

# Functional analysis of *fruitless* gene expression by transgenic manipulations of *Drosophila* courtship

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A *gal4*-containing enhancer–trap called *C309* was previously shown to cause subnormal courtship of *Drosophila* males toward females and courtship among males when driving a conditional disrupter of synaptic transmission (*shi<sup>TS</sup>*). We extended these manipulations to analyze all features of male-specific behavior, including courtship song, which was almost eliminated by driving *shi<sup>TS</sup>* at high temperature. In the context of singing defects and homosexual courtship affected by mutations in the *fru* gene, a *tra*-regulated component of the sex-determination hierarchy, we found a *C309/tra<sup>F</sup>* combination also to induce high levels of courtship between pairs of males and “chaining” behavior in groups; however, these doubly transgenic males sang normally. Because production of male-specific FRU<sup>M</sup> protein is regulated by *TRA*, we hypothesized that a *fru*-derived transgene encoding the male (M) form of an Inhibitory RNA (*fru<sup>MIR</sup>*) would mimic the effects of *tra<sup>F</sup>*; but *C309/fru<sup>MIR</sup>* males exhibited no courtship chaining, although they courted other males in single-pair tests. Double-labeling of neurons in which GFP was driven by *C309* revealed that 10 of the 20 CNS clusters containing FRU<sup>M</sup> in wild-type males included coexpressing neurons. Histological analysis of the developing CNS could not rationalize the absence of *tra<sup>F</sup>* or *fru<sup>MIR</sup>* effects on courtship song, because we found *C309* to be coexpressed with FRU<sup>M</sup> within the same 10 neuronal clusters in pupae. Thus, we hypothesize that elimination of singing behavior by the *C309/shi<sup>TS</sup>* combination involves neurons acting downstream of FRU<sup>M</sup> cells

reproductive behavior | *C309* enhancer trap | *shi<sup>TS</sup>* transgene | *tra<sup>F</sup>* transgene | inhibitory *fru* RNA transgene

Various portions of the CNS in *Drosophila melanogaster* are inferred to control separate elements of normal male courtship (e.g., refs. 1 and 2), in part by analysis of abnormal behavior (e.g., refs. 3–7). Some such studies have involved brain-behavioral analyses of the *fruitless* (*fru*) gene and its mutants (reviewed in ref. 8). Different *fru* mutants exhibit courtship subnormalities to varying degrees and at separate stages of the courtship sequence, depending on the mutant allele (e.g., refs. 9–12). Most *fru* mutants court other males substantially above levels normally exhibited by pairs or groups of wild-type males (e.g., refs. 12 and 13). The original *fruitless* mutation leads to spatially nonrandom decreases of *fru*-product presence (14, 15) within particular subsets of the normal CNS expression pattern (16, 17), which may be causally connected with the breakdown of recognition that is a salient effect of *fru<sup>1</sup>* on male behavior (9, 12).

*fru*-like courtship can be induced by the effects of a transgene that encodes GAL4 (a transcription factor derived from yeast). When this *C309* enhancer trap was combined with a GAL4-drivable factor containing a dominant-negative, conditionally expressed variant of the *shibire* gene (*shi<sup>TS</sup>*), heat treatment of doubly transgenic males caused them to court females subnormally and to court other males vigorously (18). Although this strain had been termed a mushroom body enhancer trap in terms of the *gal4* sequence it contains, being expressed “predominantly” within that dorsal-brain structure (19, 20), Kitamoto

revealed that *C309* drives marker expression in a widespread manner (18). Therefore, we sought to correlate various CNS regions in which this transgene is expressed with its effects on male behavior, emphasizing a search for “*C309* neurons” that might overlap with elements of the FRU<sup>M</sup> pattern.

We also entertained the possibility that the *C309/shi<sup>TS</sup>* combination causes a mere caricature of *fruitless*-like behavior. Therefore, what would be the courtship effects of *C309* driving a transgene that produces the female form of the *transformer* gene product? This TRA protein participates in posttranscriptional control of *fru*'s primary “sex transcript,” so that FRU<sup>M</sup> protein is not produced in females (reviewed in ref. 8; also see refs. 16 and 21). If *C309* and *tra<sup>F</sup>* are naturally coexpressed in a subset of the to-be-analyzed neurons, feminization of the overlapping cells should eliminate this protein. We extended these transgenic experiments to target *fruitless* expression specifically by *gal4* driving of an inhibitory RNA (IR) construct, which was generated with *fru* DNA by Manoli and Baker (22). Their experiments furnish one object lesson as to how “enhancer–trap mosaics” can delve into the neural substrates of a complex behavioral process, an approach commonly taken to manipulate brain structures and functions in courtship experiments (2–7). Because few genetic loci putatively identified by such transposons have been specified, the tactics we applied are in the context of CNS regions in which expression of a “real gene” is hypothesized to underlie well defined behaviors.

## Materials and Methods

**Supporting Information.** For further details, see Tables 3–5 and Figs. 5 and 6, which are published as supporting information on the PNAS web site.

**Stocks of *D. melanogaster*, Crosses, and Fly Handlings.** Cultures were maintained as in ref. 23. Pure control males came from a Canton-S wild-type (WT) stock. Other control types were male progeny of a given transgenic strain (see below) crossed to Canton-S. Adult males and females were collected and stored as in refs. 12 and 23 (see below for exceptions). The enhancer–trap line *C309* (19) is homozygous for a *gal4*-containing transposon inserted into chromosome 2; such females were crossed separately to males carrying the following transgenes: UAS-*shi<sup>TS</sup>* (homozygous on chromosome 3), which disrupts synaptic transmission in a heat-sensitive manner under the control of a given *gal4*-containing, neurally expressed transgene (24); UAS-*tra<sup>F</sup>* (homozygous on chromosome 2), which, when GAL4-driven, causes the female form of *transformer* (*tra*) mRNA to be produced (e.g., refs. 3 and 4); UAS-*fru<sup>MIR</sup>* [inserted into both the

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Abbreviations: IR, inhibitory RNA; CI, courtship index; WEI, wing extension index; ChI, chaining index; VNC, ventral nerve cord.

See accompanying Profile on page 16547.

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second and third chromosomes, the former heterozygous for the transgene and *In(2LR)O, Cy*, the latter homozygous], designed to produce a double-stranded IR that blocks production of male (M)-specific protein encoded by the endogenous *fru* gene (22); and *UAS-egfp* (homozygous on chromosome 2), which encodes an “enhanced” nuclear form of GFP (25).

Most culture rearings occurred at 25°C; but those involving *UAS-fru<sup>MIR</sup>* were effected separately at 25°C and 29°C, because the hotter condition was reported to accentuate the inhibitory effects of this transgene (22). Histochemistry involving effects of *tra<sup>F</sup>* or *fru<sup>MIR</sup>* on the presence of FRU<sup>M</sup> in *C309*-expressing neurons used females from a stock carrying both *C309* and *UAS-egfp* on the second chromosome (generated by meiotic recombination), crossed to *UAS-tra<sup>F</sup>* or to “double-insert” *UAS-fru<sup>MIR</sup>* males. Additional transgene combinations used females from a *C309/C309/Cha-gal80/In(3LR)TM6B,Hu* transgenic stock, crossed separately to *UAS-shi<sup>TS</sup>*, *UAS-tra<sup>F</sup>*, *UAS-fru<sup>MIR</sup>*, or *UAS-egfp* males; triply transgenic progeny should have *gal4* driving eliminated in neurons that coexpress *gal80* (see ref. 26) under the control of regulatory sequences from the *Choline acetyltransferase (Cha)* gene (see refs. 18 and 27).

**Behavior. Basic courtship quantification.** Audio/video recordings were obtained and processed as in refs. 12 and 23, but most of the current records were captured with a Sony VX2100 digital camera. For transgenic-male/WT-female pairings, the two types of flies were readily distinguishable despite the largely feminized external appearance of *XY* flies carrying *C309/UAS-tra<sup>F</sup>* or *C309/UAS-tra<sup>F</sup>/Cha-gal80*. For transgenic male/WT male observations involving *UAS-shi<sup>TS</sup>* or *UAS-fru<sup>MIR</sup>*, the two male types look the same, so each WT male had the tip of one wing clipped off at the time of collection. Males including *UAS-shi<sup>TS</sup>* were stored at 25°C (permissive temperature) before testing. For restrictive-temperature observations, a male- and food-containing tube was placed in a 30°C water bath for 20–40 min, then aspirated into a mating cell for recording at 30°C. For permissive-temperature controls, test males remained in food containers at 25°C before transfer into female-containing chambers at that temperature. Recordings were converted to computerized files, and behaviors were “logged” and analyzed by using LIFE-SONGX (<http://lifesong.bio.brandeis.edu>, compare ref. 28) to compute percentages of observation periods during which any interfly interactions occurred (courtship index, CI) or courtship wing displays (wing extension index, WEI).

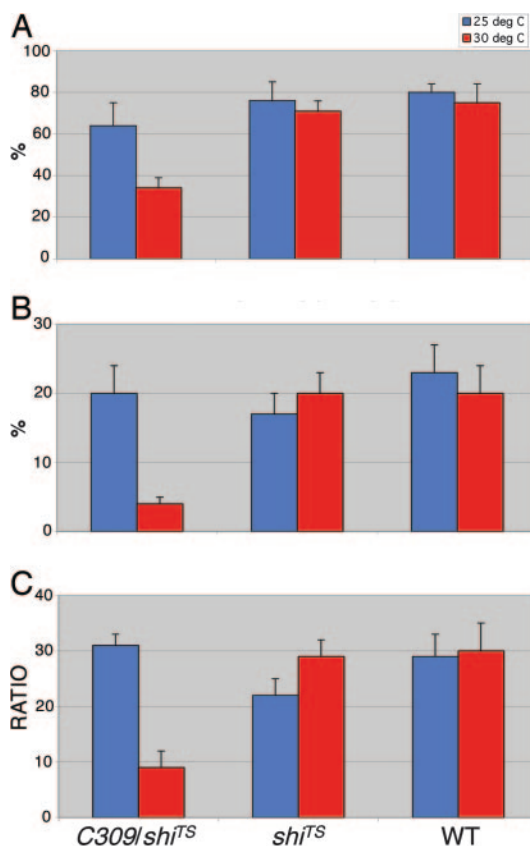
**Song sounds.** Digitized audio tracks were logged then analyzed (as in refs. 12 and 23), leading to computations of the parameters specified in Table 3.

**Mating behaviors.** Attempted copulations, Mating-initiation latencies, and copulation successes were quantified for several fly pairs in a plastic device (see ref. 1), at 25°C or at 30°C for tests involving *shi<sup>TS</sup>*.

**Courtship chaining.** Eight to 10 males of a given genotype were grouped in a food vial upon collection, stored for 3–4 days (at 25°C or 20°C), and then hand-timer recorded at 25°C for the amount of time that at least three males spent courting one another during a 10-min observation period (as in refs. 12 and 13), leading to chaining index (ChI) values. A given male group involving *shi<sup>TS</sup>* (previously stored at 20°C) was observed at 25°C for ChI determination, then shifted to 30°C for 1–2 h, after which a subsequent ChI value was obtained at that temperature.

**General locomotion and flight performances.** These were quantified as in ref. 12.

**CNS Histology.** The principal specimens were whole-mounted brains plus ventral nerve cords (VNCs) dissected from 4-day-old adult males (stored 15–20 per food vial). Additional studies involved developing *Drosophila*, as described in Fig. 6. The histological procedures were essentially those described in refs.



**Fig. 1.** Male–female courtship affected by the *C309* enhancer trap driving a temperature-sensitive *shibire* mutation. (A) CIs  $\pm$  SEM for doubly transgenic *C309/UAS-shi<sup>TS</sup>* experimental males (*C309/shi<sup>TS</sup>*) and singly transgenic *UAS-shi<sup>TS</sup>* (*shi<sup>TS</sup>*) or wild-type (WT) control males, at the 2 temperatures indicated. *C309* males paired with females at 25°C led to average CI and WEI values of  $72 \pm 3$  and  $34 \pm 2$ , respectively ( $n = 8$ ).  $n$  values for courtship values plotted: *C309/shi<sup>TS</sup>*, 9 (25°C) and 15 (30°C); *shi<sup>TS</sup>*, 10 and 15; WT, 14 and 9. A two-way ANOVA on CIs, with GENOTYPE and TEMPERATURE as the main effects, revealed significant differences for each effect ( $P$  values = 0.0003 and 0.037, respectively), but no interaction between these two factors ( $P = 0.15$ ). (B) WEIs. A two-way ANOVA with GENOTYPE and TEMPERATURE as the main effects revealed significant WEI differences for each ( $P$  values = 0.0003 and 0.01, respectively) and a significant interaction effect between the two factors ( $P = 0.0005$ ). (C) Ratios computed from the values in A and B.

15, 16, and 29 (see Fig. 6 for details). The secondary antibody applied to recognize anti-FRU<sup>M</sup> primary (see ref. 16), was Cy5-conjugated anti-rat IgG. Neuronal marking was analyzed by using a Leica TCS SP2, scanning in the Cy5 channel for singly labeled brains (anti-FRU<sup>M</sup>) or scanning for Cy5 (red) and GFP (green) in colocalization assessments, which included conversion of red to magenta and merging the two channels to create white readout. For doubly labeled specimens, *gal4/FRU<sup>M</sup>* coexpression within a given neuronal cluster was quantified by projecting a Z series of 1- to 2- $\mu$ m sections with both channels merged (see Fig. 6 for details). Coexpression values (see Table 2) were computed by taking the total numbers of white cells from the left and right side of the FRU<sup>M</sup>-cluster CNS region within a given transgenic specimen and dividing this value by the WT “adult-FRU<sup>M</sup>” neuronal count for that cluster (this denominator =  $2 \times$  the relevant hemi-brain or hemi-VNC value from the leftmost data column in Table 2).

**Statistics.** Such analyses were performed on behavioral data and histological counts, the former arcsine or log-transformed unless the raw values defined a normal distribution (compare refs. 12

**Table 1. Courtship of transgenic males directed at females or other males**

XY courters	Index	Female courtees	Male courtees
<i>C309/UAS-tra<sup>F</sup></i>		WT female ( <i>n</i> = 18)	WT male ( <i>n</i> = 15)
	CI	76 ± 3	38 ± 7
	WEI	36 ± 3	4 ± 1
WT		WT female ( <i>n</i> = 14)	<i>C309/UAS-tra<sup>F</sup></i> male ( <i>n</i> = 15)
	CI	80 ± 4	10 ± 3
	WEI	28 ± 4	1 ± 1
<i>C309/UAS-fru<sup>MIR</sup></i>		WT female ( <i>n</i> = 15)	WT male ( <i>n</i> = 20)
	CI	67 ± 3	27 ± 7
	WEI	31 ± 3	7 ± 3
WT		WT female ( <i>n</i> = 14)	<i>C309/UAS-fru<sup>MIR</sup></i> male ( <i>n</i> = 20)
	CI	80 ± 4	6 ± 1
	WEI	28 ± 4	0 ± 0
<i>C309/UAS-tra<sup>F</sup>/Cha-gal80</i>		WT female ( <i>n</i> = 11)	WT male ( <i>n</i> = 18)
	CI	63 ± 5	17 ± 3
	WEI	31 ± 4	3 ± 1
WT		WT female ( <i>n</i> = 14)	<i>C309/UAS-tra<sup>F</sup>/Cha-gal80</i> male ( <i>n</i> = 18)
	CI	80 ± 4	14 ± 3
	WEI	28 ± 4	1 ± 1

Males of the left-column genotypes had their courtships recorded in the presence of the target types indicated (courtrees; WT, wild type). CIs and WEIs (compare Fig. 1) are quoted ± SEM. Additional control values from singly transgenic males: *UAS-tra<sup>F</sup>*, CI: 80 ± 3, *n* = 10; *UAS-fru<sup>MIR</sup>*, CI: 75 ± 5, *n* = 10; *Cha-gal80*, CI: 64 ± 6, *n* = 9. For male pairs, each individual's behavior was quantified separately. A one-way ANOVA on arcsine-transformed CIs from male behavior directed at females, with GENOTYPE as the main effect, revealed significant differences among genotypes (*P* < 0.0001). Subsequent comparisons showed that *C309/UAS-tra<sup>F</sup>* and 25°C-reared *C309/UAS-fru<sup>MIR</sup>* (leading to the tabulated values) courted females at the same levels as did singly transgenic control males. *C309/UAS-fru<sup>MIR</sup>* reared at 29°C courted females to the same degree (CI: 48 ± 5, *n* = 14) as did singly transgenic *UAS-fru<sup>MIR</sup>* males (CI: 63 ± 4, *n* = 7) reared at 29°C. *C309/UAS-tra<sup>F</sup>/Cha-gal80* males courted females less vigorously than did *C309/UAS-tra<sup>F</sup>* (*P* < 0.05) but indistinguishably from *Cha-gal80*. *C309/UAS-fru<sup>MIR</sup>/Cha-gal80* males courted females (CI: 56 ± 4, *n* = 13) indistinguishably from the performance of *C309/UAS-fru<sup>MIR</sup>* (25°C) and the *Cha-gal80* type but significantly less than did another singly transgenic control: *UAS-fru<sup>MIR</sup>* (*P* < 0.05). A one-way ANOVA on (untransformed) WEIs in the presence of females, with GENOTYPE as the main effect, revealed significant differences among genotypes (*P* = 0.008); but pairwise comparisons showed that most multiply transgenic types extended their wings to the same degree (*P* values ≥ 0.05) as did WT males or corresponding singly transgenic ones (WEI for *UAS-tra<sup>F</sup>*: 29 ± 3, *n* = 10; for *UAS-fru<sup>MIR</sup>* reared at 25°C: 37 ± 4, *n* = 10; same reared at 29°C: 25 ± 3, *n* = 7; *Cha-gal80*: 26 ± 4, *n* = 9); exception: *C309/UAS-tra<sup>F</sup>* males gave significantly higher WEIs in the presence of females compared with WT male performance (*P* < 0.05). A one-way ANOVA on arcsine transformed CIs for behavior directed at males, with GENOTYPE as the main effect, revealed significant differences among genotypes (*P* < 0.0001). These comparisons factored in results from *C309/UAS-fru<sup>MIR</sup>* reared at 29°C (CI: 4 ± 1, WEI 0, *n* = 12). Subsequent pairwise comparisons showed that *C309/UAS-tra<sup>F</sup>* males courted other males more vigorously than did *C309/UAS-fru<sup>MIR</sup>* or *C309/UAS-tra<sup>F</sup>/Cha-gal80* (*P* values < 0.05). When paired with a male, the behavior of all multiply transgenic types (except for *C309/UAS-fru<sup>MIR</sup>* reared at 29°C) led to significantly higher CIs compared with singly transgenic controls (*P* values < 0.05). The latter male types did not court WT males vigorously (all CIs < 5 for: *C309*, *n* = 10; *UAS-shi<sup>TS</sup>*, *n* = 12; *UAS-tra<sup>F</sup>*, *n* = 12; *Cha-gal80*, *n* = 11; *UAS-fru<sup>MIR</sup>*, *n* = 9 and 12, 25°C and 29°C, respectively). CIs derived from male-pair recordings involving *C309/UAS-fru<sup>MIR</sup>* or *C309/UAS-tra<sup>F</sup>/Cha-gal80* males were statistically equivalent to the behavior of *C309/UAS-shi<sup>TS</sup>* courting males at 30°C; whereas those recorded for *C309/UAS-tra<sup>F</sup>* were significantly higher (*P* < 0.05). To compare courtship directed at WT males by the transgenic types to behavior of WT males directed at the transgenics, a final set of statistical comparisons (as above) revealed that most of the seven experimental types (the three multiple transgenics tabulated plus four additional ones noted above) courted WT males more vigorously than the latter courted the former (*P* values < 0.05); exceptions: *C309/UAS-tra<sup>F</sup>/Cha-gal80* and *C309/UAS-fru<sup>MIR</sup>* reared at 29°C, males of which courted WT males at the same level as the latter courted these two transgenic types.

and 23). The data were subjected to ANOVAs and subsequent planned pairwise comparisons if appropriate;  $\alpha$  levels were adjusted to correct for experiment-wise error (exemplified in ref. 12).

## Results

**Separate Components of Courtship Affected by Neurally Expressed Transgenes.** Heat-treated *Drosophila* males in which *UAS-shi<sup>TS</sup>* expression was driven by the *C309* transposon exhibited a

significant decrease in courtship toward females compared with the low-temperature control (Fig. 1A). This finding confirms that of Kitamoto (18). Reducing the extent to which *C309* drives *shi<sup>TS</sup>* spatial expression by adding the *Cha-gal80* transgene to this genotype resulted in a minimal diminution of courtship directed at females (CI, 68 ± 3; *n* = 7) compared to WT or *UAS-shi<sup>TS</sup>* (30°C) values (Fig. 1A). Spatial expression of the *Cholineacetyltransferase* (*Cha*) gene (determined by transgenic analysis) is reviewed in ref. 27. The relevance of this CNS pattern to sexual



behavior was examined by Kitamoto (18), although he observed the triply transgenic type only in terms of *Cha-gal80* effects on interactions among males in groups (compare ref. 13).

These heat-induced courtship decrements are unlikely to be caused by overall behavioral sluggishness, because general locomotion scores at 30°C for *C309/UAS-shi<sup>TS</sup>* males (numbers of line crossings, ref. 12) were  $64 \pm 6$  ( $n = 7$ ), compared to  $53 \pm 5$  ( $n = 10$ ) for singly transgenic *UAS-shi<sup>TS</sup>* controls. Moreover, heated *C309/UAS-shi<sup>TS</sup>* males courted WT males at anomalously high levels (CI  $16 \pm 5$ ,  $n = 15$ ; vs.  $4 \pm 2$  at 25°C,  $n = 8$ ), belying the notion that 30°C leads to diminished vigor of this double-transgenic type. Typical CIs for WT male pairs are  $\leq 4$  (12–14). When the spatial expression of *C309* was squeezed down by the effect of *Cha-gal80*, 30°C courtships performed by the triply transgenic males were still reasonably robust (CI,  $10 \pm 1$ ;  $n = 11$ ), compared, for example, with the behavior *UAS-shi<sup>TS</sup>* controls at 30°C (CI,  $2 \pm 0$ ;  $n = 12$ ) or *C309/Cha-gal80* controls at 25°C (CI,  $2 \pm 1$ ;  $n = 14$ ).

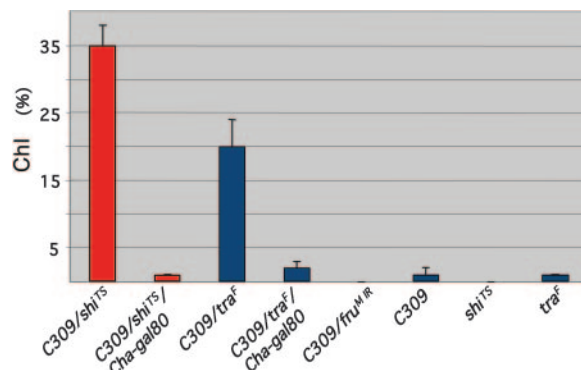
Wing extensions measured for *C309/UAS-shi<sup>TS</sup>* males (Fig. 1B) plummeted relative to the overall courtship decrement (Fig. 1C), similar to the effects on WEI values of the *fru<sup>3</sup>* or *fru<sup>4</sup>* mutation (12). These *fruitless* mutants are not generally impaired in wing usage, because their flight performances were normal (12); however, heated *C309/UAS-shi<sup>TS</sup>* males flew subnormally. Whereas 44% of singly transgenic *UAS-shi<sup>TS</sup>* control males were adhered to the top 5 cm of the cylindrical test apparatus (12) and 25% were adhered to the bottom 30 cm, the corresponding values for *C309/UAS-shi<sup>TS</sup>* at 30°C males were 3% and 86%.

Courtship-song recordings of heated *XY/C309/UAS-shi<sup>TS</sup>* flies revealed them to generate almost no tone pulses; at 25°C, all males of this type sang vigorously with acoustical parameters in the normal range (Table 3). Taking into account the  $\approx 9$ -fold reduction in wing extension for *XY/C309/UAS-shi<sup>TS</sup>* at 30°C relative to low-temperature controls (Fig. 1B) and the rate of song-pulse production for flies of this type at 25°C, one expects  $\approx 18$  pulses per minute if heat treatment would not impinge on singing. However, the 15 *shi<sup>TS</sup>*-affected males recorded at 30°C generated an average of only one pulse every 2 min (Table 3).

In context of nonmating being a key component of the *fruitless* syndrome (e.g., refs. 9 and 12), we found that only 17% of heated *C309/UAS-shi<sup>TS</sup>* males copulated; nearly all of them mated at permissive temperature (Table 4). Augmenting the current mating tests (18) showed that heated *C309/UAS-shi<sup>TS</sup>* males who copulated were not significantly different from controls in terms of mating-initiation latencies (Table 4), but quantification of attempted copulation revealed them to be substantially subnormal (Table 5). If *C309/UAS-shi<sup>TS</sup>* males performed this abdominal bending, it did not presage mating success, indicating that this transgenic type exhibits a monotonically stochastic decrease in the probability of proceeding from early, to middle, to late stages of the courtship sequence (also see Table 1).

The *fruitless*-like behaviors exhibited by *C309/UAS-shi<sup>TS</sup>* males could be a coincidence, prompting us to demasculinize *C309*-expressing neurons. We first combined *C309* with *UAS-tra<sup>F</sup>*, which encodes the TRA protein that blocks generation of FRU<sup>M</sup> in WT females (see Introduction). Despite *C309* being advertised as a predominantly mushroom body (MB) enhancer trap (19, 20), it is notable that *XY/C309/UAS-tra<sup>F</sup>* flies are largely transformed into external females (Fig. 5), one indication of *C309* being expressed well beyond the MBs in the adult CNS (also see refs. 18 and 30).

Behavioral analysis of this transgene combination showed that *XY* versions of *tra<sup>F</sup>*-affected flies courted females with no significant decrement (Table 1) and also sang normally (Table 3). However, in male-pair tests, *XY/C309/UAS-tra<sup>F</sup>* flies courted WT males at *fru*-like levels (Table 1). These double-transgenic flies also elicited anomalously high levels of courtship from WT males (Table 1). Thus, might *XY/C309/UAS-tra<sup>F</sup>* flies



**Fig. 2.** Courtship chaining. For *C309/shi<sup>TS</sup>*, 16 groups (8–10 males each) were observed; for *C309/shi<sup>TS</sup>/Cha-gal80*,  $n = 8$ . Grouped males were first measured at 25°C for ChI determinations (12, 13); these values were all 0. Fly containers were then shifted 30°C and had further ChIs determined. The *C309/tra<sup>F</sup>* ( $n = 16$ ) *C309/UAS-fru<sup>MIR</sup>* ( $n = 8$ ) types and accompanying single-transgenic controls were always at 25°C. Additional observations of *C309/UAS-fru<sup>MIR</sup>* males were made after these flies had been reared at 29°C; the average ChI was 0 ( $n = 8$  groups). One-way ANOVA on arcsine-transformed ChI values, with GENOTYPE as the main effect, revealed differences among groups [ $F(6,82) = 68.14$ ,  $P < 0.0001$ ]. Subsequent pairwise comparisons showed that *C309/shi<sup>TS</sup>* at 30°C chained significantly more than *C309/tra<sup>F</sup>* males ( $P < 0.05$ ); both types exhibited much higher levels of chaining than the single-transgenic controls ( $P < 0.05$ ). Although *UAS-tra<sup>F</sup>* males ( $n = 10$  groups) showed very brief moments of chaining, their behavior was not significantly different from that of *UAS-shi<sup>TS</sup>* ( $n = 17$ ) or *C309* ( $n = 11$ ) control groups. The behavior of *C309/fru<sup>MIR</sup>* males was statistically equivalent to those exhibited by singly transgenic control males; the very low ChI values for groups of *C309/tra<sup>F</sup>/gal80* ( $n = 9$ ) or *C309/shi<sup>TS</sup>/gal80* males were not significantly different from the behavior of control males.

court other males in part because of self-stimulation? Owing to the fact that mutations at the *fruitless* locus do not cause courtship elicitation (12, 31), we disrupted FRU<sup>M</sup> production other than via manipulation of *tra* expression by combining *C309* with *UAS-fru<sup>MIR</sup>*, surmising that this IR construct would do the job neurally but leave such flies in basically male state externally. These *C309/UAS-fru<sup>MIR</sup>* males exhibited marginal courtship decrements toward females (Table 1) but a significant reduction to 60% of the control value when *C309/UAS-fru<sup>MIR</sup>* males had been reared at 29°C (Table 1). However, there was no consequence to singing behavior of combining *C309* with *UAS-fru<sup>MIR</sup>* (Table 3). The effects of *fru<sup>MIR</sup>* included a modest intrusion on mating performance after 25°C rearing and more substantial abnormalities for latency and copulation values after rearing this double-transgenic fly at 29°C (Table 4). Regarding intermale courtship, an individual *XY* fly in which *fru<sup>MIR</sup>* was putatively knocking down FRU<sup>M</sup> expression courted his WT male partner as vigorously as did the *XY/C309/UAS-tra<sup>F</sup>* flies, but the effects of *C309*-driven *fru<sup>MIR</sup>* did not include anomalous elicitation (Table 1).

When the spatial extent of *C309*-encoded *gal4* was again reduced by the effects of *Cha-gal80*, triply transgenic males carrying *UAS-tra<sup>F</sup>* interacted with females normally but courted other males at an anomalously elevated level (Table 1). *XY/C309/UAS-tra<sup>F</sup>/Cha-gal80* flies are feminized externally (see Fig. 5) and elicited about the same level of WT-performed courtship as did the doubly transgenic *XY/C309/UAS-tra<sup>F</sup>* type (Table 1). When *Cha-gal80* was added to the *C309/UAS-fru<sup>MIR</sup>* combination, these males exhibited higher levels of courtship toward WT males than did singly transgenic controls; however, the former CI ( $11 \pm 1$ ,  $n = 18$ ) was only about three times the corresponding “baseline” values (*Cha-gal80*, CI  $4 \pm 1$ ,  $n = 11$ ; *UAS-fru<sup>MIR</sup>*,  $3 \pm 1$ ,  $n = 9$ ), compared with the  $\approx 8$ -fold enhancement caused by the *C309/UAS-fru<sup>MIR</sup>* combination (Table 1).





**Table 2. FRU<sup>M</sup>-containing neuronal groups overlapping with expression of the C309 enhancer trap**

Location	FRU <sup>M</sup> neuronal group, cluster number	Cluster name	Description of CNS region (numbers of adult FRU <sup>M</sup> neurons per hemi-brain or-VNC)	FRU <sup>M</sup> cells overlapping with C309, % (n)	<i>tra</i> <sup>F</sup> -affected FRU <sup>M</sup> cells overlapping with C309, % (n)	<i>fru</i> <sup>MIR</sup> -affected FRU <sup>M</sup> cells overlapping with C309 at 25°C/29°C, % (n)
Brain	2	<i>fru</i> -aSP2	Anterior portion of superior protocerebrum (50 ± 2, n = 7)	25 (7)	2 (6)	15 (9)/10 (5)
	5	<i>fru</i> -mAL	Medially located, just above antennal lobe (34 ± 1, n = 9)	21 (7)	2 (6)	11 (9)/6 (8)
	7	<i>fru</i> -mcAL	Anterior to mechanosensory neuropile of antennal lobe, near esophagus (36 ± 1, n = 12)	26 (9)	2 (7)	24 (8)/16 (8)
	8	<i>fru</i> -SG	Within subesophageal ganglion (10 ± 1, n = 6)	23 (5)	3 (7)	24 (3)/18 (4)
	13	<i>fru</i> -pSP2	Posterior portion of superior protocerebrum, near mushroom-body (MB) calyx (20 ± 1, n = 5)	47 (5)	7 (6)	52 (5)/30 (5)
	14	<i>fru</i> -P	Broadly scattered in posterior brain, ventral to MB calyx (78 ± 3, n = 5)	31 (5)	3 (7)	29 (5)/28 (4)
VNC	16	<i>fru</i> -Pr	Prothoracic ganglion (29 ± 1, n = 7)	14 (13)	4 (6)	11 (4)/9 (7)
	17	<i>fru</i> -PrMs	Between pro- and mesothoracic ganglion (83 ± 2, n = 8)	31 (9)	4 (6)	35 (7)/29 (6)
	18	<i>fru</i> -MsMt	Between meso- and metathoracic ganglion (37 ± 1, n = 6)	30 (5)	7 (4)	28 (6)/28 (4)
	20	<i>fru</i> -Ab	Within abdominal ganglion (99 ± 5, n = 4)	23 (5)	3 (7)	16 (8)/16 (4)

The second and third columns list FRU<sup>M</sup> cluster numbers, each followed by the name of a given such neuronal group (compare ref. 16) within the brain or ventral nerve cord (VNC). The fourth column includes novel counts of adult neurons, determined by application of anti-FRU<sup>M</sup> to CNSs of wild-type (WT) males (average numbers of immunoreactive cells per hemi-CNS region, ± SEM; n, number of specimens scored). An additional series of C309/UAS-*tra*<sup>F</sup> CNS specimens had anti-FRU<sup>M</sup> applied for single-labeling; numbers of immunoreactive neurons for the 10 clusters led to an average of 62%, comparing each *tra*<sup>F</sup>-affected count to the corresponding WT male value (the smaller percentages yielding the 62% average ranged from 42 to 86, n values for these C309/UAS-*tra*<sup>F</sup> specimens ranged from 6 to 9). Right three columns: percentages for C309/*fru* coexpression, computed as described in *Materials and Methods*. Control percentage overlaps (per CNS specimen, whose n values are in parentheses), fifth column. Experimental transgenic coexpression percentages: XY/C309/UAS-*egfp*/UAS-*tra*<sup>F</sup>, sixth column; C309/UAS-*egfp*/UAS-*fru*<sup>MIR</sup>, seventh column, based on data collected separately from males reared at 25°C or 29°C. A one-way ANOVA on (on arcsine-transformed percentages) and subsequent pairwise comparisons revealed significant *tra*<sup>F</sup>-induced overlap reductions (compared with C309/*egfp* values) for each cluster (P values < 0.05); for C309/*fru*<sup>MIR</sup> at 25°C, coexpression reductions were significant only for clusters 2, 5, and 20; at 29°C, *fru*<sup>MIR</sup> caused significantly lower coexpression for clusters 2, 5, 7, 13, and 16.

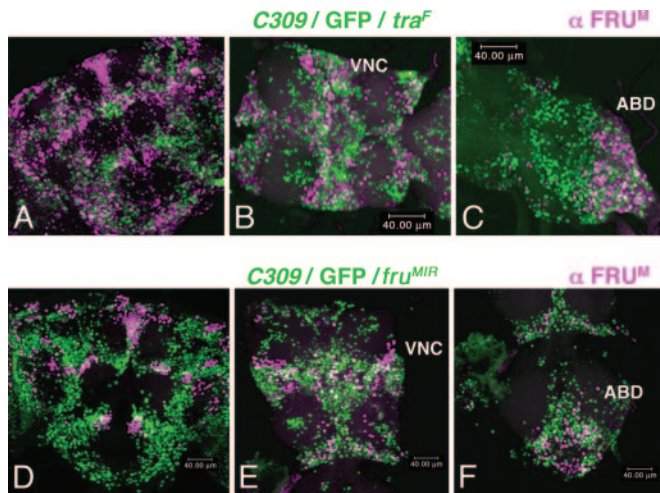
*tra*<sup>F</sup> within clusters 13 and 14. Perhaps the relevant “overlap percentages” would have had to drop from 47 and 31 to 0 for both of these clusters (Table 2) if a *tra*<sup>F</sup>-affected courtship decrement were to be realized. An alternative to viewing this matter in the context of C309/FRU<sup>M</sup> coexpression is that certain neurons in which this *gal4* driver is active could be anatomically downstream of the *fru*-expressing brain cells that influence a male’s ability to initiate and sustain courtship of a female.

This conception is relevant to the striking elimination of courtship song in recordings of C309/UAS-*shi*<sup>TS</sup> males. Once again, UAS-*tra*<sup>F</sup> had no such effect (Table 3). Neurogenetic findings pertinent to this matter are that C309 is expressed in imaginal thoracic ganglia (Fig. 3 and Table 2); *fru* is “song-involved” (12, 37); this gene makes its products within several regions of the ventral nerve cord (16, 17, 32–34), coexpressing C309 within most of them (Table 2); and genetic maleness within *Drosophila*’s VNC has been implicated in song control (reviewed in ref. 35). Thus, turning off synaptic transmission emanating from one or more subsets of the C309/UAS-*shi*<sup>TS</sup> neurons in the thoracic ganglia could be the etiology of heat-induced songlessness exhibited by these doubly transgenic males. Regarding the absence of a *tra*<sup>F</sup> effect, what if C309 was not expressed in any FRU<sup>M</sup>-containing song-relevant neurons during metamorphosis? In other words, C309 expression in VNC neurons underlying song control could be activated late in the life cycle, allowing for the *shi*<sup>TS</sup> effect to take hold after adult males are heated; however, the progenitors of such cells might not express C309 during an earlier “feminization-relevant” stage, so that post-

metamorphic activation of *tra*<sup>F</sup> would occur too late to affect singing ability. However, we found substantial coexpression of FRU<sup>M</sup> and C309 within the pupal VNC (Fig. 6). In this respect, we submit that assessing the C309’s expression pattern throughout the life cycle is a valuable object lesson as to what must be done properly to interpret the biological effects of a given enhancer trap.

As to the divergent effects C309-driven *shi*<sup>TS</sup> vs. *tra*<sup>F</sup>, recall that the former factor seems broadly to impinge on VNC functioning, in that the fly’s general ability to vibrate its wings is shut down by the synaptic disruptor; in contrast, songless *fruitless* mutants fly normally (12). Thus, consider a scenario in which C309 neurons would include those that mediate wing vibrations during flight, and that this transgene is expressed in separate VNC cells hypothetically dedicated to such vibrations during courtship. Therefore, we speculate that the expression domain of C309 includes inter- or motor-neurons functioning within and downstream of a “command center” for flight as well as neurons located in relatively distal regions of a separate anatomical pathway. The latter would originate where *fru*-expressing cells exert the gene’s crucial regulation of courtship song.

Turning to anomalous courtship interactions among males, our focus shifts back to the brain: FRU<sup>M</sup> clusters 5 and 7, where *fru*<sup>1</sup> causes an apparent absence of this protein; this mutation minimally affects the gene’s expression in other brain regions (15). It is notable that the C309/UAS-*tra*<sup>F</sup> combination knocked down driver/FRU<sup>M</sup> coexpression to ≈10% normal in cluster 5 (Table 2). Cluster 7 was similarly affected, but special attention



**Fig. 4.** Diminished *fruitless* expression in the CNS caused by a feminizing or a demasculinizing transgene. Double labeling of CNS adult male tissue from *C309/UAS-egfp/UAS-tra<sup>F</sup>* (A–C) and 25°C-reared *C309/UAS-egfp/UAS-fru<sup>MIR</sup>* (D–F). (A) Anterior view of *tra<sup>F</sup>*-affected brain (magnification,  $\times 40$ ), showing marked reductions of coexpressing white cells within clusters 2, 5, 7, and 8, compared with the roughly corresponding images in Fig. 3; similar degrees of reduction appeared within: Pro-, meso-, and metathoracic VNC regions (B, displayed from left to right, ventral views at  $\times 52$  magnification), which contain FRU<sup>M</sup> clusters 16, 17, and 18 (Table 2). (C) Abdominal ganglion, containing VNC cluster 20 (at  $\times 52$  magnification). (D) Anterior view of *fru<sup>MIR</sup>*-affected brain at  $\times 40$  magnification; the reduction of coexpressing cells is not as dramatic as in A–C, as was the case for thoracic ganglia (E,  $\times 40$  magnification) and the abdominal ganglion (F,  $\times 40$  magnification).

should be paid to cluster 5 (see below). One reason that that group was surmised to be the etiology of frantic courtship among *fru<sup>1</sup>* males (15) is that cluster 5 is located near the antennal lobes; and transgenically mediated feminization of a brain region near these structures induces intermale courtships (ref. 3, although none of the *gal4* drivers in that study included *C309*). Therefore, if proper male-specific structure or function of cluster 5 is involved in normal sex recognition, the mutation's demasculinizing effect on this brain region, or transgene-effected feminization of it, could cause this aspect of courtship to break down.

Elements of the current findings (and those in ref. 18) suggest that abnormal formation of the brain region in question is not necessary for it to mediate anomalous interactions between males (also see refs. 33 and 34); this is because deactivating synaptic transmission in cluster 5 after CNS development completed itself in a male manner is sufficient to induce intermale courtship. Perhaps this behavioral effect of driving UAS-*shi<sup>TS</sup>* involves removal of inhibitory neurotransmission relevant to the functioning of this brain region (but see ref. 7), which in normal males would block their wherewithal to sustain courtship between males. Therefore, the *fru<sup>1</sup>* effect on cluster 5 and that of driving TRA production in this region might not involve the formation of a sex recognition center (such that a hypothetical circuit involved in inhibiting intermale courtship is not present or miswired), but instead the intracellular quality and function of neurons in the mature brain.

Considering further that certain cluster 5 neurons comprise the subset of FRU<sup>M</sup>'s spatial domain for *shi<sup>TS</sup>*- or *tra<sup>F</sup>*-induced intermale courtship, the relevant cells would be those in which both *fruitless* and *C309* generate their gene products (20% of the 35 neurons within this group; Table 2). One problem with this supposition is that *C309/UAS-tra<sup>F</sup>* flies elicit fairly high levels of courtship (Table 1). Thus, groups of *C309/UAS-tra<sup>F</sup>* males may form chains for reasons extending beyond a given fly's "motivation" inappropriately to court another male: The extent to

which a *C309/UAS-tra<sup>F</sup>* fly is feminized (Fig. 5) could include self-stimulation that might contribute to intermale courting. However, recall the case of *C309/UAS-tra<sup>F</sup>/Cha-gal80* males, a transgenic type that is similarly feminine externally and elicits courtship (Table 1). The diminished extent to which *C309's gal4* is effective when combined with *Cha-gal80* led to weakened homosexual courtship in single-pair tests (Table 1) and dramatically reduced chaining behavior (Fig. 2), although there was essentially no effect of *Cha-gal80* on the basic courtship ability of these triply transgenic males. Thus, the effects of this "neurons-only" manipulation suggest that hypothetical self-stimulation, which did not cause *C309/UAS-tra<sup>F</sup>/Cha-gal80* males vigorously to court other ones, is minimally operating to induce the homosexual courtships performed by *XY/C309/UAS-tra<sup>F</sup>* flies. Males carrying *C309* and UAS-*fru<sup>MIR</sup>* are also not feminized externally; however, they courted other males robustly in single-pair tests, an effect that was diminished by adding *Cha-gal80* (Table 1). Therefore, we surmise that flies carrying a given *fruitless*-affecting transgene (Table 2) exhibit intermale courtship because the relevant CNS neurons are demasculinized.

However, what about neural structures not analyzed in the current study that could be involved in the behavioral effects of *C309* driving either *tra<sup>F</sup>* or *fru<sup>MIR</sup>*? Thus, consider that TRA affects the primary transcript emanating from the *doublesex* (*dsx*) gene (reviewed in ref. 8) and that *dsx* null mutations cause *XY* flies to exhibit modest levels of intermale courtships (38). *C309* driving of *tra<sup>F</sup>* could lead to the female (F) form of DSX (thus, no DSX<sup>M</sup>, as in *dsx<sup>-</sup>*) within brain cells connected to sex recognition other than those we analyzed. Indeed, *dsx<sup>+</sup>* is expressed in the brain (39); however, the functional significance of these cells is unknown, let alone whether any of them also express *fru<sup>+</sup>*. In this regard, it was important to home in on disruption of *fruitless*'s CNS expression alone by combining *C309* with the UAS-*fru<sup>MIR</sup>* transgenes; this was sufficient to induce courtship between a given pair of doubly transgenic males (Table 1) but led to no chaining (Fig. 2). Thus, anomalously high levels of courtship between two males has been disconnected from courtship chaining. [The same disconnect between these different kinds of intermale courtship occurred when *Cha-gal80* was added to the *C309/UAS-tra<sup>F</sup>* combination (Table 1 vs. Fig. 2).] It is as if the broad neural effects of a genetic abnormality such as a *fruitless* mutation, or combining *C309* with UAS-*tra<sup>F</sup>*, is necessary to cause sustained courtship among several variant males; however, if the impingement on *fru<sup>+</sup>* expression is more limited (right column of Table 2), only courtship between a pair of males can occur.

In this regard, the *C309/UAS-fru<sup>MIR</sup>* flies were substantially less affected in terms of numbers of brain neurons within which FRU<sup>M</sup> became undetectable, compared with the effect of the same driver combined with UAS-*tra<sup>F</sup>* (Table 2). This brings us to the matter of additional neurons that are potentially relevant to courtship and should be analyzed in context of the *C309* effects. Here, we refer to the many PNS cells recently discovered to express *fruitless* in external sensory structures (33, 34). It is unknown whether any of these neurons coexpress *C309*, such that sensory inputs relevant to courtship may have been impinged upon by combining that transgene with UAS-*shi<sup>TS</sup>* or with the sex-affecting transgenes. However, *fru<sup>+</sup>* expression in external appendages is not required for a fly to recognize, follow, and perform wing extension at a female: when these structures are genetically female in certain gynandromorphs, maleness within the brain is sufficient to trigger mosaic-with-female courtship (1, 36).

The relative inefficacy of one of the *C309*-driven transgenes just alluded to warrants further comment: The investigators who generated the *fru* IR construct reported that a *gal4* driver, when combined with two doses of UAS-*fru<sup>MIR</sup>*, knocked out detectability FRU<sup>M</sup> throughout brain cluster 7, against a backdrop of all neurons

within this group coexpressing *fru*<sup>+</sup> within the adult male's brain (22). The *C309* driver, which may be weaker than that applied by Manoli and Baker (22), leads to overlapping expression with FRU<sup>M</sup> in only a subset of cluster 7 neurons. The *C309/UAS-tra<sup>F</sup>* combination dropped the relevant coexpression percentage 13-fold (Table 2), but such a decrement did not lead to inappropriately rapid courtships and merging of courtship steps: the behavioral anomalies reported by Manoli and Baker appear to be caused by knocking out FRU<sup>M</sup> in all cluster 7 neurons by combining their driver with either *UAS-tra<sup>F</sup>* or *UAS-fru<sup>MIR</sup>*. Importantly, "cluster 7 FRU<sup>M</sup>-null" males did not court other males (22).

The current study aimed to delve into various regions of the male CNS in which the *fruitless* gene is expressed: Do certain subsets of the spatial pattern govern a male's ability to perform a discrete feature of the reproductive sequence? Using the *gal4*-containing *C309* enhancer trap was valuable, because it leads to impersonations of certain *fru*-mutant behaviors when this driver is combined with a *shi<sup>TS</sup>*-containing factor that broadly disrupts neural functioning. By limiting *C309*'s efficacy to disrupt by causing it to drive sex-related transgenes succeeded in provisionally partitioning *fru*-related "sex recognition" neurons to a subset of the normal brain pattern. By subtraction, the partitioning was further delimited by knocking out the driver's efficacy in a subset *C309*'s spatial domain: adding a neurally driven *gal80* transgene that substantially (Table 1) or completely (Fig. 2) attenuated anomalous intermale courtships. A pleasant surprise occurred when the *C309/UAS-fru<sup>MIR</sup>* combination was found not to mimic the effects on courtship among males of combining the driver with *UAS-tra<sup>F</sup>*. Thus, the broader pattern of FRU<sup>M</sup> expression, unaffected by the IR compared with the substantial decrement caused by *tra<sup>F</sup>*, takes the analysis a further step. For example, we have begun to tease out the manner by which

*fru* mutations and related factors influence courtship between two males, as opposed to the much more complicated behavioral dynamics that can occur in a group of such *Drosophila*.

However, inferences about the potentially relevant subsets of a given brain cluster do not approach specifically identifiable neurons. For this, it will be necessary to do more than quantify the cells in which a transgene driver and *fruitless* are coexpressed. Further brain-behavioral dissections will require assessing the differential connectivity patterns defining a given class of FRU<sup>M</sup> neurons, along with variations of cellular content that are likely to discriminate one category of such neurons from another. The relevant object lessons stem from analyses of, so far, only the posterior-most component of *fruitless*'s expression domain in the male CNS: partitioning certain abdominal-ganglion neurons that differentially connect with either a male-specific muscle (17, 32) or with internal reproductive organs (29, 32), and discovering that the latter type of FRU<sup>M</sup> cells uniquely contain serotonin (15, 29). Neurons containing another neurotransmitter, acetylcholine, are on point; but not all of the *C309* effects can be ascribed to neurons affected by *Cha-gal80*, because (in contrast to ref. 18) we found certain courtship defects to remain when analyzing males that carried this transgene along with *C309* and *UAS-shi<sup>TS</sup>*. This finding reinforces that notion that additional neuronal qualities must be uncovered with regard to cells expressing this enhancer-trap, the *fruitless* gene, or both.

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