

# A neuropeptide circuit that coordinates sperm transfer and copulation duration in *Drosophila*

Timothy D. Tayler<sup>a,b</sup>, Diego A. Pacheco<sup>b</sup>, Anne C. Hergarden<sup>a,c</sup>, Mala Murthy<sup>b,d</sup>, and David J. Anderson<sup>a,c,1</sup>

<sup>a</sup>Division of Biology 156-29 and <sup>c</sup>Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125; and <sup>b</sup>Princeton Neuroscience Institute and <sup>d</sup>Department of Molecular Biology, Princeton University, Princeton, NJ 08544

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Innate behaviors are often executed in concert with accompanying physiological programs. How this coordination is achieved is poorly understood. Mating behavior and the transfer of sperm and seminal fluid (SSFT) provide a model for understanding how concerted behavioral and physiological programs are coordinated. Here we identify a male-specific neural pathway that coordinates the timing of SSFT with the duration of copulation behavior in *Drosophila*. Silencing four abdominal ganglion (AG) interneurons (INs) that contain the neuropeptide corazonin (Crz) both blocked SSFT and substantially lengthened copulation duration. Activating these Crz INs caused rapid ejaculation in isolated males, a phenotype mimicked by injection of Crz peptide. Crz promotes SSFT by activating serotonergic (5-HT) projection neurons (PNs) that innervate the accessory glands. Activation of these PNs *in copulo* caused premature SSFT and also shortened copulation duration. However, mating terminated normally when these PNs were silenced, indicating that SSFT is not required for appropriate copulation duration. Thus, the lengthened copulation duration phenotype caused by silencing Crz INs is independent of the block to SSFT. We conclude that four Crz INs independently control SSFT and copulation duration, thereby coupling the timing of these two processes.

sexual dimorphism | neural circuit | *fruitless*

In many sexually reproducing animals, sperm and seminal fluids are transferred directly from males to females during copulation. In *Drosophila*, these two components function to pass along the male's genes and to prevent insemination by competing males, respectively (1–3). The transfer of these components is a complex, temporally orchestrated process (4, 5) that is coordinated with the duration of copulation behavior. The neural control of this coordination is poorly understood. Activation of neurons expressing *fruitless*, a master gene proposed to regulate all steps of male reproductive behavior (6–8), or of those expressing *doublesex* (*dsx*) was sufficient to elicit nearly all steps of mating behavior, including ejaculation (9). Serotonergic innervation of the male reproductive organs was shown to be defective in several allelic combinations of viable *fruitless* mutants (10), leading the authors to speculate that serotonin plays a role in both fertility and copulation duration. Another study demonstrated that blocking synaptic transmission in a large neuronal population including male cholinergic cells caused separate effects on the transfer of sperm and seminal fluid (11). Despite these findings, it has been difficult to determine whether the timing of transfer of sperm and seminal fluid (SSFT) is necessary and sufficient to set the duration of copulation, because of the inability to perform complementary manipulations of neuronal activity that selectively promote and inhibit the transfer process, respectively.

## Results

**Silencing *corazonin-GAL4* Neurons Blocks SSFT and Extends Copulation Duration.** In an effort to identify peptidergic neurons that control social behaviors, we expressed the inwardly rectifying K<sup>+</sup> channel, Kir2.1 (12) to block neuronal activity in ~30 different *neuropeptide promoter-GAL4* lines and performed

a battery of behavioral assays (13) (Fig. 1A). Corazonin (Crz) is a peptide previously implicated in cardiac control (14). Silencing putative Crz neurons with *corazonin-GAL4* (*crz-GAL4*) resulted in two completely penetrant phenotypes associated with mating: male infertility and dramatically increased copulation duration (Fig. 1B and C). In wild-type *Drosophila melanogaster*, as in *crz-GAL4/+* and *+/Kir2.1* controls (Fig. 1C), copulation typically lasts ~20 min (15). Strikingly, in *crz-GAL4/+;UAS-Kir2.1/+* males, the duration of copulation was increased by fivefold (to ~100 min). Courtship behavior and mating efficiency, by contrast, were normal (Fig. 1D; Fig. S1A). The increased mating duration phenotype and the fertility deficit were specific to males (Fig. S1B–D), consistent with earlier studies indicating that copulation duration in *Drosophila* is controlled primarily by males (11, 16, 17). These two phenotypes were observed using three other independent *crz-GAL4* insertions to drive *UAS-Kir2.1* expression, as well as using *UAS-hid* to ablate these neurons. To determine when the activity of *crz-GAL4* neurons is required, we restricted the expression of Kir2.1 to the adult stage using the temperature-sensitive repressor of GAL4, GAL80<sup>ts</sup> (18). Adult-specific expression of Kir2.1 in *crz-GAL4* neurons also caused male infertility and extended copulation, ruling out the possibility that these phenotypes are a consequence of developmental deficits (Fig. S2A and B).

To identify the primary cause of infertility in *crz-GAL4/UAS-Kir2.1* males, we examined whether sperm and seminal fluid were transferred to females during copulation. The reproductive organs of mated females were dissected several minutes after the completion of mating and examined using GFP reporters for the presence of sperm (don juan:GFP) or seminal fluid (Sex peptide:GFP) (19, 20), crossed into the *crz-GAL4; UAS-Kir2.1* genetic background. Such males failed to transfer either sperm or seminal fluid to females during copulation (Fig. 2K), even though both of these ejaculate components were present in male reproductive organs (Fig. S3A–D).

To determine whether the extended copulation duration phenotype was a consequence of failed SSFT, we asked whether independently blocking sperm or seminal fluid transfer affects copulation duration. After three to four successive matings, wild-type males are rendered infertile due to seminal fluid depletion (21, 22). Males born to *udor* mutant females (*son-of-udor*) lack sperm (23, 24). Surprisingly, normal copulation durations were observed using sterile males produced by both manipulations (Fig. 2J). These and additional data suggest that the extended copulation duration and block to SSFT in *crz-GAL4/UAS-Kir2.1* males are independent phenotypes.

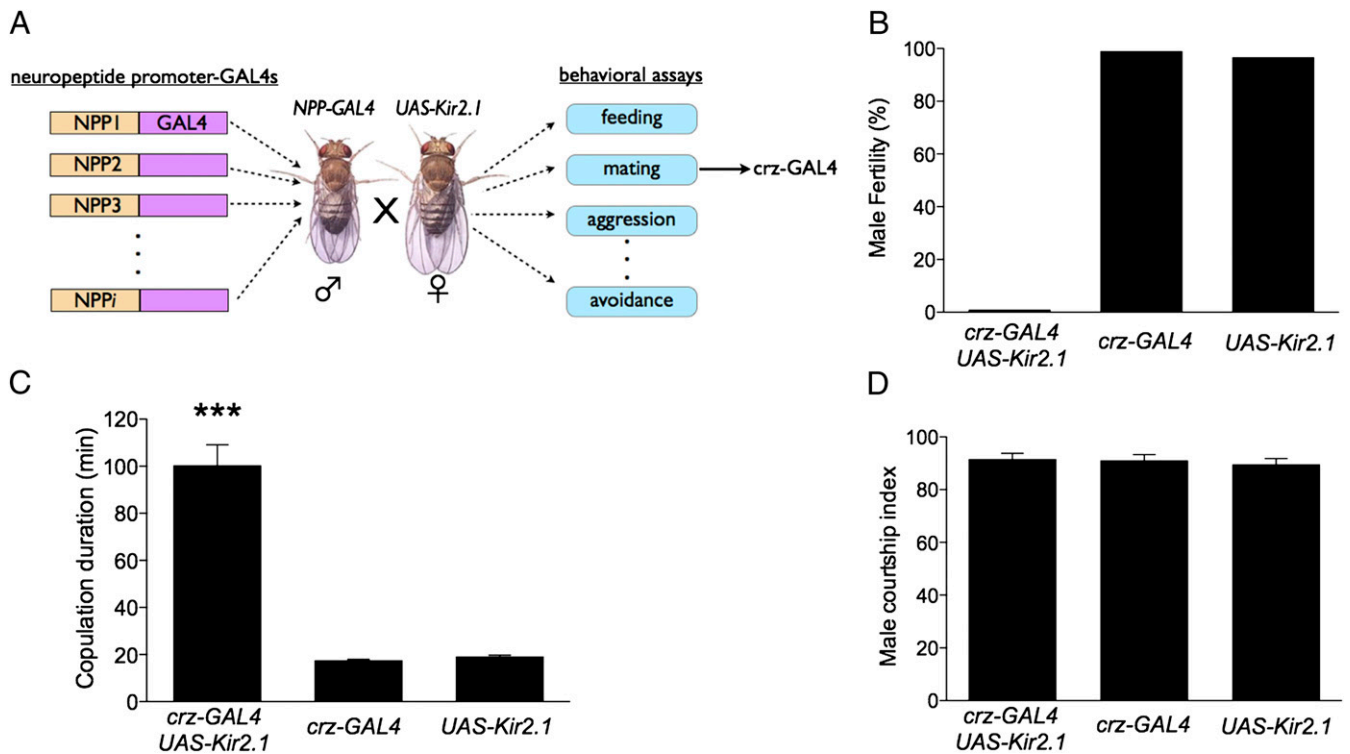
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<sup>1</sup>To whom correspondence should be addressed. E-mail: wuwe@caltech.edu.

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**Fig. 1.** Silencing *corazonin-GAL4* neurons results in male infertility and extended copulation duration. (A) Schematic illustrating neuro peptide promoter behavioral screen. (B) Fertility of *crz-GAL4 UAS-Kir2.1* (0%,  $n = 42$ ), *crz-GAL4* (96%,  $n = 28$ ), and *Kir2.1* (93%,  $n = 30$ ) males after mating with a wild-type virgin. (C) Copulation durations of *crz-GAL4 UAS-Kir2.1* ( $103 \pm 22$  min,  $n = 33$ ), *Crz-GAL4* ( $19 \pm 2$  min,  $n = 22$ ), and *Kir2.1* ( $19 \pm 2$  min,  $n = 21$ ) males paired with wild-type virgins. (D) Measure of courtship ability by males of the indicated genotype (*Materials and Methods*),  $n \geq 10$ . \*\*\* $P < 0.001$ . Error bars denote SEM. Illustration of *Drosophila* male and female (A) courtesy NASA.

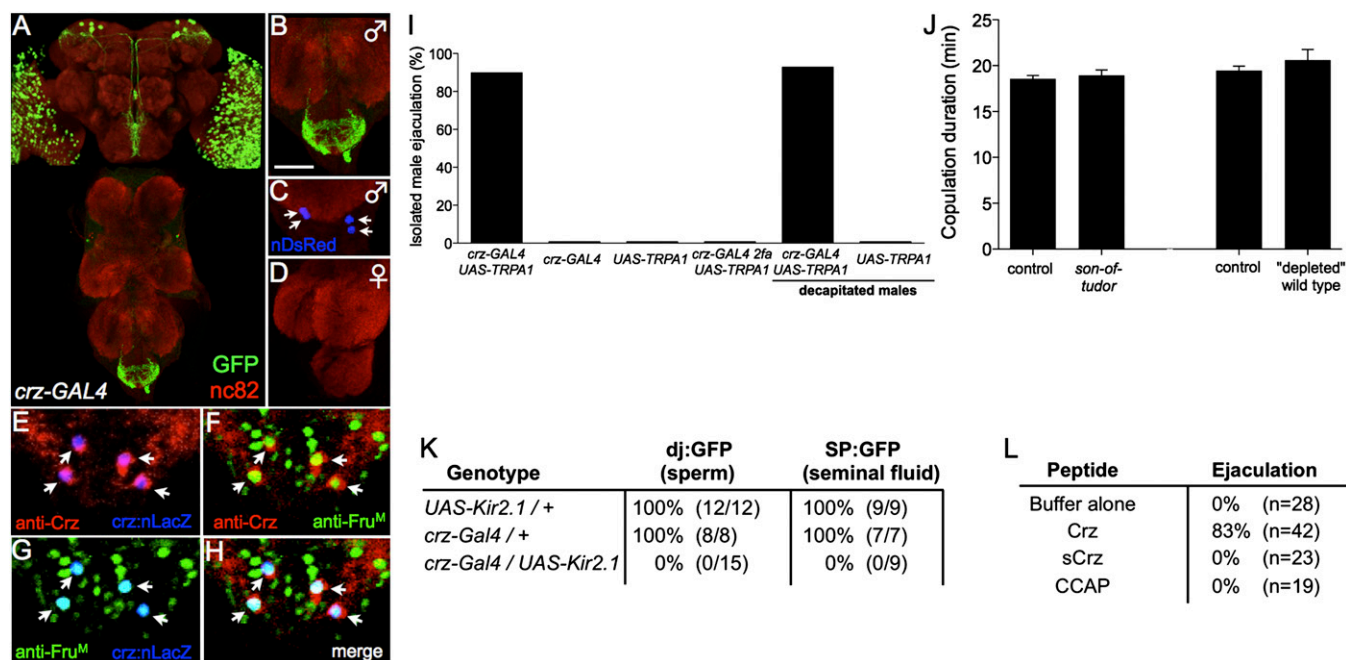
**Corazonin Is Expressed in Male-Specific Neurons in the Abdominal Ganglia.** Expression analysis of *crz-GAL4*, using a *UAS-mCD8-GFP* reporter (25), revealed four male-specific interneurons located in the abdominal ganglion (AG) (Fig. 2 A–D), as well as nondimorphic cells in the visual system and central brain (Fig. 2A; Fig. S4 A and B). An antibody raised against the corazonin peptide immunolabeled the male-specific interneurons (Fig. 2 E, F, and H) and central brain neurons, consistent with previously published reports (26), but not the *crz-GAL4*-expressing neurons in the visual system. The Crz neurons in the AG, but not those in the brain, expressed the male-specific isoform of the transcription factor Fruitless ( $Fru^M$ ), which specifies male-specific courtship behaviors (6, 8, 27) (Fig. 2 F–H; Fig. S4 A and B).

**Corazonin Neurons in the Abdominal Ganglia Promote Ejaculation.** To gain further insight into the control of sperm transfer by *crz-GAL4* neurons, we asked whether their artificial activation was sufficient to elicit ejaculation in isolated males. Individual male flies expressing the temperature-sensitive neuronal activator dTRPA1 (28) in *crz-GAL4* neurons were tethered (ventral side facing up) to a glass slide and shifted to the activating temperature (28–31 °C). This manipulation caused ejaculation in such restrained male flies within ~60 s of the temperature shift, whereas no such ejaculation was observed in genetic controls at the same temperature (Fig. 2I). To determine whether this effect is mediated by Crz itself or rather by a coexpressed classical neurotransmitter (29), we injected synthetic corazonin peptide, scrambled corazonin peptide, crustacean cardioactive peptide (CCAP, an unrelated neuropeptide), or buffer alone into restrained male flies. Corazonin peptide, but not control, injections resulted in male ejaculation (Fig. 2L). These data establish Crz as the first identified ejaculation-promoting peptide in insects.

To determine whether ejaculation was controlled by *crz-GAL4* neurons in the AG, we decapitated *crz-GAL4/UAS-dTRPA1* males before shifting to the activating temperature. Such headless males also displayed the ejaculation phenotype (Fig. 2I). In contrast, TRPA1 activation in flies expressing *crz(2fa)-GAL4*, an independent *crz-GAL4* insertion lacking expression in the AG (presumably due to chromosomal position effects; Fig. S5 A and B) failed to elicit ejaculation (Fig. 2I). Together, these data suggest that activation of the four male-specific *crz-GAL4* neurons in the AG is responsible for SSFT.

Because the copulation duration phenotype of *crz-GAL4; UAS-Kir2.1* males is independent of the SSFT deficit, we investigated whether the former was due to silencing other *crz-GAL4* neurons in the head. To do this, we decapitated *crz-GAL4; UAS-Kir2.1* males *in copulo*. These males still showed an extended copulation duration phenotype following decapitation (Fig. S6). Furthermore, expression of *Kir2.1* in *crz(2fa)-GAL4* males, which express *GAL4* in the central brain but not in the AG, failed to yield an extended copulation duration phenotype (Fig. S5C). These data suggest that the extended copulation duration phenotype of *crz-GAL4; UAS-Kir2.1* males is not due to silencing *crz-GAL4* neurons in the central brain. Consistent with this, wild-type males decapitated after the initiation of mating terminated copulation at the normal time, arguing against a critical role for the central brain in limiting copulation duration (Fig. S6).

**Corazonin Receptor Neurons Are Necessary and Sufficient for SSFT.** To identify the neurons through which Crz exerts its effect to promote ejaculation, we generated *corazonin receptor (crzR) promoter-GAL4* lines (*Materials and Methods*). These *GAL4* lines labeled projection neurons with cell bodies located in the AG and



**Fig. 2.** Male-specific *corazonin-GAL4* neurons in the abdominal ganglia control sperm transfer. (A) Confocal image of *crz-GAL4/UAS-mCD8-GFP; UAS-DsRed* (nuclear) whole-mount CNS, triple labeled with antibodies to nc82 (red), GFP (green), and DsRed (blue); higher-magnification view of the male (B and C) and female abdominal ganglia (D). (Scale bar, 50  $\mu$ m.) Arrows indicate the cell bodies of two pairs of sexually dimorphic *crz-GAL4* neurons. (A) A composite of separate images of the brain and VNC to form the full figure. (E–H) Confocal image of a *crz-GAL4 UAS-nLacZ* male abdominal ganglia triple-labeled with antibodies to Fru<sup>M</sup> (green), Crz (red), and LacZ (blue). (I) Percentage of males that ejaculate during TRPA1-induced neuronal activation,  $n \geq 25$  flies per condition. (J) Copulation duration of infertile males paired with wild-type virgins (*Materials and Methods*).  $n \geq 12$  per genotype. (K) Transfer of sperm (dj:GFP) and seminal fluid (SP:GFP) to females during mating. Males of the indicated genotype were mated to wild-type virgins. Following mating cessation, females were dissected and were examined for the presence of GFP in the reproductive organs. (L) Crz peptide promotes ejaculation. Individual male flies were injected with synthetic corazonin (Crz), scrambled corazonin (sCrz), crustacean cardioactive peptide (CCAP), or buffer and observed for ejaculation (*Materials and Methods*). Error bars denote SEM.

processes that innervate the male reproductive organs (30) (Fig. 3 G and J; Fig. S7 A–C). Immunostaining of the AG with anti-Crz antibody revealed that the arborizations of Crz neurons were closely apposed to presumptive dendrites of the CrzR neurons in the AG (Fig. 3 D–F).

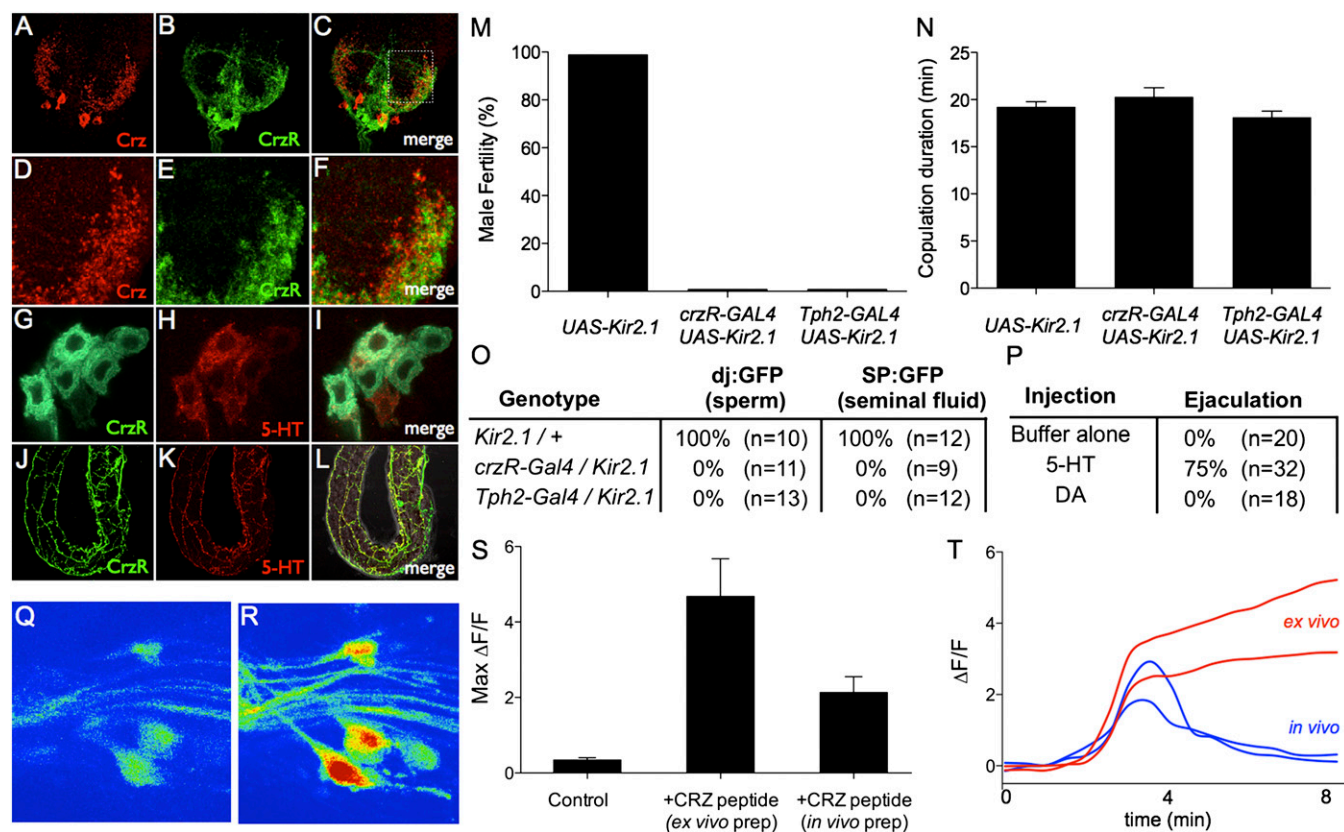
To determine whether *crzR-GAL4*<sup>+</sup> neurons in the AG are activated by Crz peptide, we performed two-photon calcium imaging experiments, using two different male preparations. In both in vivo and ex vivo preparations (*Materials and Methods*), *crzR-GAL4* neurons expressing the Ca<sup>2+</sup> indicator GCaMP5G (31) responded strongly to 50  $\mu$ M synthetic Crz peptide (Fig. 3 Q–T), producing a slow, gradual increase in fluorescence. Interestingly, the fluorescence signal in the in vivo preparation returned to baseline levels within several minutes after Crz peptide was washed out, whereas GCaMP fluorescence in the ex vivo preparation (an isolated nerve cord) continued to rise until the end of the observation period (>15 min). The reason for the difference in the postpeak response between the two preparations is not clear. It could indicate that signals from brain structures or tissues absent from the ex vivo preparation may normally terminate the Crz response. Alternatively, intrinsic changes in CrzR neuron physiology may occur ex vivo, which cause a persistent response to the peptide.

Previous studies described a small population of male-specific serotonergic neurons in the AG that innervate the reproductive organs (17, 32, 33). Double-immunostaining experiments in *crzR-GAL4; UAS-mCD8-GFP* flies revealed that these male-specific serotonergic AG neurons coexpress *crzR-GAL4* (Fig. 3 G–I; Fig. S7 C–E) and indicated a complete overlap between *crzR-GAL4* and serotonergic fibers within male internal reproductive organs (Fig. 3 J–L). Silencing of either *crzR-GAL4* neurons or serotonergic neurons (using a *Tph2-GAL4* line; *Materials and Methods*) caused infertility and blocked SSFT (Fig. 3 M and O), similar to

the effect of silencing of *crz-GAL4* neurons. Conversely, activation of *crzR-GAL4* or *Tph2-GAL4* neurons using dTRPA1 in restrained, isolated males elicited ejaculation (Fig. 4A), as did injection of 5-HT (Fig. 3P).

**Activation of *crzR-GAL4* Neurons Causes Premature Ejaculation and Shortens Copulation Duration.** The ability to accelerate SSFT via manipulation of genetically defined neural circuit elements afforded the opportunity to determine whether such a manipulation would suffice to shorten the duration of copulation. To do this, we sought to activate CrzR/Tph2 neurons *in copulo*. First, using a mating separation assay, we established that sufficient sperm transfer for fertile matings can be detected at 8 min after the initiation of copulation, but not earlier (Fig. S8), consistent with previous studies (5, 33). Next, we asked whether *crzR-GAL4* neuron activation could accelerate fertilization during active copulation with a female. *crzR-GAL4/UAS-dTRPA1* males were paired with wild-type virgins. Once copulation was initiated, the flies were shifted to the activating temperature and then manually separated after 4 min. Most females (~90%) that recovered from this manipulation were fertile (Fig. 4B), indicating that activation of *crzR-GAL4* neurons can trigger premature sperm transfer during copulation, as well as in isolated, restrained males. Similar results were obtained using the *Tph2-GAL4* driver (Fig. 4B). Activation of these CrzR projection neurons (PNs) *in copulo* also caused copulation to terminate prematurely (without manual intervention) after only ~7 min (Fig. 4C; Fig. S9). Thus, accelerating the timing of sperm transfer during active copulation is sufficient to shorten its duration. In contrast, silencing of either *crzR-GAL4* or *Tph2-GAL4* neurons had no effect on copulation duration (Fig. 3N), consistent with the results obtained using sterile males (Fig. 2J).





**Fig. 3. Corazonin receptor-GAL4 neurons innervate reproductive organs and control sperm transfer.** (A–C) Confocal image of *crzR-GAL4; UAS-mCD8-GFP* male AG stained with anti-corazonin (red). (D–F) Higher-magnification view (boxed region in C) of the AG showing the intermingling of corazonin and *crzR-GAL4* terminals. (G–L) *crzR-GAL4; UAS-mCD8-GFP* male labeled with antibodies to GFP (green) and 5-HT (red). (G–I) High-magnification view of sexually dimorphic abdominal ganglia neurons expressing both 5-HT and *crzR-GAL4*. (J and K) Overlapping projections innervating the accessory glands. (M) Fertility of the indicated genotypes after mating with wild-type virgins.  $n \geq 30$  per genotype. (N) Copulation duration of *crzR-GAL4; UAS-Kir2.1* ( $20 \pm 2$  min,  $n = 15$ ), *Tph2-GAL4/Kir2.1* ( $18 \pm 2$  min,  $n = 16$ ), and *Kir2.1/+* ( $19 \pm 2$  min,  $n = 14$ ) males. (O) Assay for transfer of sperm (dj:GFP) and seminal fluid (SP:GFP) to females during mating. (P) Individual male flies were injected with 5-HT, dopamine, or buffer and observed for ejaculation (*Materials and Methods*). (Q and R) Z-projection image of a *crzR-Gal4; UAS-GCaMP5G* abdominal ganglia before (Q) and after (R) Crz bath application. (S) Peak  $\Delta F/F$  values ( $n = 3$ –5 cells from at least three *crzR-Gal4; UAS-GCaMP5G* flies for each condition) following bath application of buffer (control) or the Crz peptide dissolved in buffer. Crz was applied either to an isolated nerve cord (ex vivo prep) or an intact fly with the AG exposed (in vivo prep). (T) Example  $\Delta F/F$  responses from ex vivo (red) and in vivo (blue) preparations. Traces have been aligned to the rise of the response. Crz peptide (or buffer alone) was added before time = 0 and washed out 300 seconds later.

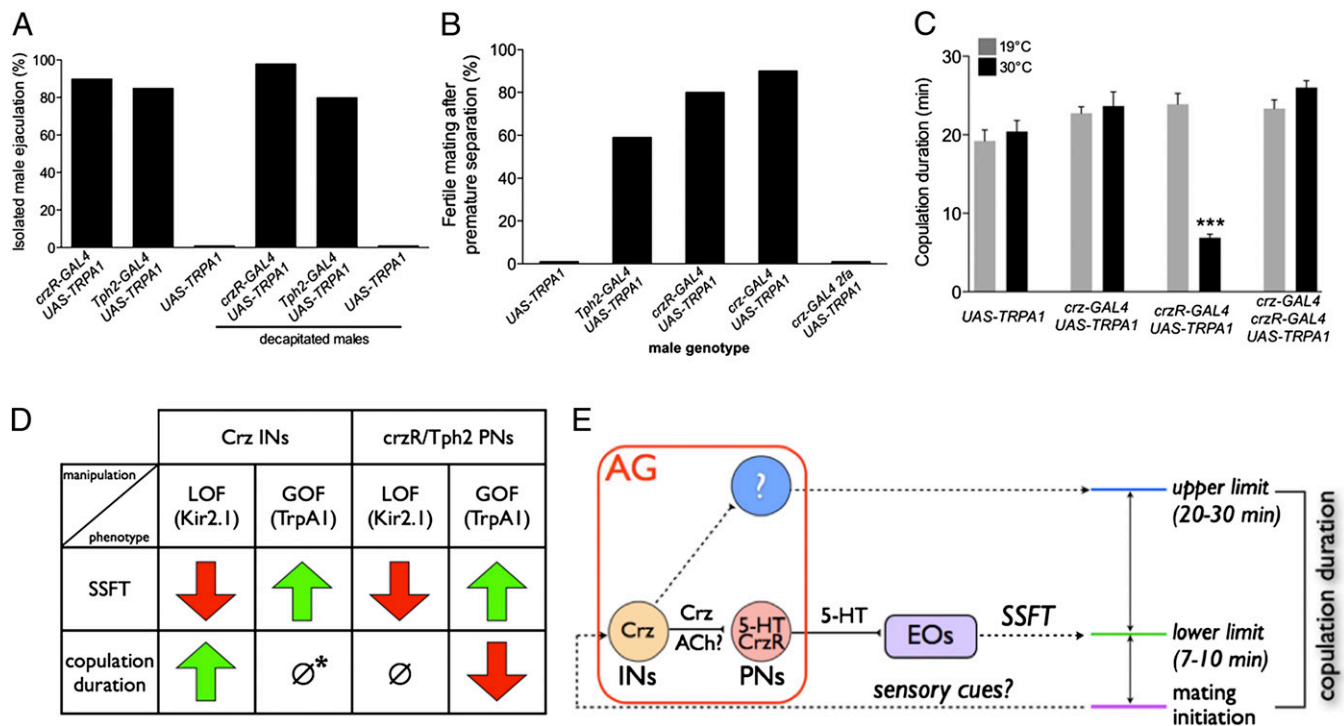
**Activation of *crz-GAL4* Neurons Causes Premature Ejaculation but Does Not Shorten Copulation Duration.** Surprisingly, although activation of *crz-GAL4* neurons *in copulo* also caused premature sperm transfer (Fig. 4B), unlike activation of *crzR-GAL4* neurons, it did not cause a shortening of copulation duration (Fig. 4C). To determine whether this difference simply reflected a less efficient or effective activation of *crz-GAL4* neurons, we combined activation of *crzR-GAL4* neurons with activation of *crz-GAL4* neurons. In this case, a normal duration of copulation was observed (Fig. 4C), indicating that coactivation of *crz-GAL4* neurons could override the effect of *crzR-GAL4* neuron activation to shorten the duration of copulation. Thus, activation of Crz neurons is not neutral with respect to copulation duration. Consistent with this conclusion, activation of Crz neurons 10 min after the initiation of copulation extended the total copulation time for an additional 15–20 min (Fig. S10).

## Discussion

We have delineated a neural pathway that coordinates copulation duration with the timing of SSFT in *Drosophila*. Using a functional screen, we identified a population of four male-specific Crz-expressing interneurons (INs) in the AG that activate, in part via release of Crz peptide, a set of CrzR-expressing

PNs that innervate the male reproductive organs (Fig. 4E). [Crz INs also express *Cha-GAL4* (Fig. S11) and therefore corelease of ACh could play a role as well (11).] These CrzR PNs in turn promote SSFT via release of 5-HT, confirming earlier suggestions of a role for this transmitter in SSFT (10). Although 5-HT exerts an inhibitory effect centrally on ejaculation in humans (34), there is pharmacologic evidence of a proejaculatory effect of 5-HT acting peripherally (35).

The ability to block or promote SSFT by complementary loss- and gain-of-function manipulations of genetically defined neuronal subpopulations has allowed us to investigate whether SSFT is necessary and/or sufficient to determine the duration of copulation (Fig. 4D). Accelerating SSFT by activating CrzR or Tph2 PNs shortened copulation duration. In contrast, silencing these same neurons blocked SSFT but did not alter copulation duration. Thus, perhaps surprisingly, SSFT is not required for the species-typical duration of mating in *Drosophila*. Paradoxically, silencing of Crz INs, which also blocks SSFT, did extend copulation duration (Fig. 4D). However, activating these INs, which caused premature SSFT, did not on its own have any effect on copulation duration (although it did neutralize the shortened copulation duration phenotype caused by activating CrzR PNs).



**Fig. 4.** Role of SSFT in determining copulation duration. (A) Percentage of isolated males of the indicated genotypes that ejaculated during TRPA1-induced neuronal activation;  $n \geq 30$  flies per condition. (B and C) *In copulo* activation of INs and PNs causes precocious SSFT. Mating pairs containing males of the indicated genotypes were shifted to the activating temperature (30 °C) 1 min after copulation was initiated. (B) Female fertility was assessed after mechanically separating mating pairs 4 min following the temperature shift;  $n \geq 15$  per genotype. (C) Copulation duration of undisturbed mating pairs;  $n \geq 15$  per genotype. Gray bars are controls mock-shifted 1 min after initiation of copulation. \*\*\* $P < 0.001$ . Error bars denote SEM. (D) Summary of main experimental results. Red arrows indicate inhibition of SSFT or shortened copulation duration; green arrows indicate promotion of SSFT or increased copulation duration.  $\emptyset$ , no change; \*, activation of Crz INs is not neutral but can override shortened copulation duration caused by premature SSFT (see C). (E) Schematic illustrating how the activity of Crz INs (perhaps triggered by sensory signals associated with the initiation of mating) may coordinate both SSFT and the duration of copulation. In the absence of Crz IN function, premature SSFT caused by hyperactivation of PNs shortens copulation duration (D), suggesting that SSFT determines the minimum duration (lower limit) of copulation. When Crz INs are active, copulation duration is extended for ~10–20 min beyond the normal time required for successful fertilization (~8 min; Fig. S8; upper limit). This influence of Crz INs may be exerted through another unknown circuit element (?). AG, abdominal ganglion; EOs, ejaculatory organs. ACh? indicates that Crz INs express Cha-GAL4 (Fig. S11), and there is evidence of cholinergic regulation of SSFT (11).

The most parsimonious interpretation of this paradox is to infer that Crz neurons do more than simply initiate sperm transfer via CrzR/Tph2 neurons; they also independently determine the duration of copulation (Fig. 4E). The observation that silencing Crz neurons caused protracted copulation implies that these neurons are required to release, encode, or receive a timing signal that normally terminates copulation ~20 min after its initiation. Such a function would explain why copulation duration is normal when SSFT is blocked by silencing CrzR/Tph2 PNs: because this block is exerted downstream of Crz neurons, these INs are presumably activated normally at the initiation of copulation (Fig. 4E), permitting them to control the duration of copulation despite the absence of SSFT. Artificial hyperactivation of CrzR/Tph2 PNs can override this putative timing signal to shorten copulation duration (presumably due to premature SSFT). Therefore, premature SSFT may establish a lower limit or minimum duration of copulation under (artificial) conditions where Crz INs are not also hyperactivated, whereas in wild-type flies, Crz INs may normally override this lower limit to set the maximum duration of copulation (Fig. 4E). Consistent with this idea, the shortened copulation duration phenotype caused by hyperactivating CrzR/Tph2 PNs can be rescued by coactivating Crz INs (Fig. 4C). Evidently, when both INs and PNs are hyperactivated, the influence of the upstream cells to determine the normal duration of copulation predominates. For the same reason, artificial hyperactivation of Crz INs alone may

rescue or bypass its own independent effect to precociously activate the PNs, explaining why mating terminates normally in this case despite premature SSFT (Fig. 4, D,  $\emptyset^*$ , and E).

Previous studies of different heteroallelic *fru* mutant combinations revealed both fertility defects and extended copulation duration among those males that did mate (10). For individual males of a given *fru* mutant genotype, all possible combinations of fertility and copulation duration phenotypes were observed, and there was no correlation between the penetrance of these phenotypes, and the different genotypes (10). These data suggested that fertility deficits and extended copulation in these partial loss-of-function *fru* mutants can be uncoupled. Here we show that *Fru*<sup>M</sup>-expressing Crz INs control both copulation duration and fertility. How can copulation duration and fertility be genetically uncoupled if the same neurons (Crz INs) control both processes? As *Fru*<sup>M</sup> is expressed in more than 2,000 neurons in the male brain (27), it is possible that the phenotypes observed previously (10) reflected an action of the gene in neurons other than those we have studied here. Alternatively, the heteroallelic *fru* combinations may indeed have affected Crz INs and/or CrzR PNs, but stochastic variation in the levels of *Fru*<sup>M</sup> in different individuals of each genotype, during development and/or adulthood, may have differentially affected different components of Crz IN function important for the two phenotypes, such as connectivity, neurotransmitter/neuropeptide phenotype, or receptor expression.

The mechanisms underlying the control of copulation duration by Crz neurons are likely to be complex and remain to be elucidated. Nevertheless, these results reveal what may be a fundamental principle in the neural control of mating behavior: the involvement of common circuit elements that control both SSFT and copulation duration. This parallel control allows the timing of these two processes to be properly coordinated. Further studies of this system may shed light on the general problem of how the nervous system coordinates coupled physiological and behavioral programs.

## Materials and Methods

Flies were reared on standard media at 25 °C, 60–70% relative humidity, under a 12-h light/12-h dark cycle. Most behavioral assays were performed with 4- to 9-d-old flies, and unless otherwise noted, mating assays were performed at 21–23 °C in 10 × 5-mm copulation chambers using a single male and female. For mating assays using TRPA1 or GAL80<sup>ts</sup>, flies were reared at 18–19 °C and then shifted to 30 °C before the experiment. Fertility was scored by isolating individual females for 48 h in a fly vial and counting the number of late-stage pupae 8 d later. Courtship indices were calculated as the fraction of time a male spent displaying a courtship behavior (e.g., chasing, wing extension, circling, licking) during the observation period. For single fly ejaculation experiments, individual males were lightly anesthetized

with CO<sub>2</sub> and then glued to a glass coverslip. After a 1-h recovery period in a humidified chamber, flies were observed at the activating temperature (30 °C) on a custom-made Peltier device. Peptides and biogenic amines were solubilized in saline injection buffer and injected into flies using an Eppendorf FemtoJet. Graphs were generated using GraphPad Prism software (GraphPad Software). A detailed description of fly stocks, antibodies, experimental designs, and construction of *crz-GAL4*, *crzr-GAL4*, and *tph2-GAL4* is provided in [Supporting Information](#).

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