Supporting Information

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SI Materials and Methods

Samarkand and *DTS-3*. Samarkand is one of the wild-type laboratory strains of *Drosophila melanogaster*. *DTS-3* was isolated from EMS-treated isogenic Samarkand stock (1), as one of the dominant-lethal mutations that are lethal when heterozygous at 29 °C. Regarding the lethal phenotype of *DTS-3*/+, 22 and 29 °C are permissive and restrictive temperature, respectively. The temperature 27 °C is sublethal temperature (2). *DTS-3* is likely a mutation involved in ecdysone biosynthesis, because its developmental defect can be rescued when flies are fed 20E (2, 3). At 22 °C, ecdysteroid levels in 10-day-old *DTS-3*/+ females are lower than those of wild-type Samarkand (2), suggesting that *DTS-3*/+ flies has a defect in the ecdysone synthesis even at nonlethal temperature. *DTS-3* is cytogenetically mapped at 84.5 on 3rd chromosome. Simon et al. (4) suggested, based on personal communication, that *DTS-3* encodes a protein with Krüppel Zn-finger DNA-binding domains.

***UAS-EcR* RNAi Transformants.** The UAS-*EcR* RNAi construct was created using the pWIZ vector developed by Lee and Carthew (5) with a transgene containing inverted repeats of partial *EcR* cDNA. An *EcR* target sequence was amplified by PCR using the following primers. Forward: 5′-GAA GAA TTC CCT AGG GTC GCG ATG ATC TCT CGC CTT C-3′; and reverse: 5′-AGA ATC TAG ACCS TAG GCG GAA TAG TGG CAT GCT GGG-3′. Transformed fly lines were established by injecting the DNA construct in "white" mutant embryos using standard procedure (6).

Courtship Conditioning Assay and Data Presentation. Courtship conditioning assays were carried out as described in Sakai et al. (7) with minor modifications. Unreceptive, mated-females were prepared as "trainers" a day before they were used for courtship conditioning, by placing a 3-day-old virgin Canton-S (CS) female, and a 3- to 5-day-old CS male in a courtship chamber made of transparent acrylic plastic (15 mm in diameter × 3 mm in depth) for copulation. For the 1-h, 5-h, and 7-h training, a male and a trainer female were introduced in a conditioning chamber (15 mm in diameter × 5 mm in depth) containing food medium. During the training period, male's courtship activity toward the trainer female was significantly reduced (8). After training, the male flies were kept individually in a glass test tube (12 mm in diameter × 75 mm in depth) containing food medium, until test. For memory tests, a trained or naive male was placed in the courtship chamber with a target freeze-killed virgin female. Flies were videotaped for 10 min by using a digital video camera (DCR-DVD105, SONY), and the courtship index was determined as described previously (7, 9).

Because courtship index values are often not distributed normally, the data were analyzed nonparametrically using the Mann–Whitney *U* test, and are presented using box plots, in which the first quartile, the median, and the third quartile are represented by the lower edge, the middle line, and the upper edge, respectively, of each box. The outliers are shown as dots in the figures. In this study, an outlier was a data point outside of either lower or upper fences. The lower and upper fences were defined as follows. Lower fence, Q1 − 1.5 × IQ; upper fence, Q2 + 1.5 × IQ, respectively (Q1, Q2, and IQ are the lower quartile, the upper quartile, and the interquartile range, that is, the difference between the 25th and 75th percentiles). The lowest and highest values that are not outliers are shown as whiskers.

Immunohistochemical Staining. Adult brains were dissected from 3-day-old flies in PBS, and fixed overnight with 4% formaldehyde at 4 °C, in PBS containing 0.05% Triton X-100. The brains were incubated for 60 min at room temperature, in PBS containing 0.05% Triton X-100 and 0.1% goat serum, and then overnight at 4 °C with mouse monoclonal antibodies, followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG.

Western Blot Analysis. Five fly heads were homogenized in 50 μL of PBS-T (0.01% Tween-20 in PBS) and dissolved in SDS sample buffer. Samples were analyzed on an 8% SDS polyacrylamide gel, and blotted to a PVDF membrane. The blot was blocked with 5% skim milk in PBS-T for 30 min, and incubated with anti-EcR antibody DDA2.7 (1:100) or anti-tubulin antibody (1:1,000; Developmental Studies Hybridoma Bank at the University of Iowa) overnight at 4 °C. Alkaline phosphatase-conjugated anti-mouse goat IgG (1:1,500, Santa Cruz Biotechnology) was used as the secondary antibody. Immunoreactivity was visualized by using the CDP-star system (Roche), according to the manufacturer's instructions. Images were digitized to analyze signal intensity using the ImageJ processing program (National Institutes of Health).

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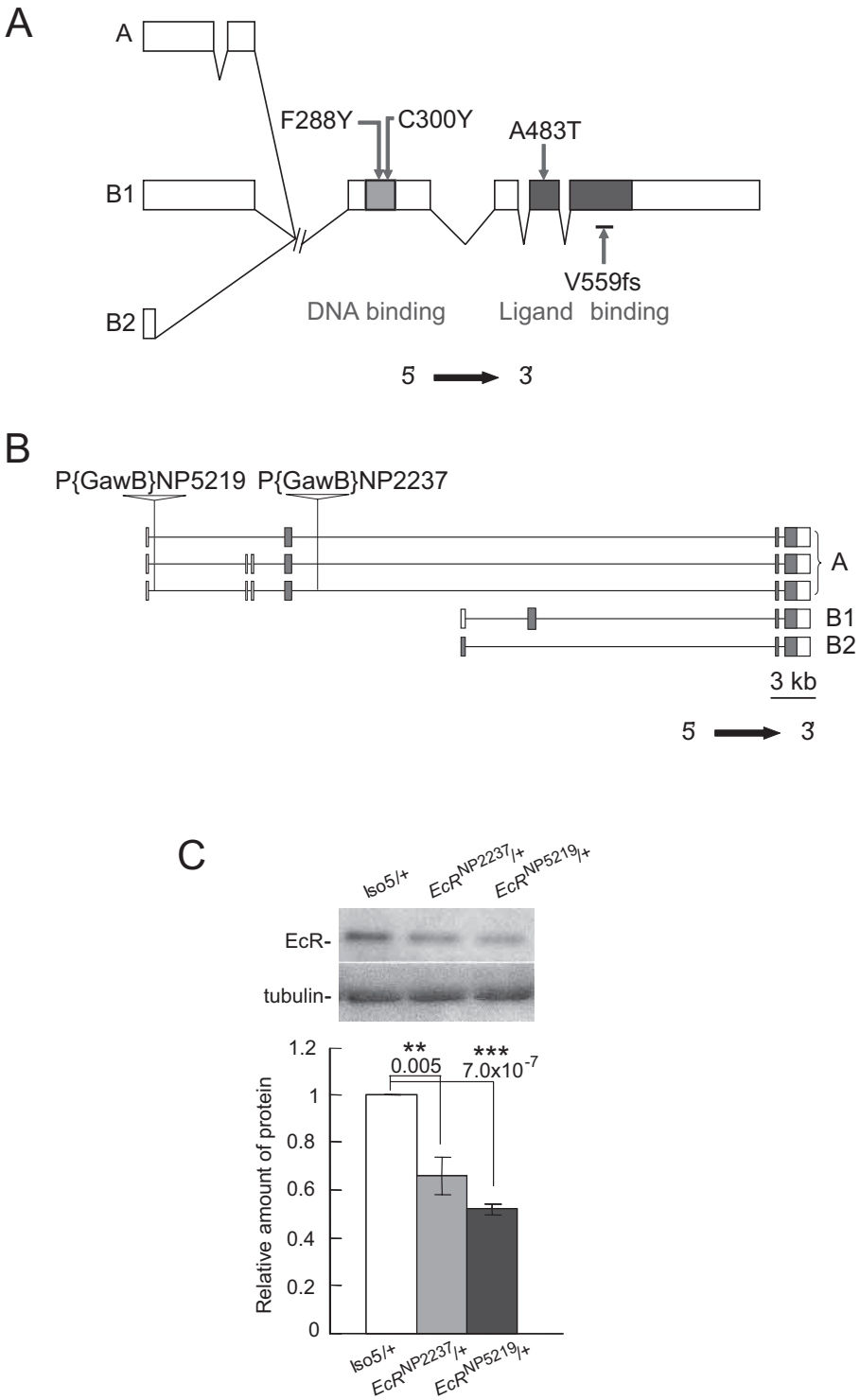

Fig. S1. *EcR* mutations and RNAi strains. (*A*) A schematic representation depicting the positions of chemically-induced *EcR* mutations. They were generated by EMS mutagenesis in a *cn bw* background (10). All 3 EcR isoforms (EcR-A, EcR-B1, and EcR-B2) share common DNA-binding and ligand-binding domains. (*B*) The positions of P-element induced *EcR* mutations. Open and filled boxes indicate untranslated and protein-coding regions of the exons, respectively. (*C*) Western blot analysis demonstrates that EcR protein levels are reduced in the heads of adult P-element insertion mutants. The decrease in the relative amount of EcR protein in the mutants is significant. Error bars represent the mean ± SEM. Data were analyzed using Student's *t* test. **, $P < 0.01$; ***, $P < 0.001$.

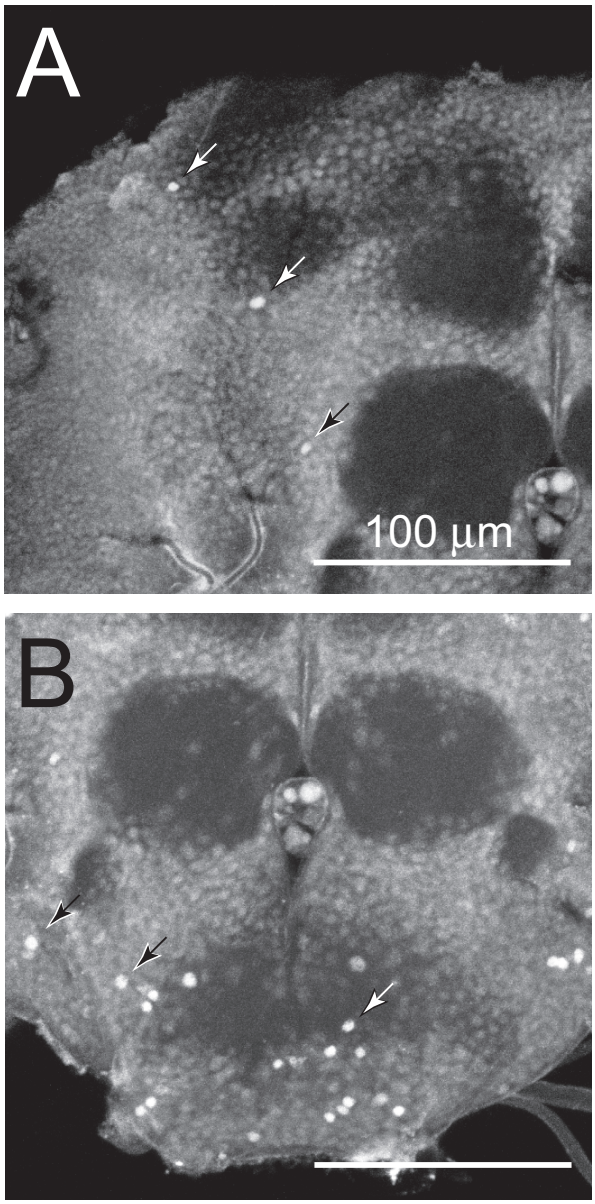

Fig. S2. Immunohistochemical detection of ecdysone receptors in the adult *Drosophila* brain. Magnified images of superior lateral protocerebrm (*A*) and suboesophageal ganglion (*B*), with arrows indicating strongly stained cells. (Scale bar, 100 μm.)

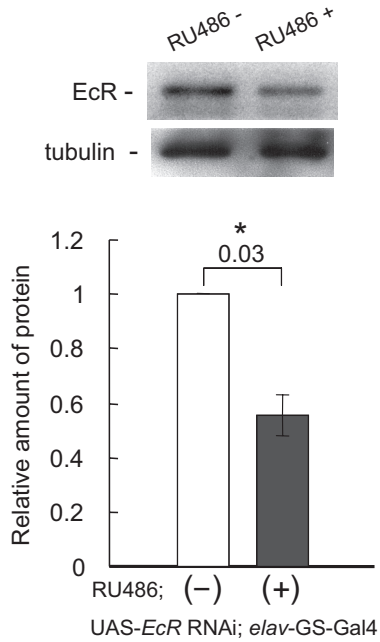

Fig. S3. Western blot analysis demonstrates that EcR protein levels in the heads of adult flies of the genotype *EcR* RNAi; *elav*-GS-Gal4 flies are reduced after driver activation. The reduction in the relative amount of EcR protein is significant, as indicated by the quantitation shown in the lower panel. Error bars represent mean ± SEM. Data were analyzed using Student *t* test. *, $P < 0.05$.