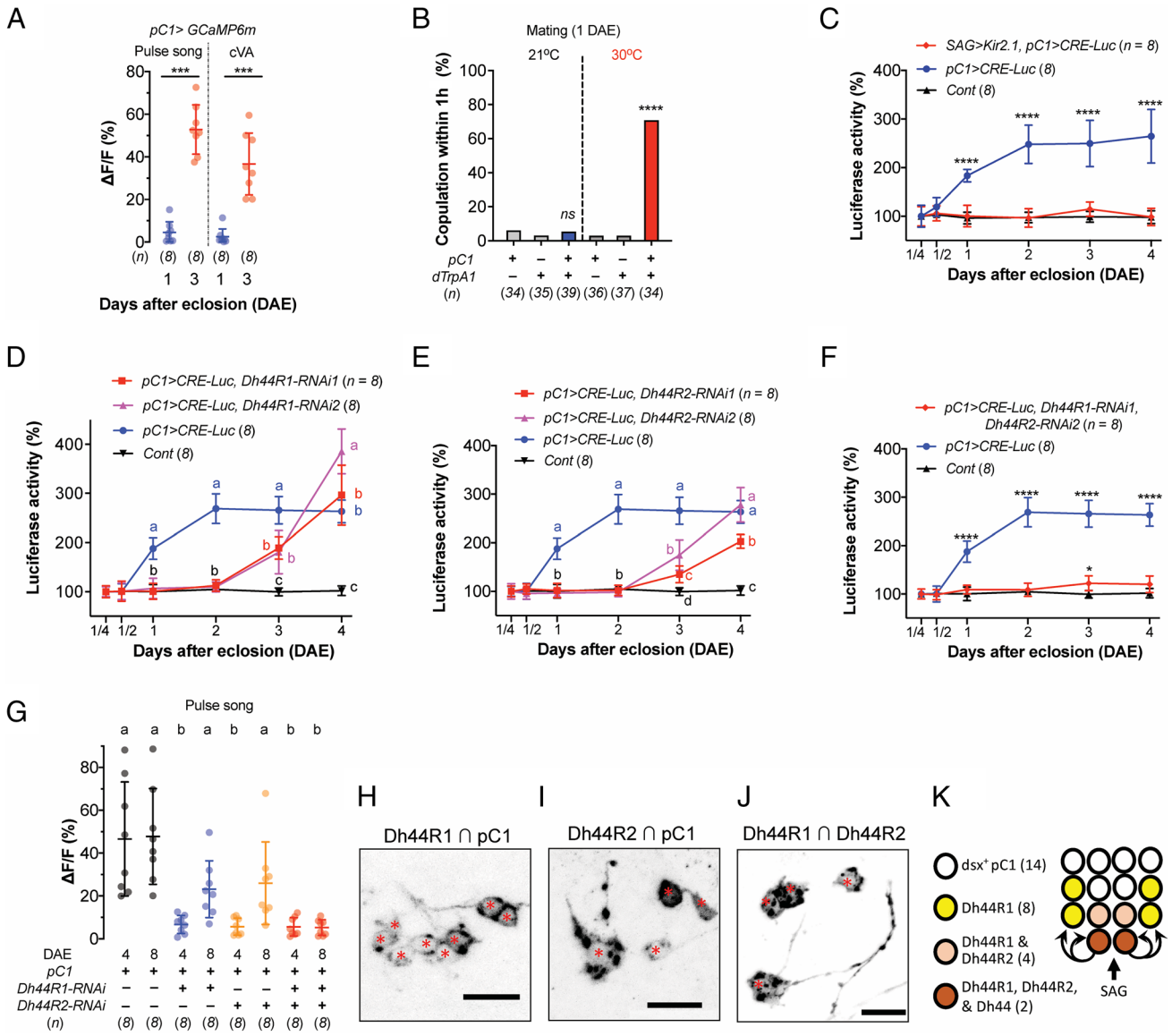

Fig. 1. Increased excitability and CREB activity during sexual maturation requires the expression of both Dh44R1 and Dh44R2 in pC1 neurons. (*A*) The peak ΔF/F in the lateral protocerebral complex (LPC) projections of pC1 neurons from 1- and 3-d-old virgin females carrying *pC1-GAL4* and *UAS-GCaMP6m* (*pC1 > GCaMP6m*) in response to male olfactory (cVA) and auditory (pulse song) courtship stimuli. (*B*) Mating frequencies for 1-d-old virgin females of indicated genotypes, scored as the percentage of females that copulated within 1 h. For specific genotypes, see also *SI Appendix*, Table S3. (*C*) The relative *CRE-Luciferase* reporter activity of pC1 neurons from virgin females of the indicated genotypes. To calculate the relative luciferase activity, we set the average luminescence unit (LU) values of each group at the first measurement time point (1/4 DAE) to 100%. (*D–F*) Relative *CRE-Luciferase* reporter activity in pC1 neurons from females of the indicated genotypes. To aid comparisons, we used the same positive (blue) and negative (black) control data in all panels. (*G*) The peak ΔF/F in the LPC projections of pC1 neurons from 4- and 8-d-old virgin females of the indicated genotypes in response to male auditory courtship stimuli (pulse song). (*H*) A confocal image showing Dh44R1-positive pC1 neurons from females carrying *pC1-GAL4, Dh44-R1-LexA, LexAOP-FLP*, and *UAS>stop>smGFP-HA* stained with anti-HA. In each brain hemisphere, eight of fourteen pC1 neurons were positive for *Dh44R1-LexA* activity (red stars). See also *SI Appendix*, Fig. S1*E*. (Scale bars for *E–G*, 10 μm.) (*I*) A confocal image showing Dh44R2-positive pC1 neurons from females carrying *pC1-LexA, Dh44R2-GAL4, LexAOP-FLP*, and *UAS>stop>smGFP-HA* stained with anti-HA. In each brain hemisphere, four of fourteen pC1 neurons were positive for *Dh44R2-GAL4* activity (red stars). (*J*) A confocal image showing pC1 neurons double-positive for Dh44R1 and Dh44R2 from females carrying *Dh44R1-LexA, Dh44R2-GAL4, LexAOP-FLP*, and *UAS>stop>smGFP-HA* stained with anti-HA. In each brain hemisphere, four of fourteen pC1 neurons were positive for both Dh44R1-LexA and Dh44R2-GAL4 (red stars). (*K*) Schematic showing pC1 neurons expressing Dh44, Dh44R1, or Dh44R2 in each brain hemisphere. The numbers in parentheses indicate the number of pC1 neurons expressing the indicated molecules. Statistical analysis: Mann–Whitney test (*A*); chi-square test (*B*); One-way ANOVA with Dunnett's test (*C* and *F*) and Tukey's test (*D* and *E*) for multiple comparisons; Kruskal–Wallis test with Dunn's test for multiple comparisons (*G*). Data are presented as means ± SD (****$P < 0.0001$; ***$P < 0.001$; ns or no labeling, $P > 0.05$). In *A* and *G*, each dot represents an individual female. The numbers in parentheses indicate *n*. The letters above the plots in panels *D*, *E*, and *G* indicate significant differences ($P < 0.05$) between genotypes and DAE.

SAG neurons supply excitatory synaptic inputs to pC1 neurons (27), and their neural activity progressively increases as females age after eclosion (34). We, therefore, speculated that SAG neurons modulate pC1 neurons, potentially increasing pC1-CREB activity. Indeed, we found neural silencing of SAG neurons via expression of the inward rectifying potassium channel Kir2.1 almost completely inhibited pC1-CREB activity (Fig. 1*C*). This result links SAG neurons with the modulatory inputs responsible for the gradual increase in pC1-CREB activity during sexual maturation.

Increased Excitability and CREB Activity Require the Expression of Dh44 Receptors. cAMP is a major intracellular second messenger in neurons that mediates GPCR signals activated

by neuromodulators such as amines and neuropeptides (63). Therefore, we looked for the GPCR(s) responsible for the increase of pC1-CREB activity during sexual maturation and found that the knockdown of *Dh44R1* or *Dh44R2* caused the greatest decrease in pC1-CREB activity (Fig. 1 *D* and *E*). In *Dh44R1-* or *Dh44R2-RNAi* females, pC1-CREB activity was suppressed by 2 days after eclosion (DAE). However, it rebounded to control levels or above by 4 DAE. This suggests the existence of homeostatic mechanisms that keep pC1-CREB activity within a target range. Since both Dh44R1 and Dh44R2 are highly selective for the same neuropeptide ligand, Dh44 (64, 65), we speculated that one might compensate for the loss of the other. Indeed, we found simultaneous knockdown of both receptors almost completely blocked pC1-CREB activity and prevented any rebound until the end of the experiment (Fig. 1*F*).

Next, we asked whether Dh44R1 or Dh44R2 is required for pC1 neurons to exhibit Ca^{2+} transients in response to male courtship cues. Hereafter, we refer to the presence of these transients as pC1 neuron excitability. We found that RNAi knockdown of either *Dh44R1* or *Dh44R2* suppressed pC1 neuron excitability to its lowest level at 4 DAE. Like what we observed with pC1-CREB activity, however, the inhibitory effect of single gene knockdown was transient, with pC1 neuron excitability showing a significant rebound (compare the results from 4 and 8 DAE in Fig. 1*G*). In contrast, double knockdown of *Dh44R1* and *Dh44R2* inhibited pC1 neuron excitability until 8 DAE. Therefore, we concluded that Dh44R1 and Dh44R2 are necessary for the increases in both pC1-CREB activity and pC1 neuron excitability normally observed as females develop sexual maturity. We also concluded that each of the two receptors can complement the other's function in pC1 neurons.

We next asked whether pC1 neurons produce Dh44R1 and Dh44R2. We combined two binary expression systems to visualize neurons simultaneously expressing two transgenes. For example, the combination of *Dh44R1-GAL4, pC1-LexA, LexAOP-Flipase*, and *UAS-FRT-stop-FRT-smGFP-HA* allowed only the subset of pC1 neurons with simultaneous *Dh44R1-GAL4* and *pC1-LexA* activity to produce HA-tagged smGFP. Once produced, this HA-tagged smGFP could then be stained with an anti-HA antibody (66). Using this method, we found that 8 pC1 cells in each brain hemisphere produced Dh44R1 and 4 pC1 cells produced Dh44R2 (Fig. 1 *H* and *I* and *SI Appendix*, Fig. S1*F*). The 4 pC1 cells expressing Dh44R2 were a subset of the 8 Dh44R1-positive pC1 cells (Fig. 1*J*). In summary, the fourteen pC1 cells in each brain hemisphere are divided into three subsets according to their expression of Dh44R1 and Dh44R2: 4 cells express both Dh44R1 and Dh44R2, 4 cells express Dh44R1 but not Dh44R2, and 6 cells do not express either receptor (Fig. 1*K*).

The pC1b/c Subtype Expresses Dh44 and Its Receptors Dh44R1 and Dh44R2. Silencing SAG neurons inhibits the increase in pC1-CREB activity during sexual maturation (Fig. 1*C*). Thus, we initially suspected that SAG neurons secrete Dh44, which would activate Dh44R1 and Dh44R2 in pC1 neurons. We found, however, that SAG neurons exhibit no anti-Dh44 signal (*SI Appendix*, Fig. S2*A*). Instead, we found two pairs of pC1 neurons (henceforth, Dh44-pC1) that showed anti-Dh44 staining (Fig. 2*A*). Moreover, when we used *Dh44-RNAi* to knock down Dh44 in pC1 neurons, pC1-CREB activity and pC1 neuron excitability were almost completely lost (Fig. 2 *B* and *C*). As we saw with the *Dh44R1* and *Dh44R2* double knockdown, *Dh44* knockdown inhibited both pC1-CREB activity and pC1 neuron excitability without a rebound. Two of the four Dh44R1- and Dh44R2-double positive pC1 cells per brain hemisphere also showed anti-Dh44 staining (Fig. 2*D*). We therefore propose that Dh44 secreted by Dh44-pC1 neurons activates the Dh44-pC1 neurons themselves, along with other pC1 neurons, creating a positive feedback loop that progressively increases pC1-CREB activity over the 2 d required to reach sexual maturity (Fig. 1*K*).

Existing high-resolution brain EM databases showed that pC1 neurons are composed of five anatomically distinct subtypes referred to as pC1a-e (27, 28). Although *pC1-SS2-GAL4* is expressed in cells of all five anatomical subtypes, it is expressed in only five of the fourteen pC1 cells in each brain hemisphere (27). As with *pC1-GAL4*, *Dh44* knockdown in the more restricted pC1 subset labeled by *pC1-SS2-GAL4* also completely inhibited pC1-CREB activity (*SI Appendix*, Fig. S2*B*).

To evaluate the function of a smaller subset of pC1 neurons positive for Dh44 (i.e., Dh44-pC1), we set out to generate a split-GAL4 transgene specifically targeting Dh44-pC1. First, we generated four *Dh44-GAL4* lines (*Dh44^{A}*, *Dh44^{B}*, *Dh44^{C}*, and *Dh44^{D}*), each carrying separate ~1 kb-long genomic fragments that tile the 5′-upstream cis-regulatory region of the *Dh44* gene (*SI Appendix*, Fig. S2*C*). Of these four lines, we found that silencing brain *Dh44^{A}-GAL4* neurons almost completely blocked mating receptivity in virgin females (*SI Appendix*, Fig. S2 *D–I*). Next, we combined *Dh44^{A}-AD* with the *dsx-DBD* to make a *Dh44-pC1-GAL4* with more restricted expression (*SI Appendix*, Fig. S2*J*). By comparing the resulting *Dh44-pC1-GAL4* expression with the anatomical description of the pC1 subtypes in the Hemibrain EM database, we identified the highest and second highest similarities to one pC1c neuron (neuprint body ID, 267551639) and one pC1b neuron (neuprint body ID, 267214250) (Fig. 2*E* and *SI Appendix*, Table S1). In each brain hemisphere, *Dh44-pC1-GAL4* labeled 2.9 ± 0.53 pC1 cells (n = 10 brains), two of which were also positive for anti-Dh44 (*SI Appendix*, Fig. S2*K*). We therefore concluded that the two Dh44^{+} pC1 neurons correspond to pC1b and pC1c, respectively. *Dh44-pC1-GAL4* also labeled a pair of Dh44-positive local interneurons that arborized exclusively in the AbG (*SI Appendix*, Fig. S2*L*).

Finally, like what we observed with *Dh44^{A}-GAL4* silencing, we confirmed that transient silencing of *Dh44-pC1-GAL4* neurons completely blocked mating receptivity in virgin females (Fig. 2*F*).

The Activity of Dh44-pC1 Neurons Affects the Rate of Sexual Maturation. To investigate the role of Dh44-pC1 neurons in sexual maturation, we conducted two experiments. First, we over-activated *Dh44-pC1-GAL4* neurons during the first day of sexual maturation and examined mating receptivity in 1-d-old immature virgin females. We used the light-activatable cation channel CsChrimson to activate neurons in the presence of all-*trans*-retinal (ATR) (67). Virgin females expressing CsChrimson in Dh44-pC1 neurons were divided into ATR-fed or vehicle-fed groups and exposed to light from 0 to 1 DAE. After light exposure, while vehicle-fed control females exhibited low mating receptivity, ATR-fed females exhibited increased mating receptivity (Fig. 2*G*). When we exposed virgin females to light only during the mating assay, however, the flies exhibited low mating receptivity regardless of their ATR status (Fig. 2*G*). We further corroborated this observation when we found dTrpA1-induced thermal activation of Dh44-pC1 neurons during mating assays did not enhance mating receptivity in 1-d-old females (*SI Appendix*, Fig. S2*M*). Given that thermal activation of all pC1 neurons significantly enhanced mating receptivity in 1-d-old females (Fig. 1*B*), the behavioral effects of acute activation require the engagement of all or at least those expressing either Dh44R1 or Dh44R2.

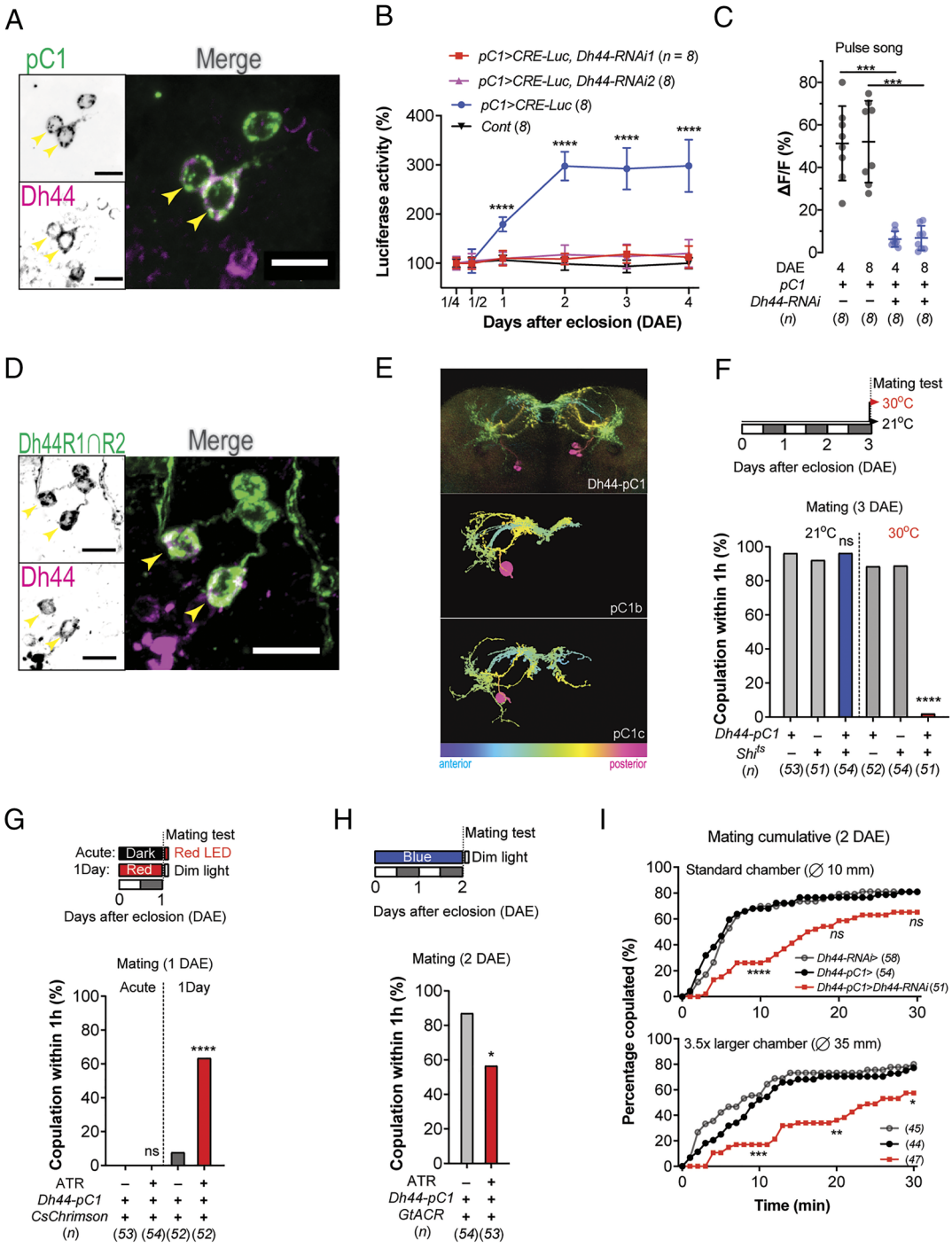

Fig. 2. The pC1b/c subtypes express Dh44 neuropeptide and its receptors Dh44R1 and Dh44R2. Their activity is required for the increased CREB activity, pC1 neuron excitability, and mating receptivity of females during sexual maturation. (*A*) Confocal images showing a pair of Dh44-expressing pC1 neurons (yellow arrowheads) from a female carrying *Dsx-GAL4*, *pC1-LexA, LexAOP-FLP,* and *UAS>stop>smGFP-HA,* stained with anti-HA (green) and anti-Dh44 (magenta). The negative images (*Left*) show the respective anti-HA (above) and anti-Dh44 (below) staining. (Scale bars for *A* and *D*, 10 μm.) (*B*) Relative *CRE-Luciferase* reporter activity in pC1 neurons from females of the indicated genotypes. (*C*) The peak ΔF/F in the LPC projections of pC1 neurons from 4- and 8-d-old virgin females of the indicated genotypes in response to male auditory courtship stimuli (pulse song). (*D*) Confocal images showing a pair of pC1 neurons expressing Dh44R1, Dh44R2, and Dh44 neuropeptide (yellow arrowheads) from a female carrying *Dh44R1-LexA, Dh44R2-GAL4, LexAOP-FLP,* and *UAS>stop>smGFP-HA* stained with anti-HA (green) and anti-Dh44 (magenta). The negative images (*Left*) show the respective anti-HA (above) and anti-Dh44 (below) staining. (*E*) An anatomical comparison between *Dh44-pC1-GAL4* neurons (above) and pC1b/c neurons (below; neuprint body ID, pC1b: 267214250; pC1c: 267551639). The panel above shows the maximum intensity projection image (MIP) of an aligned confocal image of the brain from a female carrying *Dh44-pC1-GAL4* and *UAS-myrEGFP* stained with anti-GFP and anti-nc82. (*F–H*) Mating frequencies for virgin females of the indicated genotypes, examined at the indicated time, scored as the percentage of females that copulate within 1 h. Above, experimental protocols. (*I*) Cumulative mating rate within 30 min for 2 DAE virgin females of the indicated genotypes in the standard 10 mm diameter chamber (above) and the 35 mm diameter chamber (below). Statistical analysis: One-way ANOVA with Tukey's test for multiple comparisons (*B*); Mann–Whitney test (*C*); chi-square test (*F–I*) [****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; no labeling (*B* and *C*) or ns (*F–I*), $P > 0.05$]. Data are presented as means ± SD. The numbers in parentheses indicate *n.* In *C*, each dot represents an individual female.

In our second experiment, we silenced Dh44-pC1 neurons in virgin females during sexual maturation and examined mating receptivity. We used the light-activatable anion channel GtACR1 to silence neurons in the presence of ATR (68). After 2 d of light exposure post-eclosion, we found ATR-fed virgin females expressing GtACR1 in Dh44-pC1 neurons exhibited roughly 60% mating receptivity, while vehicle-fed control females showed 80% mating receptivity (Fig. 2*H*). These results suggest that the activity of Dh44-pC1 neurons can affect the rate of sexual maturation, with increased Dh44 neuron activity accelerating sexual maturation and reduced Dh44 neuron activity slowing sexual maturation. Our findings also suggest Dh44-pC1 neurons play multiple roles in regulating female mating receptivity during and after sexual maturation. Silencing Dh44-pC1 neurons during mating assays after sexual maturation almost completely blocked mating receptivity (Fig. 2*F*), whereas silencing them only during sexual maturation produced only a partial block (Fig. 2*H*). Notably, we observed similar effects when silencing SAG neurons (*SI Appendix*, Fig. S3). Virgin females whose SAG neurons were silenced via GtACR1 during sexual maturation exhibited roughly 60% mating receptivity, compared to 90% in control females. Furthermore, silencing SAG neurons during mating assays had a greater effect on mating receptivity after sexual maturation than during the maturation phase.

We next asked whether *Dh44* knockdown in Dh44-pC1 neurons affects the development of sexual drive during maturation. We found suppression of pC1 neuron excitability via *Dh44* knockdown did not consistently affect mating receptivity (*SI Appendix*, Fig. S4*A*). We propose that the reduced pC1 neuron excitability achieved during *Dh44* knockdown remains sufficient for mating because our assay conditions limit female avoidance of male courtship by holding the mating pair for an hour in a confined chamber. When we instead measured a time-lapse of cumulative mating receptivity, we found a significant delay in mating onset in 2-d-old females with *Dh44* knockdown (Fig. 2*I*). Increasing the chamber size amplified these effects, indicating that reduced pC1 neuron excitability would induce stronger behavioral effects under more "natural" conditions (Fig. 2*I* and *SI Appendix*, Fig. S4*B*). In addition, despite a more than 90% reduction in anti-Dh44 levels, older, 3-d-old females did not exhibit the behavioral *Dh44* knockdown effect (*SI Appendix*, Fig. S4 *C–E*). Notably, however, the combined knockdown of *Dh44R1* and *Dh44R2* in all dsx^+ neurons abolished mating receptivity in 3-d-old females (*SI Appendix*, Fig. S4*F*). This suggests the involvement of additional redundant circuit components in sexual drive development or the influence of Dh44R signaling in other dsx^+ neurons on various behavioral aspects of female mating, collectively affecting the mating rate.

The Pyx Trp Channel Is Essential in pC1 Neurons for Their Increased Excitability. We hypothesized that increased CREB activity would stimulate synthesis of new proteins, such as cation channels, to make pC1 neurons more excitable. With an in silico search, we identified 14 cation channel genes that contain the CRE regulatory region (Fig. 3*A*). Using transgenic RNAi to knock down each of these channels in pC1 neurons, we found that two independent RNAi lines targeting *pyx* significantly reduced pC1 excitability in response to both cVA and pulse song (Fig. 3 *B* and *C* and *SI Appendix*, Fig. S5 *A* and *B*). The *pyx* gene, which has been implicated in high temperature stress tolerance in fruit flies, encodes two isoforms of a non-selective cation channel, Pyx-A and Pyx-B, which are orthologous to human TrpA1 (i.e., transient receptor potential subfamily A member 1) (69) (*SI Appendix*,

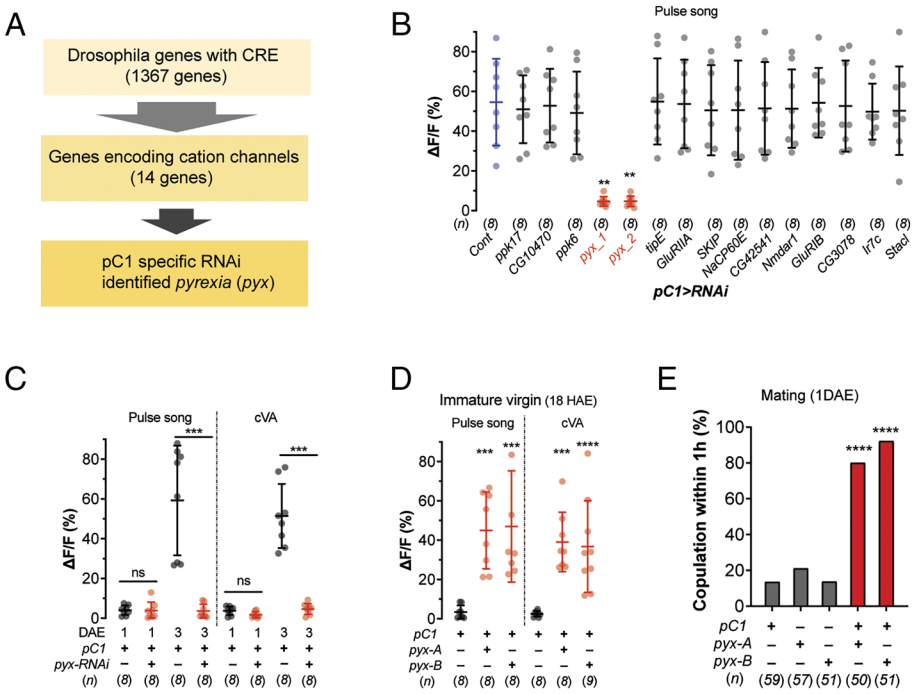

Fig. 3. Expression of a transient receptor potential cation channel *pyx* is essential for the increased excitability of pC1 neurons, and its overexpression induces precocious mating receptivity in females. (*A*) The experimental strategy used to search for CRE-regulated cation channel genes required for pC1 neuron excitability. (*B*) A cation channel-RNAi screen identified *pyx* as an ion channel required for pC1 neuron excitability. The peak ΔF/F in the LPC projections of pC1 neurons from 3-d-old virgin females of the indicated genotypes in response to male auditory courtship stimuli (pulse song). For *pyx*, two independent RNAi lines were examined (*Pyx_1* and *Pyx_2*). (*C* and *D*) The peak ΔF/F in the LPC projections of pC1 neurons from virgin females of the indicated genotypes at the indicated times after eclosion in response to male olfactory (cVA) and auditory (pulse song) courtship stimuli. (*E*) Mating frequencies for 1-d-old immature virgin females of the indicated genotypes, scored as the percentage of females that copulate within 1 h. Statistical analysis: Kruskal–Wallis test with Dunn's test for multiple comparisons (*B*); Mann–Whitney test (*C* and *D*); chi-square test (*E*); (****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; no labeling (*B* and *E*) or ns (*C* and *D*), $P > 0.05$). Data are presented as means ± SD, with each dot representing an individual female. The numbers in parentheses indicate *n*.

Fig. S5*A*). We next asked whether overexpression of Pyx elicits precocious responses to male courtship cues in immature virgin females. When we overexpressed each Pyx isoform in their pC1 neurons, we found 18-h-old females exhibited pC1 excitability like that of mature virgins (Fig. 3*D*). Furthermore, Pyx expression increased mating receptivity by up to 80% in 1-d-old virgin females, compared to age-matched controls, which showed a receptivity of 20% (Fig. 3*E*).

pC1 Neuron-Specific Dh44R Activation in Immature Females Induces Earlier Acquisition of Sexual Drive via Pyx. Our findings suggest the Dh44-Dh44R pathway increases CREB activity during sexual maturation, resulting in higher Pyx expression in pC1 cells. This, in turn, makes the pC1 cells more responsive to male courtship signals. To further investigate this mechanism, we developed a transgene capable of constitutively activating the Dh44-Dh44R signal independent of sexual maturity or mating experience. Inspired by the strategy that was used to construct a constitutively active human CRHR1, a mammalian ortholog of Dh44R1 (70), we created a constitutively active Dh44R1 (Dh44R1-CA) by fusing the receptor activation domain of Dh44 [Dh44(1-23)] with an N-terminally truncated Dh44R1 (Dh44R1-ΔN) (Fig. 4*A*). When we expressed Dh44R1-CA in Chinese hamster ovary cells, we observed robust CRE-Luciferase reporter activity comparable to that of cells expressing wild-type Dh44R1 treated with 10 nM Dh44 (*SI Appendix*, Fig. S6). Furthermore, we found expression of Dh44R1-CA in pC1 neurons increased pC1-CREB activity, resulting in higher CRE-Luciferase expression immediately after eclosion than similar flies expressing the Dh44R1-ΔN control (Fig. 4*B*). In addition, Dh44R1-CA expression caused the pC1 neurons of 18-h-old virgin females to exhibit Ca^{2+} responses like those of mature females in response to cVA and pulse song (Fig. 4*C*). Dh44R1-CA expression also increased mating receptivity, leading to higher rates of precocious mating in 1-d-old immature virgin females (Fig. 4*D*). Importantly, RNAi knockdown of *pyx* completely blocked the physiological and behavioral effects of Dh44R1-CA in immature females (Fig. 4 *E* and *F*). Together, our results strongly suggest the Dh44-Dh44R pathway induces sexual maturation by increasing Pyx expression, thereby making pC1 neurons more responsive to male courtship signals.

Mating or SP Decreases CREB Activity and pC1 Neuron Excitability. *Drosophila* females exhibit a significant drop in sexual drive or mating receptivity after mating. This drop occurs in two phases: an earlier SP-independent phase that lasts only 6 to 8 h after mating and a later SP-dependent phase that can last

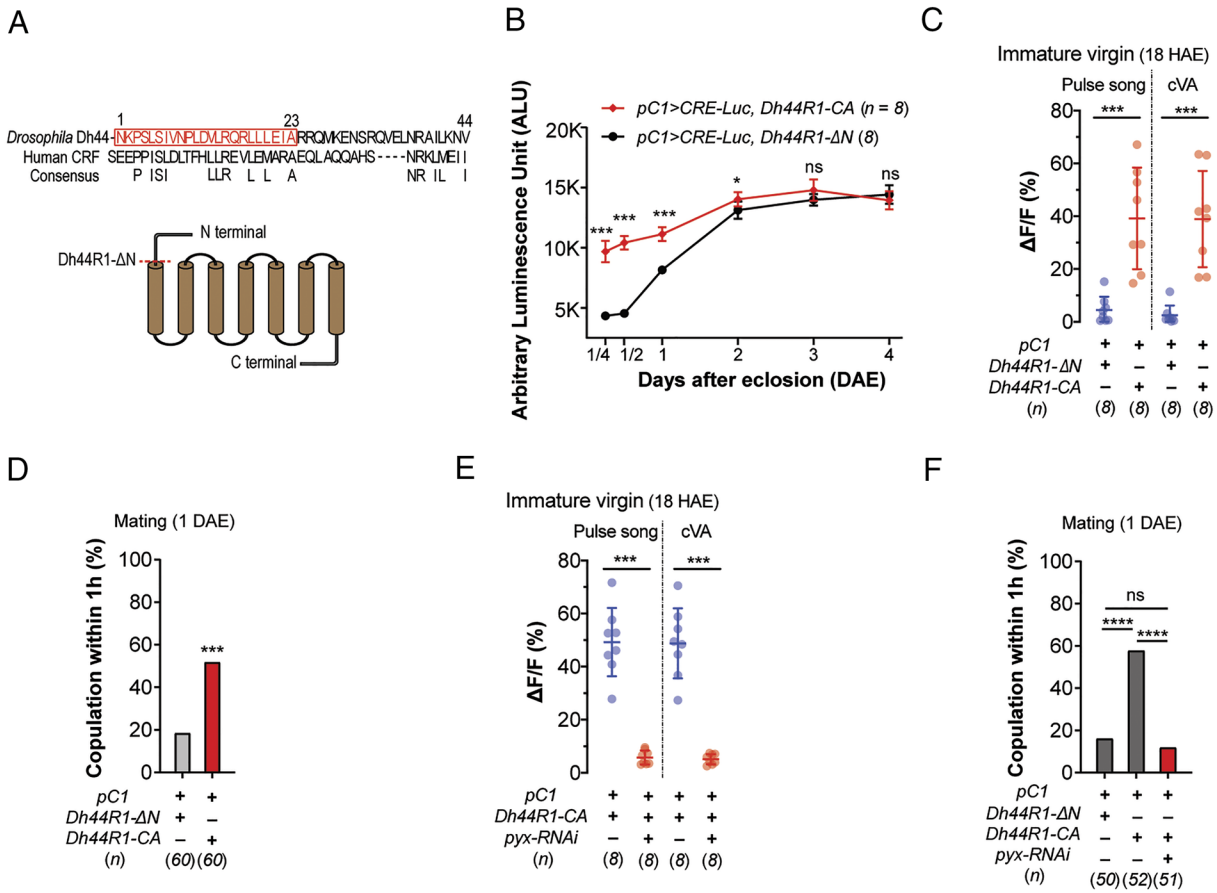

Fig. 4. Activation of pC1 neuron-specific Dh44R1 increases CREB activity and *pyx* expression, leading to higher pC1 neuron excitability and earlier mating receptivity in immature virgin females. (*A*) Schematic showing the generation of a constitutively active form of the Dh44 receptor (Dh44R1-CA). In humans, the N-terminal half of CRF or CRH functions as a receptor activation domain, while the C-terminal half is involved in receptor binding. To produce a Dh44R1-CA, we attached the N-terminal half of *Drosophila* Dh44, Dh44(1-23), to the N-terminus of Dh44R1-ΔN, which lacks the N-terminal extracellular domain. (*B*) *CRE-Luciferase* reporter activity in pC1 neurons from females of the indicated genotypes recorded for 4 d after eclosion. Expression of Dh44R1-CA in pC1 neurons resulted in a significant increase in CRE-Luciferase activity, as compared to the control (*pC1 > Dh44R1-ΔN*). (*C* and *E*) The peak ΔF/F in the LPC projections of pC1 neurons from 18-h-old immature virgin females of the indicated genotypes in response to male olfactory (cVA) and auditory (pulse song) stimuli. (*D* and *F*) Mating frequencies for 1-d-old immature virgin females of the indicated genotypes, scored as the percentage of females that copulate within 1 h. Statistical analysis: Mann–Whitney test (*B*, *C*, and *E*); chi-square test (*D* and *F*) (***$P < 0.001$; *$P < 0.05$; ns, $P > 0.05$). Data are presented as means ± SD. The numbers in parentheses indicate *n*. In *C* and *E*, each dot represents an individual female.

days or even weeks (40, 71). We found that females mated with SP-producing males showed a marked inhibition of pC1-CREB activity lasting for days, whereas females mated with males that lacked SP showed no such inhibition (Fig. 5*A*). Mating or SP also inhibited pC1 Ca^{2+} responses to male courtship cues, with pC1 neurons from mated females showing a significantly attenuated response at 2 d after mating (Fig. 5*B*).

pC1 Neuron-Specific Dh44R Activation in Mated Females Enables a Second Round of Mating via Pyx. To determine whether activation of Dh44-pC1 neurons increases sexual drive in mated females, as we observed in immature virgin females, we activated pC1 neurons with CsChrimson starting 4 h before performing the mating assays. This intervention increased sexual drive in mated females, enabling them to engage in a second round of mating more frequently than controls (Fig. 5*C*). Optogenetic activation during the mating assays alone did not have the same effect. We also asked whether Dh44R1-CA expression could increase pC1-CREB activity and pC1 excitability in mated females in the same way it did in immature virgin females. Indeed, we found that Dh44R1-CA expression maintained high pC1-CREB activity even after mating (Fig. 5*D*) and increased Ca^{2+} responses in pC1 neurons to courtship cues (Fig. 5*E*). This increased sexual drive in mated females, enabling them to engage in a second round of mating more frequently than controls (Fig. 5*F*).

Last, we asked whether Pyx expression alone in post-mating females could increase pC1 excitability and second mating frequency. We found, however, that Pyx expression alone did not alter pC1 responsiveness or mating behavior in mated females (*SI Appendix*, Fig. S7 *A* and *B*). Nevertheless, we found that Pyx plays a crucial role in mediating the action of Dh44R1-CA in pC1 neurons, even in mated females, because *pyx-RNAi* almost completely blocked the physiological and behavioral effects of Dh44R1-CA in the pC1 neurons of mated females. (Fig. 5 *G* and *H*). Overall, our findings suggest the Dh44-Dh44R pathway controls sexual drive by controlling the physiological responsiveness of the pC1 neurons. It does this by increasing Pyx expression and by regulating other processes, such as synapse remodeling (*Discussion*).

Discussion

The Dh44-Dh44R-PKA-CREB-Pyx Signaling Axis in pC1 Neurons during Sexual Maturation. The pC1 neurons play a critical role in connecting sensory inputs and motor outputs related to female mating behavior (25–27). Initially, we found that the expression of a *CRE-Luciferase* reporter increased in the pC1

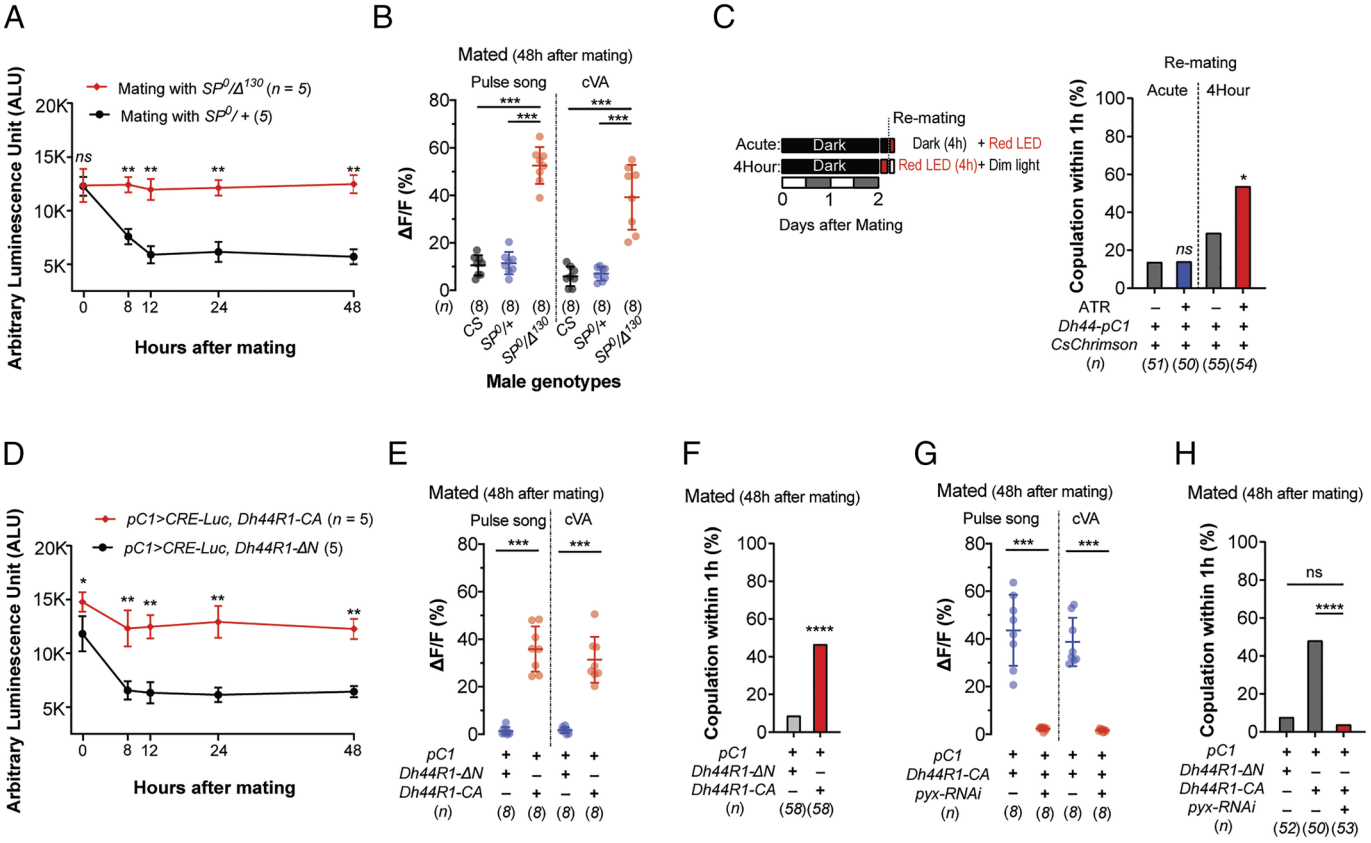

Fig. 5. Mating decreases CREB activity and the excitability of pC1 neurons via SP, whereas activation of pC1 neuron-specific Dh44R1 in mated females increases CREB activity and *pyx* expression, resulting in higher pC1 neuron excitability and additional mating. (*A*) *CRE-Luciferase* reporter activity in pC1 neurons from *pC1>CRE-Luciferase* females for 48 h after mating with SP-less males (SP^0/Δ^{130}) or control males ($SP^0/+$). (*B*) The peak ΔF/F in the LPC projections of pC1 neurons from *pC1>GCaMP6m* females 48 h after mating with SP-less males (SP^0/Δ^{130}) or control males (*CS*, $SP^0/+$) in response to olfactory (cVA) and auditory (pulse song) courtship stimuli. (*C*) Re-mating frequencies of females of the indicated genotypes 48 h after mating with *CS* males, scored as the percentage of females that copulate within 1 h. The *Left* panel shows the experimental protocols: Females were kept in darkness for 48 h after mating and exposed to light activation (Red LED) 4 h before or only during re-mating assays. (*D*) *CRE-Luciferase* reporter activity in pC1 neurons from females of the indicated genotypes for 48 h after mating with *CS* males. (*E* and *G*) The peak ΔF/F in the LPC projections of pC1 neurons from females of the indicated genotypes after mating with *CS* males in response to olfactory (cVA) and auditory (pulse song) courtship stimuli. (*F* and *H*) Re-mating frequencies of females of the indicated genotypes 48 h after mating with *CS* males, scored as the percentage of females that copulate within 1 h. Statistical analysis: Mann–Whitney test (*A*, *B*, *D*, *E*, and *G*), chi-square test (*C*, *F*, and *H*) (****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; *ns*, $P > 0.05$). Data are presented as means ± SD. The numbers in parentheses indicate *n*. In *B*, *E*, and *G*, each dot represents an individual female.

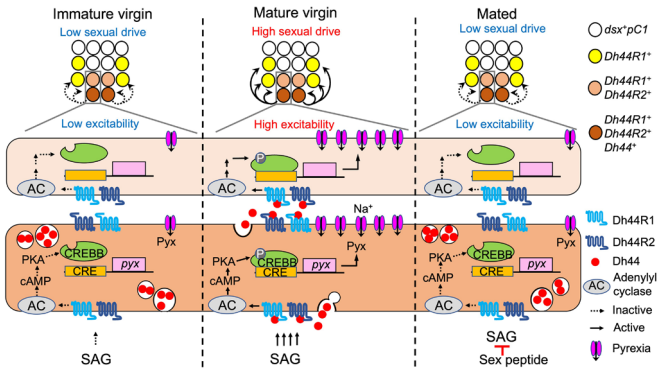

Fig. 6. A model for the long-term modulation of sexual drive circuitry through the Dh44-Dh44R-CREB-Pyx signaling axis. (*Top*) There are 14 *dsx*+ pC1 neurons with four expressing both Dh44R1 and Dh44R2 (light and dark brown circles), and the remaining four expressing Dh44R1 alone (yellow circles). Two pairs of Dh44R1 and Dh44R2-double positive neurons also express their ligand, Dh44 (dark brown circles). (*Left*) In newly emerged females, limited SAG activity and Dh44 secretion result in low CREBB phosphorylation and Pyx expression, reducing the excitability of pC1 neurons. As a result, male courtship cues cannot activate them, leading to low sexual drive. (*Center*) During sexual maturation, SAG neurons progressively increase their activity, stimulating Dh44 secretion from Dh44-pC1 neurons, which express both Dh44R1 and Dh44R2. Dh44 activates the cAMP-PKA-CREB pathway in more pC1 neurons expressing Dh44R1 or Dh44R2, inducing Pyx expression and increasing pC1 neuron excitability. This results in increased sexual drive. Dh44 secreted from Dh44-pC1 neurons can also activate the Dh44-pC1 neurons themselves, creating a positive feedback loop that progressively increases Dh44 secretion and CREBB activation. (*Right*) After mating, SP from the male ejaculate silences SAG neurons, which in turn suppresses the Dh44-Dh44R-cAMP-PKA-CREB pathway. This leads to a decrease in Pyx expression, ultimately causing a blockade of pC1 excitability and a decrease in sexual drive.

neurons during sexual maturation and required expression of both Dh44R1 and Dh44R2 in the same cells. Dh44R1 and Dh44R2 couple to trimeric G-proteins with Gαs, which triggers the production of cAMP via adenylate cyclase (56, 64, 65). This suggests Dh44-Dh44R signaling in pC1 neurons increases intracellular cAMP levels, activating PKA, which is responsible for the phosphorylation and activation of the transcription factor CREB (72). Phosphorylated CREB binds to CREs, activating the transcription of target genes like *CRE-Luciferase* transgene and *pyx*, which have CREs in their 5′-cis regulatory regions. Our results suggest *pyx* expression in pC1 neurons during sexual maturation increases sexual drive by making the pC1 neurons more responsive to male courtship cues (Fig. 6).

A previous study suggested a role for the PKA-CREB pathway in sexual maturation (73). *PKA-RNAi* in pC1 neurons before sexual maturation greatly reduced female mating receptivity, whereas the same intervention after sexual maturation only partially reduced mating receptivity. Similarly, RNAi knockdown of the adenylate cyclase *rutabaga* and CREB also significantly reduced mating receptivity. Although this study also found that *PKA-RNAi* altered the morphology of pC1 neurites, we did not observe significant changes in pC1 neuron morphology upon double knockdown of *Dh44R1* and *Dh44R2*, which should have inhibited PKA and CREB activity (*SI Appendix*, Fig. S8).

Recent advances in high-resolution EM connectomics have uncovered descending neurons that control female mating behavior and inspired work describing their complex circuits. For instance, the vpoDNs that control VPO and enable vaginal coitus receive synaptic inputs from pC1 neurons (26). Silencing vpoDNs completely inhibits female mating. Similarly, the silencing of Dh44-pC1 neurons after sexual maturation almost completely inhibited mating. Dh44-pC1 neurons thus seem to serve two separate functions in regulating female mating behavior. First, Dh44-pC1 neurons participate in assembling sexual drive during sexual maturation. Second, they gate downstream neurons like vpoDNs, which are responsible for the execution of mating behavior, even after sexual maturation is complete.

JH and Sexual Maturation. JH plays a critical role in triggering sexual maturation by stimulating processes such as egg maturation, pheromone production, and the development of mating receptivity (31). The exact mechanism by which JH activates the Dh44-Dh44R1 pathway in pC1 neurons is still unclear. One previous study documented an increase in SAG neuron activity in the 3 d following eclosion (34). SAG neurons provide excitatory inputs to pC1 neurons, including the pC1a, pC1b, and pC1c subtypes (27). The pC1b/c subtype is extremely similar to Dh44-pC1 neurons. Thus, we propose that during sexual maturation, SAG neurons increase excitatory inputs to Dh44-pC1 neurons, causing them to increase their secretion of Dh44. This would further activate the Dh44R-cAMP-PKA-CREB-Pyx pathway in pC1 neurons expressing Dh44R1 or Dh44R2. Dh44-pC1 neurons also express Dh44R1 and Dh44R2, creating a positive feedback loop that sustains the gradual and prolonged augmentation of Dh44 secretion and Dh44R activation. Thus, we speculate that JH should be involved in activating SAG neurons to drive this process. SAG neurons will continue to activate Dh44-pC1 neurons even after sexual maturation is complete (Fig. 6). Thus, the SP mating signal silences SPSNs and SAG neurons, reducing Dh44R-cAMP-PKA-CREB-Pyx activity in pC1 neurons (see below). We remain curious how a transient increase in JH titer during eclosion leads to long-lasting changes in the properties of SPSNs, SAG neurons, and other target neurons.

Shared Principles and Divergent Mechanisms between Sexes. Like females, males also undergo a gradual increase in sexual motivation over a span of 4 to 5 d post-eclosion (74). Despite significant sexual dimorphism in both numbers and arborizations, male pC1 neurons, specifically the subset named P1 neurons, integrate multiple sex-related sensory inputs, initiating courtship activity (75). We noted that both sexes share commonalities in the involvement of CREB signaling. In males, NPF/pCd neurons, forming a recurrent excitation loop and providing excitatory inputs to P1 neurons through motivation-promoting dopamine neurons, peak in CREB activity at eclosion, then decreasing significantly over 2 d post-eclosion (74, 76). Consequently, this heightened CREB activity induces the expression of an ion channel with inhibitory conductance (i.e., the potassium leak channel Task7) in NPF/pCd neurons, suppressing P1 excitability in immature males. As males age, decreased CREB activity diminishes Task7 expression, thereby allowing increased P1 activity. This stands in stark contrast to females, where pC1 neurons themselves undergo a gradual post-eclosion increase in CREB activity, resulting in elevated expression of the excitatory ion channel Pyx and increased pC1 neuron excitability. These parallels, alongside opposing molecular mechanisms, highlight the intricate interplay of evolutionary pressures and adaptation to distinct reproductive roles.

The Dh44-Dh44R-CREB-Pyx Signaling Axis in the PMR. Our study revealed a role for the Dh44-Dh44R-CREB-Pyx pathway in pC1 neurons in suppressing female sexual drive after mating. We found that *CRE-Luciferase* reporter expression in pC1 neurons decreased after mating with SP-producing males, but not with SP-less males. SP transferred to the uterus silences SPSNs and SAG neurons, the latter of which form excitatory synapses with pC1 neurons.

Before mating, SAG neurons are continuously active, stimulating Dh44 secretion from Dh44-pC1 neurons, activating the Dh44-Dh44R-CREB-Pyx pathway in pC1 neurons. Mating silences the SAG neurons, leading to a downregulation of this pathway and eventually ceasing Pyx expression (Fig. 6). In addition to altering Pyx expression, Dh44R activation seems to induce other unknown changes in pC1 neurons. This was evident from the fact that the expression of Dh44R-CA, but not Pyx alone, induced changes in pC1 neuron activity in mated females. One recent study using ExLLSM (i.e., tissue expansion and light sheet microscopy) found 28% fewer SAG neuron-to-pC1 neuron synapses in mated females (77), suggesting Dh44R activation remodels pC1 neuronal synapses.

A Potential Relevance in the Mammalian System. The *Drosophila* Dh44 peptide and its receptors share sequence homology with the CRH peptide and its receptors in mammals, implying conserved functions (57, 78). Since female transgenic mice that overexpress CRH do not mate and CRH injection suppresses estrous behavior (79, 80), our findings underscore a contrasting role for Dh44 in promoting sexual activity in female fruit flies. Notably, in certain mammals, CRH reportedly promotes gonadotropin secretion or sexual activity (81, 82).

Like fruit flies, mice rely heavily on their sense of smell in their reproductive behaviors. Estrogen fluctuations during the estrous cycle are linked to behavioral and physiological responses to male pheromones (83). Kisspeptin neurons in the rostral periventricular area of the third ventricle (RP3V) in the hypothalamus respond to male pheromones and are necessary for expressing mate preference and lordosis. Most of the hypothalamus kisspeptin neurons express CRHR, particularly those in the anteroventral periventricular nucleus, periventricular nucleus continuum, and arcuate nucleus (84). It is therefore feasible that CRH modulates the RP3V kisspeptin neurons or those related to them.

Materials and Methods

Fly Stocks. Fly stocks were raised at 25 °C and 60% humidity in 12 h:12 h light:dark cycle with the standard fly media. Following stocks were generated in this study: *Dh44^{A}-GAL4, Dh44^{B}-GAL4, Dh44^{C}-GAL4, Dh44^{D}-GAL4*, *pC1-FLP, Dh44-pC1-GAL4, UAS-Dh44R1-ΔN*, *UAS-Dh44R1-CA*, *UAS-Pyx-A, UAS-Pyx-B, Dh44R1-GAL4* and *Dh44R1-LexA*.

CREB Downstream Cation Channel Search. To identify CREB-regulated cation channel genes, we performed a keyword search of the Flybase gene summaries (http://flybase.org) of the previously reported 1,367 CREB target gene candidates (76).

EM-Based Anatomy Analysis. A confocal image of the brain from a female carrying *Dh44-pC1-GAL4* and *UAS-myrEGFP* stained with anti-GFP and anti-nc82 (*SI Appendix*, Fig. S3*J*) was registered onto the JRC2018 unisex brain template (85) using the Computational Morphometry Toolkit (https://github.com/jefferis/fiji-cmtk-gui) and then converted into a Color-Depth MIP (maximum intensity projection) image (Fig. 2*E*). Color-depth MIP masks of *Dh44-pC1-GAL4* neurons or pC1 subtype neurons in a hemibrain (86, 87) were generated using the ColorMIP_Mask_Search plugin (88) for Fiji (https://github.com/JaneliaSciComp/ColorMIP_Mask_Search) and NeuronBridge (89).

Additional information on *Fly Stocks*, *Cloning*, *Transgenesis*, *RT-PCR*, *Behavioral Assays*, *Immunohistochemistry*, *Calcium Imaging*, *Luciferase Assays*, and the *Statistical Analyses* used in this study is available in *SI Appendix*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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