Supporting Information

Tayler et al. 10.1073/pnas.1218246109

SI Materials and Methods

Fly Stocks and Genetics. The following lines were obtained for this study: *UAS-eGFP-Kir2.1* (1), *UAS-dTRPA1* (P. Garrity, Brandeis University, Waltham, MA), *Sex Peptide-GFP* (2), *fru-GAL4* (B. Dickson, IMP), and *UAS-TRPM8* (B. White, National Institutes of Heath). The following stocks were obtained from Bloomington *Drosophila* Stock Center at Indiana University: Canton-S (#1), w^{1118} from Exelixis (#6326), *UAS-mCD8-GFP* (#5130), *UAS-tubGAL80*^{ts} (#7017 and #7018), *tudor*^{1} (#1786), *UAS-nuclear-LacZ* (#3956), *don juan*-GFP (#5417), and *UAS-Stinger* (#28863). UAS-GCaMP5G lines were obtained from Janelia Farm Research Campus [Howard Huges Medical Institute (HHMI)].

Molecular Biology. GAL4 lines were generated by PCR amplifying the putative enhancer/promoter region (correct sequence verified by DNA sequencing) and then subcloning the PCR fragment into a GAL4 vector (pC3G4; *Drosophila* Genomics Resource Center). Enhancer fragments were chosen by identifying upstream sequences that are conserved with other *Drosophila* species (http://flybase.org/blast/) and/or by the positional constraints of neighboring genes. Constructs were microinjected into w^{1118} embryos (BestGene). *Corazonin-GAL4* was generated using a 391-bp DNA fragment upstream of the putative transcriptional start site of *Drosophila corazonin* (NCBI RefSeq NM_079626.2). *Corazonin receptor-GAL4* (also known as GRHRII) was generated using a 3.5-kb DNA fragment upstream of the putative transcriptional start site of *Drosophila corazonin receptor/GRHRII* (NCBI RefSeq NM_140314.3). Tph2-GAL4 (tryptophan hydroxylase, *Trh*) was generated using a 1.0-kb DNA fragment upstream of the putative transcriptional start site of *Drosophila Trh/Tph2* (NCBI RefSeq NM_138236.1).

Immunohistochemistry. Tissue was dissected from 4- to 8-d-old adult flies and fixed in 2% paraformaldehyde in PBS for 45–60 (brain and nerve cords) or 15 min (reproductive organs) and then washed in PBT (PBS + 0.05% Triton X-100), blocked in 5–10% normal goat serum, and incubated in primary antibody for 24–48 h. Tissue was then washed in PBT and incubated in secondary antibodies for 16–24 h. Samples were mounted on glass slides in Vectashield (Vector Laboratories) and imaged on a Zeiss LSM 510 confocal microscope. Primary antibodies and concentrations: nc82 (DSHB), 1:25; anti-Fru (B. Dickson), 1:1,000; anti-Crz (J. Veenstra), 1:1,000; anti-5-HT (Sigma), 1:500. Secondary antibodies (AlexaFluor; Invitrogen) were used at a concentration of 1:500–1:1,000.

Adult *Drosophila* Injections. Corazonin [(pGLU)TFQYSRGWT (ASN)], scrambled corazonin [(pGLU)SRYTTFGQW(ASN)], and crustacean cardioactive peptide (CCAP) (RP10572) peptide were obtained from GenScript. Serotonin and dopamine were obtained from Sigma. Peptides and biogenic amines were resuspended in injection buffer (108 mM NaCl, 5 mM KCl, 8.2 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO_4, 15 mM ribose, 5 mM Hepes). Flies were briefly anesthetized on ice and affixed to a glass slide with nontoxic commercial glue. After recovering for ~60 min, flies were injected (FemtoJet microinjector; Eppendorf) and observed under a Zeiss dissecting microscope. For corazonin, ejaculation was observed at 100 and 1,000 nM (100 nM is the concentration presented in Fig. 2*M*). For serotonin, ejaculation was observed at injection concentrations of 1.0, 10, and 100 mM (1.0 mM is the concentration presented in Fig. 3*P*).

Behavioral Assays. All flies were generated in or outcrossed (at least six generations) to the Canton-S or Exelixis genetic background. To avoid homozygous mutations commonly found in inbred lines, only F_1 hybrid flies were used in the behavioral studies. As the *white* gene is known to influence behavior (3), all flies used in behavioral experiments contained at least one wild-type copy of *white*. A small percentage of mating pairs displayed a "stuck" phenotype (4, 5) (remaining coupled while actively attempting to disengage for several minutes or more). Flies displaying an extended stuck phenotype were not used in our analysis. Video was obtained with a Basler GigE camera (piA640), Fire-i digital camera (Unibrain), or a commercial Sony HD camcorder via StreamPix 4 (Norpix) or Windows Movie Maker (Microsoft) acquisition software.

Male courtship index. A single male and virgin were introduced into a 10 × 5-mm chamber (using a mouth aspirator) and video recorded at high magnification. Courtship indices were calculated by observing the time each male spent displaying courtship behaviors (orientation, tapping, chasing, singing, licking, or attempted copulation) and dividing this by the observation period (5 min or until copulation was achieved).

Isolated male ejaculation assay. Anesthetized males (brief CO_2 anesthesia or ice anesthesia) were affixed to a glass coverslip using a small drop of nontoxic commercial glue and then placed in a humidified chamber for recovery (~60 min). Glass slides were placed on a custom-built Peltier device (Mike Walsh and Tim Heitzman, Division of Biology, Electronics Shop) and raised to the desired temperature. Ejaculation behavior was observed under a Zeiss dissecting scope. A thin razor blade was used for decapitations.

In copulo *activation.* A single male and virgin female were aspirated into a thin-walled, acrylic mating chamber (25 × 25 mm, round, open top) and capped with a thin acrylic cover. After copulation was initiated, the mating chamber was moved to a temperature- and humidity-controlled chamber (within 1 min) and video recorded. Videos were then scored for duration of copulation. 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.

In copulo *decapitation.* A single male and virgin female were paired in a 25 × 25-mm fly vial. After copulation was initiated, the mating pair was gently tapped onto a surface of double-sided tape to prevent female locomotion. Microscissors (tip diameter 0.05 mm; Fine Science Tools) were inserted between the head and thorax of males and then clipped (or removed without clipping for controls) and assayed for total copulation duration. Female flies were recovered and transferred to individual fly vials to assess fertility.

Separation assay. Pairs were mated in a 5 × 10-mm acrylic chamber and then separated with two soft-tip paintbrushes at the desired time point. Mated females were transferred to a standard fly vial, and presence of progeny was assessed several days later.

Calcium Imaging. ***Ex vivo preparation.*** Ventral nerve cords were dissected from male flies and affixed to a custom imaging slide. ***In vivo preparation.*** Male flies were anesthetized on ice and waxed into a custom imaging platform. The dorsal side of the fly, including the entire head, was kept dry beneath the platform, whereas the ventral side was immersed in saline (103 mM NaCl, 3 mM KCl, 5 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM trehalose, 10 mM glucose, 9 mM sucrose, pH = 7.25, 275 mOsm) that was perfused with 95% oxygen, 5% CO_2. After removing the legs, a small piece of cuticle

was removed at the base of the ventral thorax to visualize the abdominal ganglia. Calcium responses were imaged with an LSM 710 two-photon laser scanning microscope (Zeiss) at 920 nm. $\Delta F/F$ was calculated by averaging a baseline fluorescence period before injection of buffer or buffer + peptide (F0) and then subtracting and dividing F0 from each time point.

Statistical Analysis. GraphPad Prism software (version 5.0) was used for statistical analysis and generation of graphs. Statistical significance was determined using Kruskall-Wallis one-way ANOVA with Dunn's correction and one-way or two-way ANOVA with Bonferroni correction (for temperature shift experiments).

1. Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M (2001) Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. *J Neurosci* 21(5):1523–1531.
2. Villella A, Peyre JB, Aigaki T, Hall JC (2006) Defective transfer of seminal-fluid materials during matings of semi-fertile fruitless mutants in *Drosophila*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 192(12):1253–1269.
3. Zhang SD, Odenwald WF (1995) Misexpression of the white (w) gene triggers male-male courtship in *Drosophila*. *Proc Natl Acad Sci USA* 92(12):5525–5529.
4. Hall JC, Siegel RW, Tomkins L, Kyriacou CP (1980) Neurogenetics of courtship in *Drosophila*. *Stadler Genet Symp Proc* 12:43–82.
5. Kuniyoshi H, et al. (2002) lingerer, a *Drosophila* gene involved in initiation and termination of copulation, encodes a set of novel cytoplasmic proteins. *Genetics* 162(4):1775–1789.

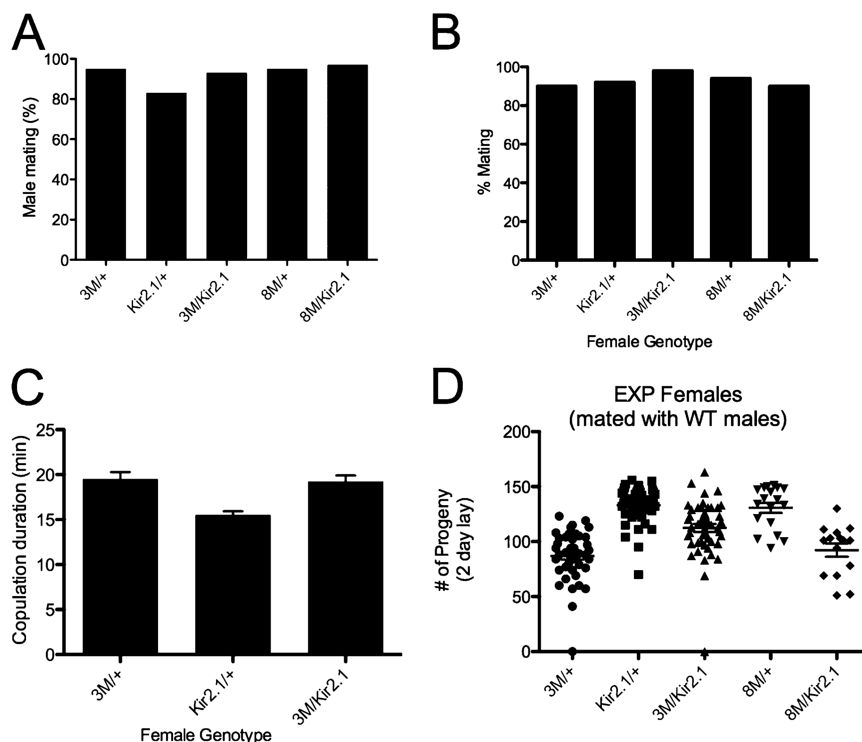

Fig. S1. Effect of neuron silencing on mating. (*A*) Expression of *UAS-Kir2.1* in *crz-GAL4* neurons in males has no effect on the ability to mate within 1 h. (*B*) Females expressing *UAS-Kir2.1* in *crz-GAL4* neurons mate normally within 1 h, display normal copulation durations (*C*), and are fertile (*D*). 3M and 8M represent independent inserts of *crz-GAL4*. $n \geq 20$ for each genotype.

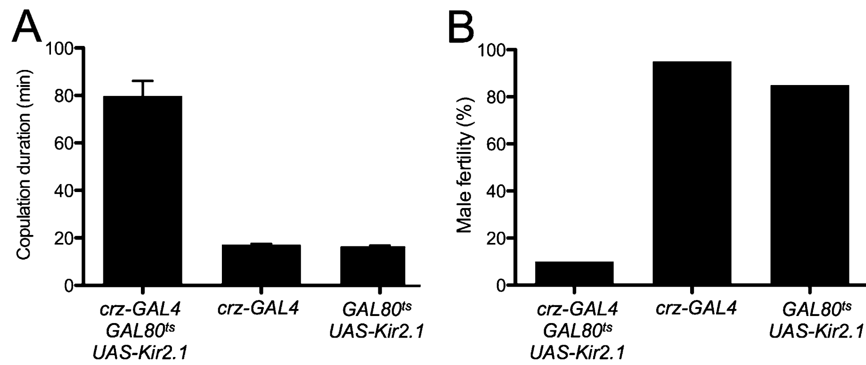

Fig. S2. Corazonin neuron activity is required during the adult stage for normal copulation duration. Expression of Kir2.1 was restricted to adult stage using a temperature-sensitive version of GAL80. Flies were reared at 18 °C and then shifted to 30 °C for 24–48 h, several days after eclosion, and assessed for copulation duration (*A*) and male fertility (*B*). $n \geq 10$ in *A* and $n \geq 20$ in *B*.

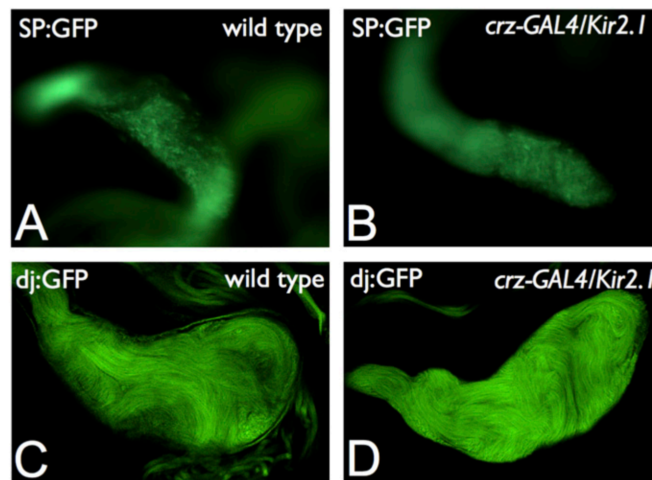

Fig. S3. Sperm and seminal proteins are present and appear normal in *crz-GAL4 UAS-Kir2.1* males. Sex peptide:GFP fusion protein in wild-type males (*A*) and *crz-GAL4 UAS-Kir2.1* males (*B*). don juan:GFP labels sperm in wild-type males (*C*) and *crz-GAL4 UAS-Kir2.1* males (*D*).

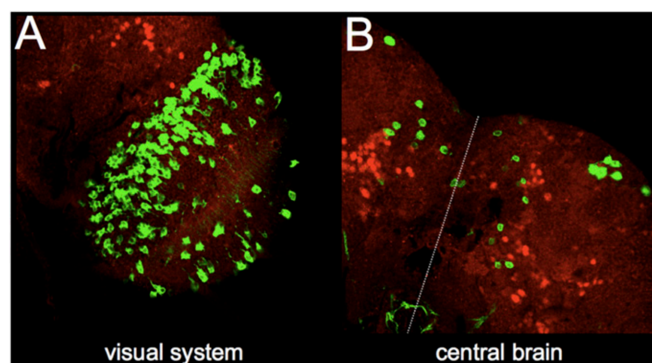

Fig. S4. Expression of *corazonin-GAL4* and Fru^{M} in the adult brain. Confocal image of crz-GAL4 neurons (green) and anti-Fru^{M} (red) in the adult visual system (*A*) and central brain (*B*) of an adult male fly. No overlap is observed between the two molecularly defined cell types.

Fig. S5. Abdominal ganglia neurons are absent from an independent insertion of *crz-GAL4* (2fa) males, and expression of Kir2.1 in *crz-GAL4 2fa* neurons has no effect on copulation duration. (*A*) Confocal image stack of *crz-GAL4(2fa) UAS-mCD8-GFP UAS-DsRed* (nuclear) whole-mount CNS labeled with antibodies to nc82 (blue) and GFP (green). (*B*) Higher-magnification view of the third thoracic segment (T3) and abdominal ganglia (AG). Arrow indicates the AG. (*C*) *crz-GAL4(2fa) UAS-Kir2.1* males display normal copulation durations. $n = 10$.

Fig. S6. Male decapitation during mating. crz-GAL4 UAS-Kir2.1 and control males were paired with wild-type virgins. After mating was initiated, males were decapitated and then scored for copulation duration (*Materials and Methods*). ***$P < 0.001$. Error bars denote SEM.

Fig. S7. *crzR-GAL4* neurons innervate the male internal reproductive structures. Confocal image of the internal reproductive organs of *crz-GAL4* (*A*) or *crzR-GAL4* (*B*) male expressing *UAS-mCD8-GFP*, stained with anti-GFP (red). No expression of *crz-GAL4* is detected. *crzR-GAL4* projections innervate all of structures associated with sperm and seminal fluid transfer. (*C–E*) Confocal image of a *crzR-GAL4 UAS-mCD8-GFP* male abdominal ganglion stained with anti-GFP and anti-5-HT.

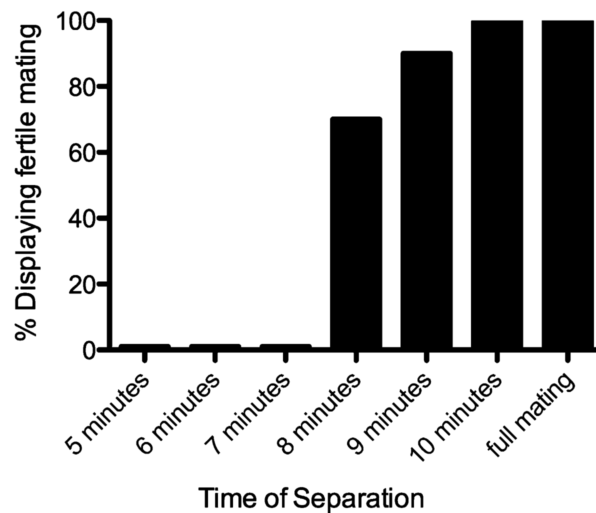

Fig. S8. Timing of sperm transfer. Wild-type pairs were mated and then separated at the indicated time. Females were then individually sorted into fly vials containing food and scored for the presence or absence of progeny. In most individuals, sperm transfer begins to occur between 7 and 8 min after copulation.

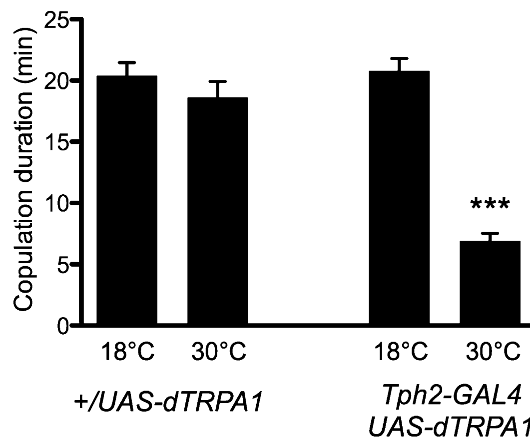

Fig. S9. Activation of serotonergic neurons during mating shortens copulation duration. *Tph2-GAL4 UAS-TRPA1* males were mated to wild-type females. Approximately 60 s after mating, flies were shifted to the activating temperature (28–31 °C) and scored for mating duration. $n \geq 12$ per genotype. $^{***}P < 0.001$. Error bars denote SEM.

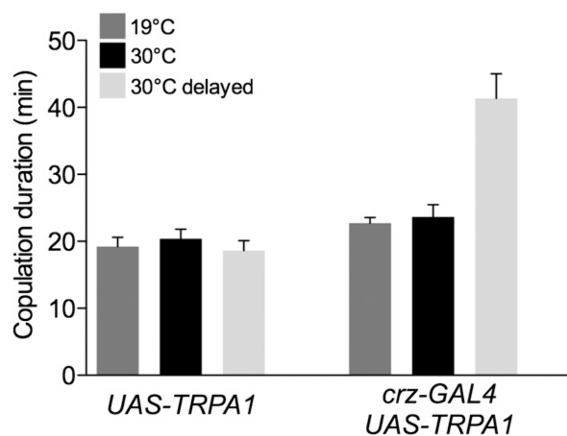

Fig. S10. Delayed activation of *crz-GAL4* neurons during mating increases copulation duration. *crz-GAL4 UAS-TRPA1* males were mated to wild-type females. Approximately 60 s after mating (black columns) or ~10 min after mating (light gray columns), flies were shifted to the activating temperature (28–31 °C) and scored for mating duration. $n \geq 10$ per genotype. Error bars denote SEM.

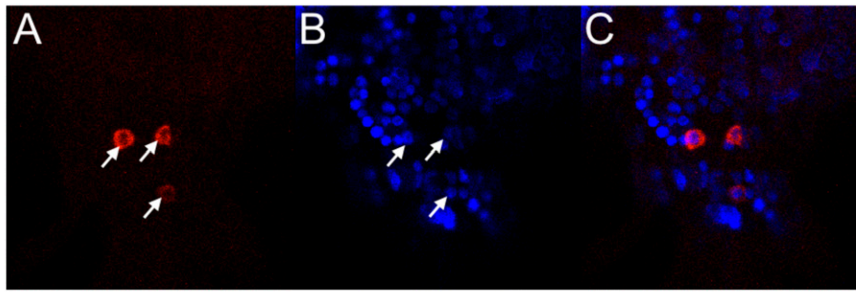

Fig. S11. *Cha-GAL4* is expressed in corazonin AG neurons. Confocal image (single slice) of a *Cha-GAL4 UAS-nLacZ* male abdominal ganglion stained with anti-LacZ (blue) and anti-Crz (red). (*A*) anti-Crz. (*B*) anti-LacZ. (*C*) Merged image. Arrows indicate the cells with overlapping expression.