A Gene Necessary for Normal Male Courtship, *yellow*, Acts Downstream of *fruitless* in the *Drosophila melanogaster* Larval Brain

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ABSTRACT: **The *fruitless* (*fru*) gene is a member of the *Drosophila melanogaster* somatic sex determination genetic pathway. Although it has been hypothesized that the primary function of *fru* is to regulate a genetic hierarchy specifying development of adult male courtship behavior, genes acting downstream of *fru* have not yet been identified. Here we demonstrate that the *yellow* (*y*) gene is genetically downstream of *fru* in the 3rd-instar larval brain. Yellow protein is present at elevated levels in neuroblasts, which also show expression of male-specific FRU proteins, compared to control neuroblasts without FRU. A location for *y* downstream of *fru* in a genetic pathway was experimentally demonstrated by analysis of *fru* mutants lacking transcription of zinc-finger DNA binding domains, and of animals with temporal, spatial, or sexual mis-expression of male-specific FRU. A subset of *fru* and *y* mutants is known to reduce levels of a specific behavioral component of the male courtship ritual, wing extension, and FRU and Yellow were detected in the general region of the brain whose maleness is necessary for development of that behavior. We therefore hypothesized that ectopic expression of Yellow in the 3rd-instar brain, in a *y* null background, would rescue low levels of wing extension and male competitive mating success, and this was found to be the case. Overall, these data suggest that *y* is a downstream member of the *fru* branch of the *D. melanogaster* sex determination hierarchy, where it plays a currently unknown role in the development of adult male wing extension during courtship.** © 2003 Wiley Periodicals, Inc. J Neurobiol 55: 53–72, 2003

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INTRODUCTION

Courtship behavior is central to the divergence and diversity of animal species and is of obvious adaptive significance. Arguably, the two species in which the molecular genetic and cellular mechanisms underlying courtship behavior are best characterized are the fruit fly *Drosophila melanogaster* and the nematode worm *Caenorhabditis elegans* (Goodwin, 1999). Our knowledge of the developmental genetic basis of

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courtship behavior, or for that matter any animal instinct, is meager in comparison to better-studied morphological traits, however.

D. melanogaster has a well-characterized male courtship ritual consisting of six steps: following, orienting, horizontal wing extension, wing vibration ("singing"), genital licking, and attempted copulation (Bastock and Manning, 1955; Bastock, 1967; Hall et al., 1982; Hall, 1994a; Yamamoto et al., 1997; Greenspan and Ferveur, 2000). Many genes have been identified that, when mutated, disrupt specific aspects of the male courtship ritual (Hall et al., 1982; Hall, 1994a; Greenspan, 1997; Yamamoto et al., 1997, 1998; Goodwin, 1999; Yamamoto and Nakano, 1998, 1999; Orgad et al., 2000; Gaines et al., 2000; Greenspan and Ferveur, 2000; Sokolowski, 2001).

Although these "male courtship genes" are good candidate genes for the development of behavior, their developmental function, if any, remains to be demonstrated experimentally. Currently, no experimentally determined genetic or molecular pathway exists that connects these genes to each other in relation to the development of *D. melanogaster* male courtship behavior. Furthermore, we do not know how such a pathway or gene network would be structured, or how it would operate at a molecular or cellular level.

Studies of the *fruitless* (*fru*) gene have arguably shed the most light on the developmental genetics of the *D. melanogaster* male courtship ritual. The first *fru* mutant characterized, fru^1, had an unusual sexual behavior phenotype: male-male courtship and "chaining" behavior (Gill, 1965; Hall, 1978). Since then, behavior genetic studies of numerous *fru* alleles have demonstrated that *fru* function is necessary for most aspects of the male courtship ritual, including wing extension (Ryner et al., 1996; Villella et al., 1997). The *fru* gene has been cloned and examined at the nucleotide level by two research groups (Ito et al., 1996; Ryner et al., 1996), who have each found it to encode a predicted BTB-ZF (*Bric-a-brac*, *Tramtrack* and *Broad Complex*-like Zinc-Finger)-family transcription factor, and *fru* was later shown to be a downstream member of the *D. melanogaster* somatic sex determination hierarchy (Heinrichs et al., 1998), "legitimizing" its theorized role in sexual differentiation in the form of male courtship behavior.

The organization and functions of the about 140 kb *fru* locus are complex by *D. melanogaster* standards: It has at least four promoters, it codes for male-specific, female-specific, and sex-nonspecific transcripts, which are selectively translated, and the 3′ region of *fru* encoding the DNA-binding zinc fingers is alternatively spliced (Ito et al., 1996; Ryner et al., 1996; Usui-Aoki et al., 2000; Anand et al., 2001). Recently (Lee et al., 2000; Lee and Hall, 2001), the use of an antibody specific to male-specific *fru* protein isoforms (henceforth called FRU^M) has demonstrated the spatial and temporal distribution of FRU^M in the CNS to be consistent with both the timing of the critical period for programming maleness (Belote and Baker, 1987; Arthur et al., 1998) and, roughly, the regions of the CNS whose maleness is necessary for normal development of male courtship behaviors (Hall, 1979; von Schlicher and Hall, 1979).

The available evidence suggests that *fru* is the ultimate regulator of a developmental genetic hierarchy responsible for many aspects of the male courtship ritual in *D. melanogaster* (reviewed in Baker et al., 2001; Greenspan and Ferveur, 2001; Sokolowski, 2001). The looming genetic question now is, what genes are downstream of *fru*, or more specifically, FRU^M zinc finger transcription factors, and how and where do these downstream genes work together during development to build the neural circuitry underlying adult behavior (Drapeau, 2001a)?

We recently reported the immunohistochemical colocalization of FRU^M and the protein product of the *yellow* (*y*) gene, Yellow (Radovic et al., 2002). In the dorsal posterior male 3rd-instar larval brain, Yellow was present at very high levels in and around neuroblasts expressing FRU^M, compared to neuroblasts in the same brains not exhibiting anti-FRU^M staining (Radovic et al., 2002). This association between FRU^M and Yellow is particularly significant, because mutations in *y* have long been known to disrupt male mating success under a number of experimental conditions (e.g., Sturtevant, 1915; Bastock, 1956; Threlkeld et al., 1974; Dow, 1975; Wilson et al., 1976; M.D. Drapeau and A.D. Long, unpublished observations). More specifically, null mutants of *y* disrupt a specific male courtship behavior, called wing extension, in which the male horizontally extends his wing 90° (Bastock, 1956; Burnet et al., 1973; M.D. Drapeau and A.D. Long, unpublished data). Some *fru* mutants severely disrupt wing extension (Villella et al., 1997).

From these data and from circumstantial behavior-genetic evidence, we hypothesized that *y* is downstream of *fru* in the somatic sexual differentiation genetic pathway. Furthermore, we hypothesized that *y* expression is regulated in a sex-specific manner by FRU^M (possibly with intermediates or cofactors) in the CNS during development. These hypotheses fol-

low from the following specific experimental observations:

1. Courtship wing extension is sex-limited to males; that is, *D. melanogaster* females are not known to exhibit 90° wing extension behavior in any context.
2. *fru* mutant males show multiple severely disrupted aspects of the courtship ritual (Villella et al., 1997), while *y* mutant males have only wing extension replicably disrupted (Bastock, 1956; Burnet et al., 1973; M.D. Drapeau and A.D. Long, unpublished data).
3. *fru* mutants reducing wing extension reduce it to approximately <5% of its wild-type level (Ryner et al., 1996; Villella et al., 1997), while *y* mutants reducing wing extension reduce it to only roughly 50% of its normal level (Bastock, 1956; Burnet et al., 1973; M.D. Drapeau and A.D. Long, unpublished data).
4. *y* is most likely not *upstream* of *fru*, because the genes upstream of *fru* in the sex determination hierarchy are well-characterized (reviewed in Hall, 1994a; Cline and Meyer, 1996), and *y* does not appear to be a transcription factor of known type (Flybase, 1999; Drapeau, 2001b; Wittkopp et al., 2002a; M.D. Drapeau, unpublished data).

In this study, we analyze patterns of Yellow in the brain in adult flies carrying *fru* mutations with known behavioral and molecular phenotypes, or in adult flies with mis-expressed FRU^{M} via the GAL4/UAS system, in order to demonstrate that *y* is downstream of *fru* in the 3rd-instar developing brain. Furthermore, we present behavioral evidence that *y* expression in the 3rd-instar brain is sufficient for normal levels of wing extension during the male courtship ritual. These data suggest that *y* plays a developmental role in the 3rd-instar CNS, leading to an increased rate of wing extension in adult males during courtship.

MATERIALS AND METHODS

Fly Stocks, Crosses, and GAL4/UAS Mis-Expression

We used *Df(1)y-ac*^{22}, a complete *y* deletion (obtained from the Umea *Drosophila* Stock Center, Sweden), and *y*^{1}, a mis-sense point mutation (obtained from the Bloomington *Drosophila* Stock Center, Bloomington, IN), as null mutants of the *y* locus, and Oregon-R as a standard inbred wild-type strain. *fru* mutant stocks *fru*^{1}, *fru*^{3}, and *fru*^{4} were originally obtained over the *MKRS* balancer (obtained from Ralph Greenspan and Herman Dierich, The Neurosciences Institute, La Jolla, CA). We crossed the *fru* alleles into a genetic background containing the balancer chromosome *TM6B* and a dominant-stage mutant larval marker, *Tubby*^{1} (*Tb*^{1}). In our experiments, *fru/fru* homozygotes were selected at the 3rd-instar larval stage as those that were non-*Tb*^{1}. All of the above mutants and balancers are described in Lindsley and Zimm (1992) and/or online at Flybase (1999). We note the *fru* mutant strains that we used are the same as those used by Lee and Hall (2000) to study male aggressive behavior, and that as in that study, no effort was made to backcross the above mutant stocks into a common genetic background. Previous to their experiments, Lee and Hall (2000) outcrossed the *fru* mutant strains to other strains, and then re-extracted the mutant alleles, so as to avoid inbreeding effects on behavior.

To mis-express *fru*$^{+}$ in the developing CNS, the widely used GAL4/UAS mis-expression system was employed (Brand and Perrimon, 1993). Here, a *fru* transcript was put under control of yeast UAS (Upstream Activating Sequence), which is activated by the yeast GAL4 protein. In turn, in the same fly, GAL4 is expressed under the control of different *D. melanogaster* regulatory sequences, for instance, those only expressing in the CNS. UAS-*fru*$^{+}$ stocks were generously donated to us by K. Usui-Aoki and D. Yamamoto.

We used the pan-neural *elav*-GAL4 driver (Robinow and White, 1991; Kazantsev et al., 2002) to drive *fru*$^{+}$ transcripts from responding UAS-*fru*$^{+}$ cDNA transgenes. We used two distinct UAS-*fru* stocks. Each stock generates either the "AM" or the "BM" male-specific *fru* transcript (Usui-Aoki et al., 2000; Davis and Ito, 2001). Here, we name these two protein products $FRU^{M\{AM\}}$ and $FRU^{M\{BM\}}$, respectively, to indicate that they are male-specific FRU proteins ("FRU^{M}") of a specific isoform ("{AM}" or "{BM}"). *elav*-GAL4/UAS-*fru*$^{+}$ larvae were recovered from a cross between *w; P{w*$^{+}$*; elav*-GAL4*}/ CyO, P{w*$^{+}$; *Actin*-GFP*}* males (obtained from Larry Marsh and Rui Sutton de Sousa-Neves, UC-Irvine, Irvine, CA) and *w; P{w*$^{+}$*;* UAS-*fru*$^{+}$*}* females (Usui-Aoki et al., 2000) by selecting non-GFP-expressing larvae.

Larval Dissection and Antibody Staining

Second- and third-instar larvae were sexed based on presence of male genital discs, and their brains were dissected and processed as previously described (Hough et al., 1997; Parmentier et al., 2000). Primary anti-FRU antibodies were rat anti-FRU^{M}, rat anti-FRU^{COM} (1:300; obtained from Jeffery Hall and Troy Carlo, Brandeis University, Waltham, MA; see Lee et al., 2000; Lee and Hall, 2001; our Fig. 8), and guinea pig anti-DLG (1:1000). The anti-FRU^{M} antibody recognizes only male-specific FRU proteins, while the anti-FRU^{COM} antibody recognizes all FRU proteins ("COM" stands for "common").

We previously raised a polyclonal rabbit anti-Yellow antibody against all but the first seven amino acids of the Yellow protein (Radovic et al., 2002; Wittkopp et al.,

2002a), and it was used at a dilution of 1:150. Secondary Cy2, Cy3, or Cy5 conjugated antibodies were used (1:100; Jackson ImmunoResearch, Inc.). Images were taken using a BioRad MRC 1024 confocal microscope. Utilization of the *discs large* (DLG) neural cell membrane marker (Woods and Bryant, 1991) allowed visualization of different cell types within the brain, and DNA stained with propidium iodide was used to visualize chromosomes during different cell cycle stages. In combination, we were able to use DLG and propidium iodide to partially determine the subcellular localizations of FRU^{M}, FRU^{COM}, and Yellow.

Western Blotting

Third-instar larval heads were dissected in PBS 1X, placed in 100 m*M* Tris pH 7.5, 1% NP 40, homogenized, and centrifuged at 14,000 rpm for 15 min. The concentration of total protein in the soluble fraction was measured by the Bradford method. Twenty micrograms of total protein from each extract was separated on SDS-PAGE using 15% gradient Tris-HCl precast polyacrylamide gels (BioRad Life Sciences) and electroblotted to the Immobilon P transfer membrane (Immobilon). The membrane was then blotted in 5% skim milk blocking reagent in PBS-T, incubated overnight with rabbit anti-Yellow antibody (dilution 1:300), washed, and incubated with peroxidase-conjugated goat antirabbit secondary antibodies (1:5000; Jackson ImmunoResearch). Detection of Yellow protein was performed using the ECL Western Blotting analysis system (Amersham Life Sciences), followed by autoradiography. IgG was used as a loading control.

Courtship Behavior

We ectopically expressed the *y* gene in males null for the endogenous *y* locus in order to determine if CNS expression of *y* could reinstate wild-type levels of wing extension behavior during the male courtship ritual. Behavior assays were performed in the "Copulatron", a multichambered Plexiglas apparatus for observing insect courtship behaviors (Drapeau and Long, 2000). Flies were placed in the Copulatron using light and brief (15–30 s) CO_2 anesthesia about 24 h before the start of an assay. Males and females were separated by a piece of lightly lubricated plastic, and had fresh fly media placed in a small adjoining chamber to eat during this 24 h lag time (see details in Drapeau and Long, 2000). Single adult males 4 days posteclosion were videotaped in chambers with single adult inbred wild-type Oregon-R females, also 4 days posteclosion. Videotaping was carried out with a Hitachi CCD color video camera, a JVC VHS VCR, and a Hitachi Color Video Monitor.

We analyzed three male genotypes for the level of wing extension performed during courtship. In each case, we crossed virgin *y; UAS-y, Ser /UAS-y* females (Calleja et al., 1996) to another strain and collected males that were non-*Ser* (a dominant notched-wing phenotype). As described above for *fru*, the *y; UAS-y, Ser /UAS-y* strain allows the control of *y* expression by GAL4 constructs inserted in *trans*. Flies were crossed *en masse* in glass half-pint food bottles in groups of about 20 males and 20 females, and females were allowed to lay eggs. To engineer males to act as negative controls, we crossed the *y; UAS-y, Ser /UAS-y* females to wild-type males from an iso-female line derived from the "Ives" population (e.g., Ives, 1970). The resulting male progeny with genotype *y; Ives/UAS-y* had a *y* phenotype. We used two GAL4 drivers to direct *y* expression. The first, a "ubiquitous" driver, was obtained from the Bloomington Stock Center as stock #3954: y^1 *w; P{*w^+*; Actin5c-GAL4}/TM6B,* Tb^1. This *Actin5c-GAL4* driver served as a positive control: because we know that *y* expression in *some* tissue(s) is necessary for normal male wing extension and mating success, expression of *y* in virtually all tissues throughout development should rescue these phenotypes.

The second GAL4 driver we used has been reported to drive the expression of UAS transgenes throughout the 3rd-instar larval brain lobes in a "diffuse" pattern, and has been reported to *not* drive expression in the embryo, in egg chambers, or in larval imaginal discs (Manseau et al., 1997). This driver was obtained from the Bloomington Stock Center as stock #3738: w^{1118}*; P{*$w^{+mW.hs}$*=GawB}c81*, or *w; c81-GAL4*, as will be the shorthand used in this article. While we cannot absolutely rule out expression of *y* in tissues outside the 3rd-instar CNS in this experiment, it is sufficient as a first step in examining the function of Yellow in the 3rd-instar CNS that *c81-GAL4* elicits a relatively restricted pattern of UAS-regulated gene expression. Like the Ives strain, each of the GAL4 strains used were crossed to generate male progeny with a genotype of either y^1 *w; Act5c-GAL4/UAS-y* or y^1 *w; c81-GAL4/UAS-y*. All construct insertions are on the third chromosome.

We performed the experiment in three "blocks" on 3 days, with each genotype being tested on each day, in a standard "fully-crossed" analysis-of-variance (ANOVA) design. Each block consisted of flies collected from different bottles, to isolate any effects on behavior due to developmental/environmental conditions rather than genotype. Five males of each genotype were typed for their wing extension behavior in each block, giving a total of 15 individuals for each genotype. A standard two-way ANOVA was performed.

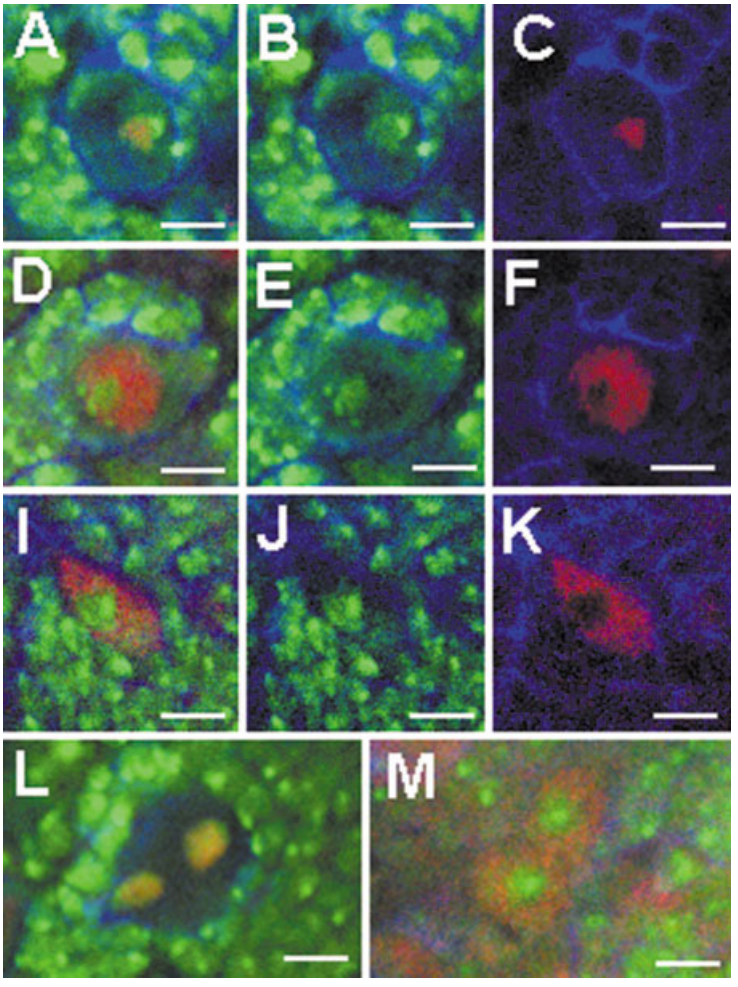
RESULTS

Localization of FRU Proteins in Neuronal Cells

Based on protein sequences, FRU proteins are predicted to function as zinc-finger transcription factors (e.g., Usui-Aoki et al., 2000). We wished to determine the subcellular localization of male-specific FRU, using anti-FRU^{M}, and the staining pattern of an antibody against a portion of FRU common to male-, female-, and sex-nonspecific proteins, anti-FRU^{COM}, in the brain. We detected anti-FRU^{M} staining in a small number of neuroblasts in the dorsal part of

wild-type *Drosophila* 3rd-instar larval brains, while anti-FRU^{COM} recognized FRU proteins in a larger number of neuroblasts, as well as in other cells in different areas of the brain, which we presume to be postmitotic neurons. This is generally consistent with previously published data (Lee et al., 2000; Usui-Aoki et al., 2000).

Our results are shown in Figure 1. In male telophase and early prophase neuroblasts, anti-FRU^{M} staining clearly overlaps with DNA staining [Fig. 1(A–C,L)]. However, in both neuroblasts and non-neuroblasts of females, anti-FRU^{COM} never substantially overlaps DNA staining [Fig. 1(D–K,M)]. These data suggest that, during these stages, male-specific



COLOR

Figure 1 Confocal images of subcellular localization of FRU^{M} (A–C, L) and FRU^{COM} (D–K, M) in wild-type 3rd-instar larval brains. Both FRU^{M} and FRU^{COM} are shown in red. DNA stained with propidium iodide (green) was used to visualize chromosomes during different cell cycle stages. DLG (blue) was used as a marker to outline neural cell membranes. (A–F) show neuroblasts in early prophase when chromosomes become visible. It is clear that anti-FRU^{M} staining overlaps DNA (orange color represents colocalization), while FRU^{COM} is largely in the nuclear matrix and appears to be mutually exclusive with DNA. (I–K) show a similar distribution of FRU^{COM} in a different cell type, possibly a postmitotic neuron. The easiest phase in which to visualize colocalization of FRU and DNA is during telophase/cytokinesis (L,M). In late prophase to early anaphase, staining with both anti-FRU antibodies is very pale, and neither seems to colocalize with DNA (data not shown). Male and female brains have a slightly different distribution of FRU^{COM} (Lee et al., 2000), but the subcellular localization and the cell types in which it is detected are indistinguishable. Here we show a female brain to avoid overlap of anti-FRU^{M} and anti-FRU^{COM} staining (see Fig. 8). Female brains were also stained with anti-FRU^{M} as control (not shown). All scale bars on this and other figures are 5 μ long.

FRU binds to DNA, while nonmale-specific FRU does not. Our finding of unequivocal nuclear localization of FRU^M is consistent with the fact that FRU proteins are predicted to be zinc-finger transcription factors. With the same reasoning, in the case of the nonmale-specific proteins recognized by anti-FRU^{COM}, we predict that in neuroblasts [Fig. 1(D–F,M)] such proteins are mainly localized in the nuclear matrix and not the cytoplasm. Indeed, the pattern of anti-FRU^{COM} staining resembles that of a nuclear marker in equivalent neuroblasts (Kazantsev et al., 2002, their Fig. 6).

Yellow Is Associated with Male-Specific FRU in the CNS

We hypothesized that *y* was downstream of *fru* in a "wing extension branch" of the sex determination pathway. Previous to this and our earlier study (Radovic et al., 2002), nothing was known about Yellow presence or distribution in the CNS. Using immunohistochemistry, we followed the expression of FRU^M and Yellow in the larval brain.

The late 3rd-instar is the start of the critical period for programming male-specific behavior (Arthur et al., 1998). At this stage, Yellow is present throughout the brain in the cytoplasm of various cell types of both males and females. In male larvae, numerous dorsal posterior neuroblasts in the environs of the brain region known to be involved in the development of wing extension (Hall, 1979; Greenspan and Ferveur, 2001) were studied. Those that show anti-FRU^M staining also show a dramatic increase in Yellow in both the cytoplasm and in the area surrounding the neuroblast (Fig. 2). The latter is perhaps a consequence of Yellow being secreted into and/or inherited by the progeny of the FRU^M-expressing neuroblast. Yellow was long ago reported to be nonautonomous across short distances (Hannah, 1953), and it is known to be secreted to the exterior of cuticle cells, possibly to become a structural component (Kornezos and Chia, 1992; Wittkopp et al., 2002a). FRU^M is only present in a small subset of cells in the male 3rd-instar larval brain (Lee et al., 2000; Baker et al., 2001; our unpublished data), and wild-type male neuroblasts in the same brains not showing anti-FRU^M staining did not show a correlated increase of anti-Yellow levels (Fig. 2). Wild-type 2nd-instar larval brains did not show reactivity with anti-FRU^M nor with anti-Yellow [see Fig. 4(D)], consistent with the timing of anti-FRU^M staining beginning in the 3rd-larval instar (Lee et al., 2000) and additionally with the critical period for programming male behavior (Arthur et al., 1998). Our cellular model for FRU^M and Yellow action in the developing brain is shown in Figure 2(E).

Several FRU^M neuroblasts were observed with less dramatic increases in Yellow levels, which may be due to these cells being newly formed. Qualitatively, Yellow protein appeared to be present at higher levels in larger, and likely more mature, FRU^M neuroblasts. Also, a few neuroblasts without clear FRU^M expression, but with Yellow expression, were noted, and we hypothesize that these cells were undergoing mitosis.

We detected Yellow protein distributed across both male and female brains that was not associated with FRU^M-expressing cells. In addition, there was no correlated presence of Yellow in or near male or female brain cells associated with the staining pattern of anti-FRU^{COM} (not shown). We speculate that this non-FRU-regulated, non-sex-specifically regulated Yellow protein may be involved in non-sex-limited behaviors that *y* mutants have been reported to affect, such as olfactory learning (Tully and Gergen, 1986) or locomotion (Wilson et al., 1976; Dow, 1977).

These data lead to the following hypothesis: If Yellow is up-regulated by FRU^M, then we would expect that Yellow would fail to be up-regulated in brains of males carrying mutant *fru* alleles lacking zinc fingers and having low levels of wing extension.

fru Wing Extension Mutants Lack Yellow Up-Regulation

We examined anti-FRU^M and anti-Yellow staining patterns in three *fru* mutants with varied behavioral, molecular, and biochemical phenotypes (Ryner et al., 1996; Villella et al., 1997; Goodwin et al., 2000; Lee and Hall, 2001) (Fig. 3). The *fru* gene in all three mutants produces male-specific FRU proteins (i.e., FRU^M) whose relatively 5′ male-specific regions are recognized by the FRU^M antibody (Lee et al., 2000; Lee and Hall, 2001; see also Fig. 8). We found that males carrying fru^3 and fru^4, transposable element insertion mutants whose relatively 3′ *fru* zinc finger DNA binding region is not transcribed (Goodwin et al., 2000), do not have elevated Yellow levels in FRU^M neuroblasts compared to non-FRU^M control neuroblasts in the same males. Wing extension is almost completely abolished in both fru^3 and fru^4 males (Villella et al., 1997).

In contrast to the above mutants, fru^1 males, which have wild-type but mis-expressed FRU^M (Goodwin et al., 2000; Lee and Hall, 2001) and wild-type levels of wing extension (Ryner et al., 1996; Villella et al., 1997), showed correlated FRU^M and Yellow in neu-

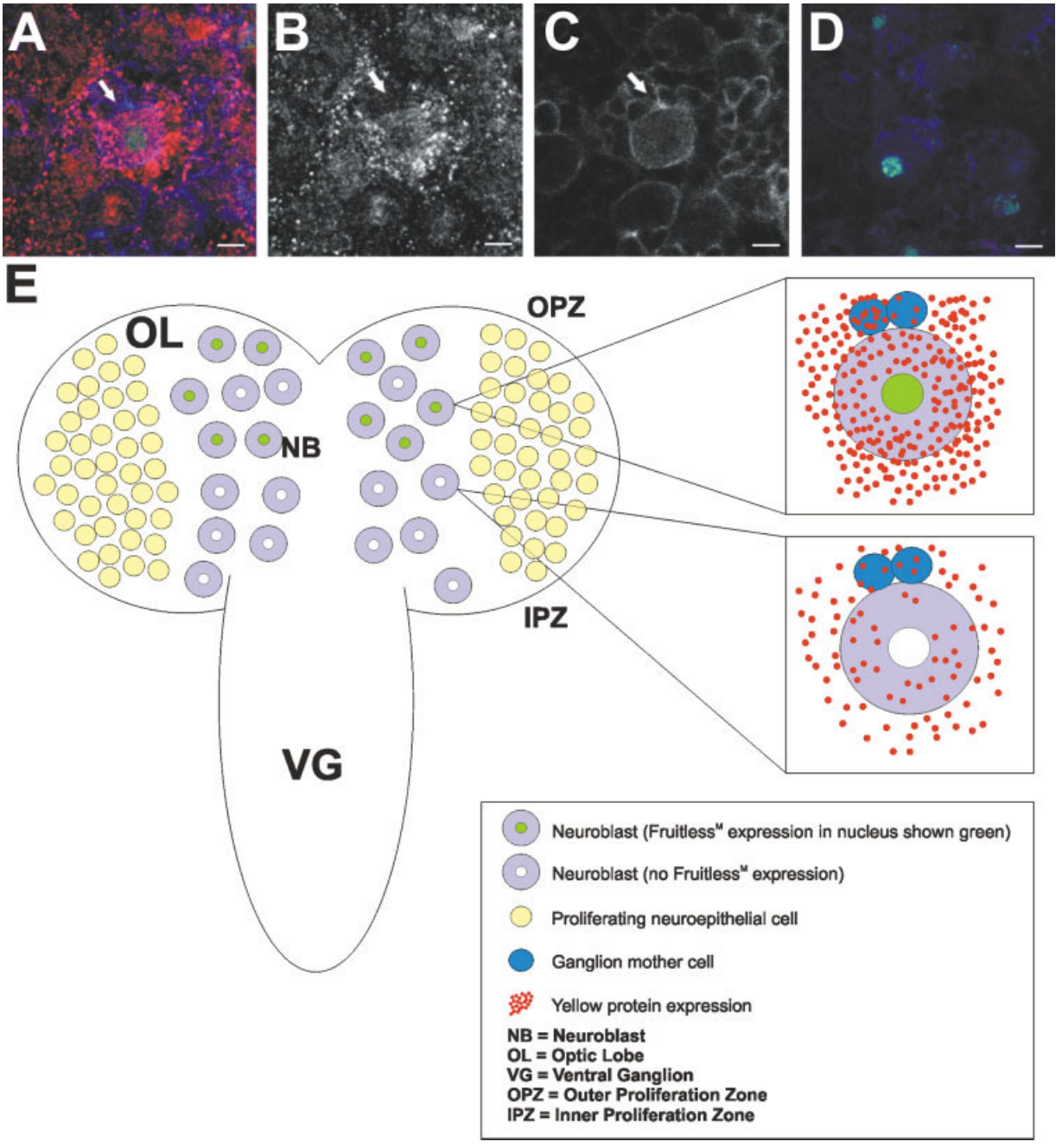


Figure 2 FRU^{M}-dependent increase in Yellow (Y) level in wild-type *Drosophila* 3rd-instar larval male brains. (A–D) Confocal images showing expression of Yellow and FRU^{M} (DLG was used as a marker to outline neural cell membranes) in Oregon-R (wt) (A–C) and *Df(1)y-ac*^{22} (complete *y* deletion) male dorsal brain (MDB). (A) Merged triple-labeled image of anti-FRU^{M} (green), anti-Y (red), and anti-DLG (blue); (B) anti-Y only, from (A). (C) Anti-DLG only, from (A). (D) *Df(1)y-ac*^{22} MDB. Merged triple-labeled image of anti-FRU^{M}, anti-Y, and anti-DLG, showing the specificity of anti-Y. Neuroblast showing FRU^{M} expression in the nucleus is indicated by an arrow. Surrounding non-FRU^{M} neuroblasts have dramatically lower levels of Yellow. All scale bars on this and other figures are 5 μ long. (E) Schematic drawing of the 3rd-instar larval male brain demonstrating different cell types (dividing neuroepithelium in the proliferation zones, and neuroblasts and ganglion mother cells in the area where asymmetric divisions and neuronal differentiation occur). Color scheme is roughly the same as in (A–D) with green representing FRU^{M} and red representing Yellow protein expression. On the left the whole brain is shown, with FRU^{M+} neuroblasts localized at the upper middle part. On the right are magnified FRU^{M-} and FRU^{M+} neuroblasts, where the difference in Y level is shown.

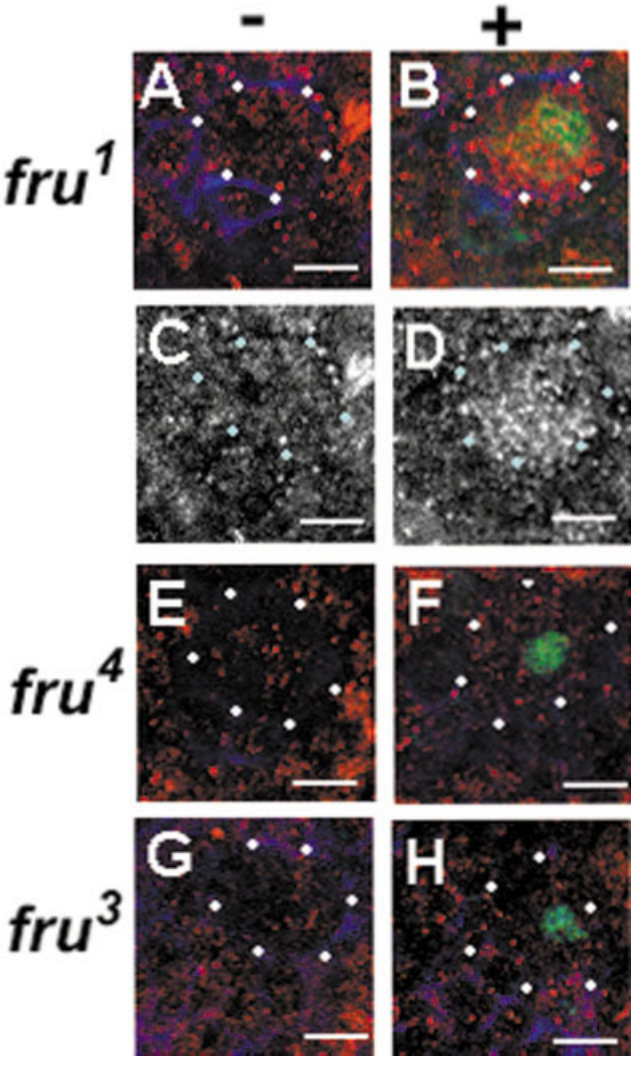


Figure 3 Mutant Fruitless proteins lacking zinc-finger domains do not up-regulate Yellow. Shown are confocal images of single neuroblasts from late 3rd-instar male larval brains. Neuroblast membranes are outlined by the dots. Comparison of cells not showing anti-FRU^{M} staining (A, C, E, G) to anti-FRU^{M} staining cells (B, D, F, H) shows up-regulation of Yellow in *fru^{1}* males but not in *fru^{3}* or *fru^{4}* males. Contrast these *fru* mutants to the wild-type control cells in Figure 2. (A–D) Neuroblasts from *fru^{1}* males. *fru^{1}* cellular phenotype is altered expression of a structurally wild-type FRU^{M} protein. (A–B) Merged triple-labeled images of anti-FRU^{M} (green), anti-Yellow (red), and anti-DLG (blue). (C, D) Single-labeled anti-Yellow from (A) and (B), respectively. (E–H) Brain cells from *fru^{4}* (E, F) and *fru^{3}* (G, H) males, showing merged triple-labeled images of anti-FRU^{M} (green), anti-Yellow (red), and anti-DLG (blue). *fru^{4}* and *fru^{3}* protein products lack the region of FRU^{M} protein containing two zinc-finger DNA-binding domains.

roblasts (Fig. 3). These data suggest a functional link between FRU^{M}, Yellow, and wing extension, as follows.

Because the *fru^{3}* and *fru^{4}* mutants all but abolish wing extension (Ryner et al., 1996; Villella et al., 1997), but *y* null mutants such as *y^{1}* reduce it to roughly half of wild-type level (Bastock, 1956; Burnet et al., 1973; Hall, 1994a; M. D. Drapeau and A.D. Long, unpublished observations), these data are consistent with a developmental genetic model in which *y* and other genes downstream of *fru* form a wing extension branch of the male sexual behavior differentiation pathway. Additionally, the *fru* zinc finger

domain is implicated in the direct or indirect control of *y* transcription.

FRU$^{M\{BM\}}$ Is Sufficient for Yellow throughout the 3rd-Instar CNS

In order to determine if FRU^{M} is sufficient for Yellow in the 3rd-instar larval CNS, where FRU^{M} and Yellow colocalize in wild-type males, we ectopically expressed one isoform of FRU^{M} formed by alternative splicing, FRU$^{M\{BM\}}$ (Usui-Aoki et al., 2000), in most 3rd-instar larval neural cells *in vivo* by utilizing the GAL4-UAS system (Brand and Perrimon, 1993) with an *elav*-GAL4 driver (Robinow and White, 1991; Kazantsev et al., 2002). Nearly every FRU$^{M\{BM\}}$-expressing cell at the 3rd-instar stage was associated with high levels of Yellow (Fig. 4; see also Fig. 6). This includes cells in the symmetrically-dividing neural epithelium, where anti-FRU^{M} staining is not normally found (Lee et al., 2000; our unpublished data). We independently verified that there is more Yellow in the CNS of 3rd-instar larvae overexpressing FRU$^{M\{BM\}}$ by Western blotting, using larval head extracts from wild-type and *elav*-GAL4/UAS-*fru*$^{+}$ *{BM}* animals [Fig. 4(G)].

FRU$^{M\{BM\}}$ Is Sufficient for Yellow in the 2nd-Instar CNS

We also followed FRU$^{M\{BM\}}$ expression at an incorrect stage of neural development, the 2nd-instar. FRU$^{M\{BM\}}$ presence in wild-type males begins in the late 3rd larval instar (Lee et al., 2000). To accomplish this mis-expression, we again used the *elav*-GAL4 driver. Similar to our mis-expression experiment in 3rd-instar larvae, we found that FRU^{M} expression was positively correlated with Yellow expression (Fig. 5). Neither protein is normally detected during this stage [Fig. 5(D)]. This result bolsters the hypothesis that *y* is downstream of FRU$^{M\{BM\}}$, and in addition it suggests that any cofactors and/or intermediates that are required for FRU$^{M\{BM\}}$-mediated Yellow up-regulation are already present in 2nd-instar larvae.

FRU$^{M\{BM\}}$ Is Sufficient for Yellow in the Female CNS

We used the *elav*-GAL4 driver to coerce male-specific FRU expression in the female CNS. Again, we found that in most FRU$^{M\{BM\}}$ neural cells, Yellow was found at high levels, relative to those of wild-type

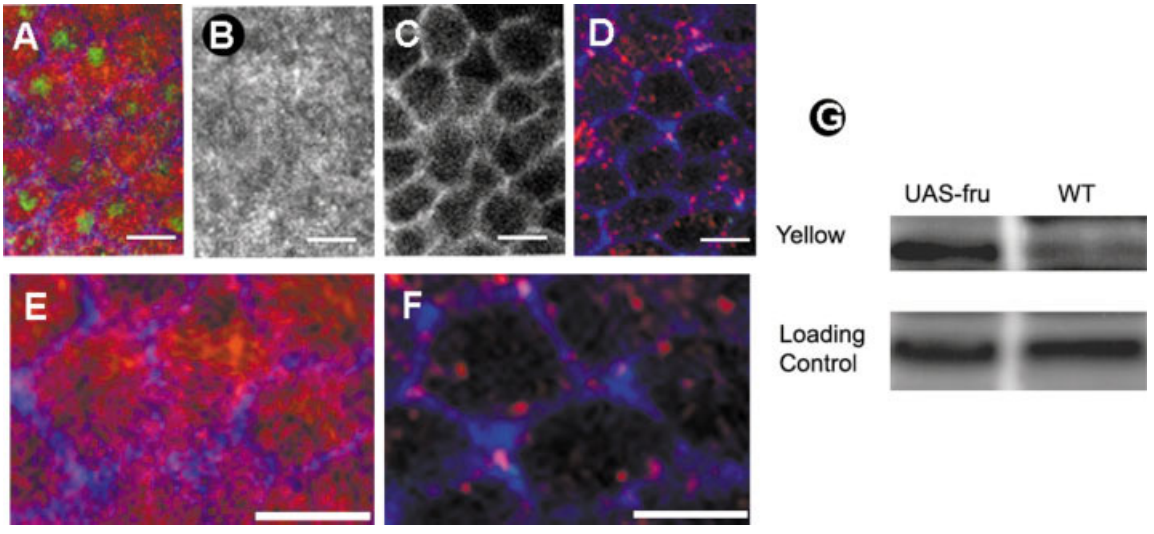


Figure 4 Mis-expressed FRU$^{M\{BM\}}$ up-regulates Yellow (Y) in the wrong cell type within the developing nervous system. (A–F) Neuroepithelial cells of the proliferation zone are shown. We never detected FRU^{M}, nor elevated Yellow, in the proliferation zone of Oregon-R (wt) males or females. (A–D) Proliferation zone of *elav*-GAL4/UAS-*fru*$^{+}$ {BM} male: (A) triple-labeled anti-FRU^{M} (green), anti-Y (red), and anti-DLG (blue); (B) anti-Y only; (C) anti-DLG only; (D) Oregon-R (wt) female, triple-labeled as in (A). (E) and (F) are magnified versions of (A) and (D), respectively, without anti-FRU^{M} labeling. (G) Western blot showing increase of Yellow in the larval head following overexpression of FRU^{M} in *elav*-GAL4/UAS-*fru*$^{+}$ {BM} flies. Our experimentally determined Yellow molecular weight is consistent with previously published data (Kornezos and Chia, 1992).

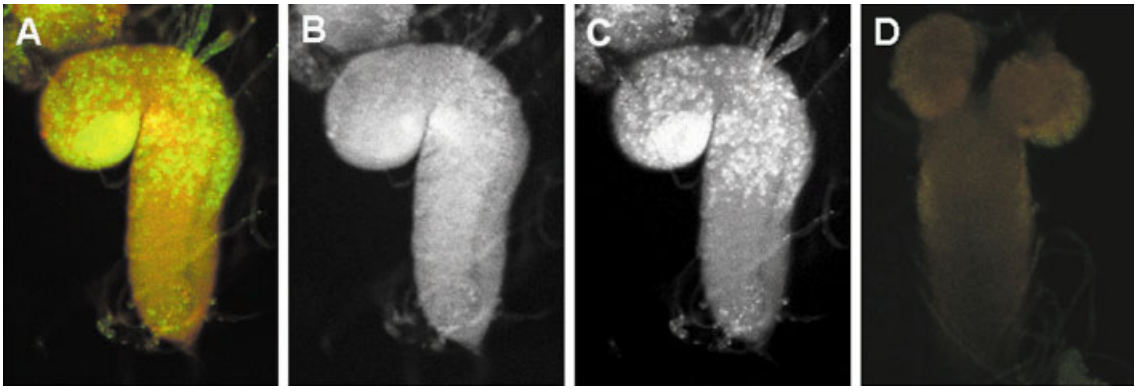


Figure 5 $FRU^{M\{BM\}}$ expression is sufficient for Yellow (Y) up-regulation in an incorrect developmental stage. (A–D) 2nd-instar larval brains. (A–C) *elav*-GAL4/UAS-*fru*$^+$ {BM} female: (A) double-labeled anti-FRU^M (green) and anti-Y (red); (B) anti-Y only; (C) anti-FRU^M-only. (D) Double-labeled anti-FRU^M and anti-Y Oregon-R (wt) male. Neither FRU^M nor Y is detectable in wild-type flies at this stage.

control females (Fig. 6). This result again provides strong evidence that *y* is downstream of the BM male isoform of *fru*. In addition, it strongly suggests that any cofactors and/or intermediates that are required for $FRU^{M\{BM\}}$-mediated Yellow up-regulation are not specific to males. This is interesting because the developmental pathways and phenotypes we have in mind, the FRU^M-controlled male differentiation pathway and male sexual behavior, are quite male specific.

$FRU^{M\{AM\}}$ Is Not Sufficient for Yellow

We repeated each of the experiments described above for $FRU^{M\{BM\}}$ with a UAS transgene for a different

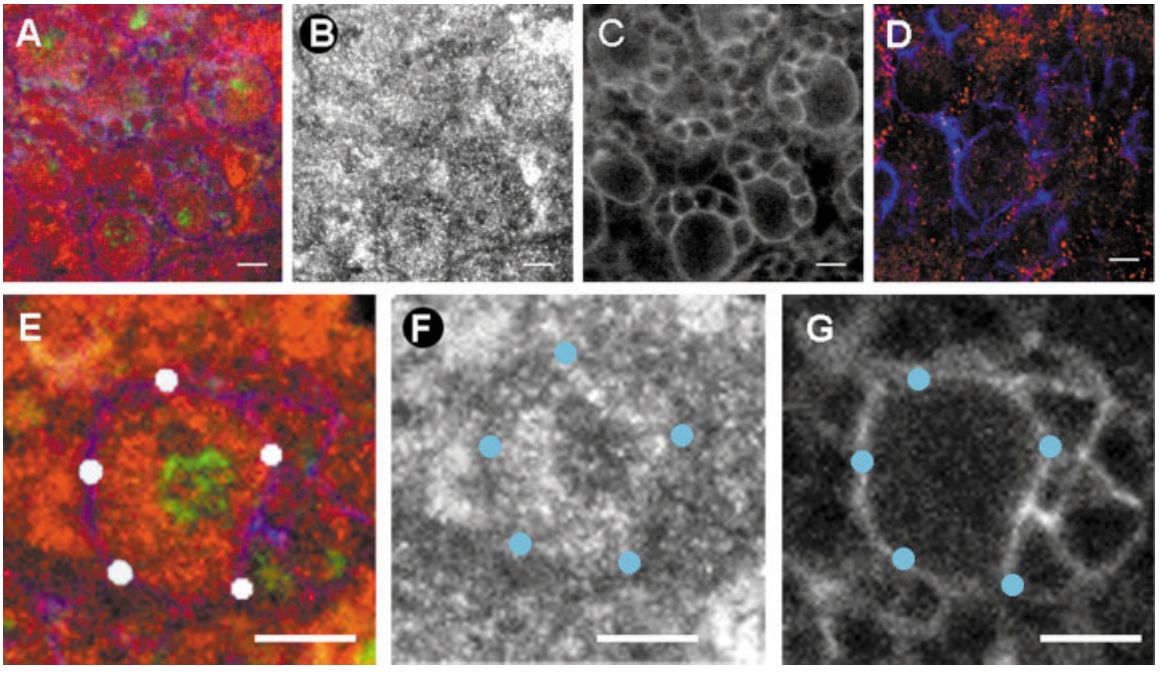


Figure 6 Mis-expressed $FRU^{M\{BM\}}$ up-regulates Yellow in the wrong gender, females. (A–D) 3rd-instar larval brains. (A–C and E–G) *elav*-GAL4/UAS-*fru*$^+$ {BM} female: (A) triple-labeled anti-FRU^M (green), anti-Y (red), and anti-DLG (blue); (B) anti-Y only; (C) anti-DLG only; (D) Oregon-R (wt) female, triple-labeled as in (A). (E–G) Single neuroblasts: (E) like (A); (F) like (B); (G) like (C).

male-specific isoform of FRU, $FRU^{M\{AM\}}$. Our working hypothesis was that different male-specific FRU isoforms, with distinct zinc finger protein sequences (Usui-Aoki et al., 2000; Baker et al., 2001; Davis and Ito, 2001), have different downstream targets. These different targets may play distinct roles in the development of different aspects of the male courtship ritual that *fru* function is known to be necessary for (Hall et al., 1982; Ryner et al., 1996; Villella et al., 1997). Because *y* is only known to reliably affect a single aspect of the male courtship ritual, wing extension (Bastock, 1956; Burnet et al., 1973; M.D. Drapeau and A.D. Long, unpublished observations), we predicted that *y* would only be downstream of a single FRU^{M} zinc finger, in this case, $FRU^{M\{BM\}}$ (see above).

In accordance with our hypothesis, we did not find a single instance of $FRU^{M\{AM\}}$-mediated up-regulation of Yellow. This result held in the CNS of both males and females at different developmental stages based on immunohistochemistry. This result is significant because it suggests that different male isoforms of FRU (Usui-Aoki et al., 2000) have different downstream targets in distinct genetic pathways (Baker et al., 2001; Sokolowski, 2001).

Ectopic Expression of Yellow in the 3rd-Instar Larval CNS Increases Adult Male Wing Extension during Courtship

We have thus far shown that Yellow is present in the male CNS at elevated levels, and that *y* function is downstream of male-specific *fru* proteins. However, these data by themselves do not demonstrate a role for *y* CNS expression in the development of male courtship behavior, and specifically, wing extension. Based on the coexpression patterns of $FRU^{M\{BM\}}$ and Yellow reported here, and on information in the literature, we predicted that ectopically expressing *y* in the developing CNS in a *y* null background would increase the amount of wing extension during male courtship relative to that found in *y* mutant males (Bastock, 1956; Burnet et al., 1973; M.D. Drapeau and A.D. Long, unpublished data). A corollary of this hypothesis is that the poor male mating success of *y* null males would be rescued to wild-type levels.

In order to test this hypothesis, we again used the GAL4/UAS system, but rather than expressing FRU proteins, we ectopically expressed Yellow protein in progeny of crosses between a *y; UAS-y* strain and strains with GAL4 drivers. We engineered three types of males: A negative control with no GAL4 driver, a positive control with the ubiquitous driver *Actin5c-GAL4*, and a 3rd-instar CNS-specific driver, *c81-GAL4* (see Materials and Methods).

We phenotyped these males for their Wing Extension Index (WEI), the percentage of time during courtship that a male has his wing extended at approximately 90° (see, for example, Ryner et al., 1996; Villella et al., 1997). The purpose of wing extension is to vibrate the wing and "sing" a song to the female in order to stimulate her to receptivity. Courtship songs are also species-specific, and therefore act as an important identification signal, particularly in situations in which indiscriminate males that look similar or identical, but belong to different sibling species, court a female simultaneously.

Our WEI data are shown in Figure 7. We found that males carrying either the *Actin5c* or the *c81* driver had relatively high levels of wing extension in comparison to the *y* males carrying no driver, and males with either of the two GAL4 drivers had very similar WEI. A two-way ANOVA with factors "genotype" and "block" revealed no effect of block (day-of-assay and developmental bottle) on WEI [F (2, 36) = 0.1636; $p \leq 0.85$], but showed a highly significant

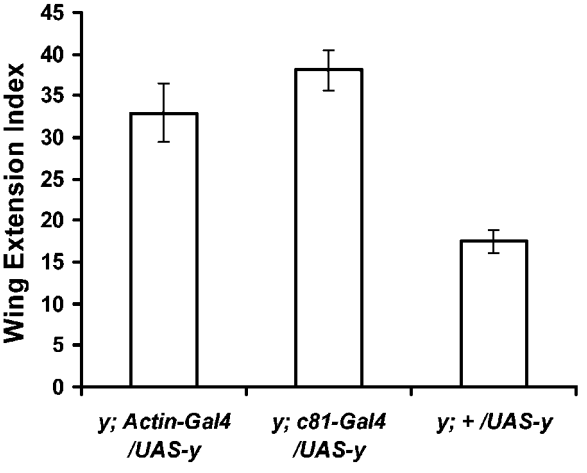


Figure 7 Ectopic expression of *yellow* (*y*) in the 3rd-instar CNS via GAL4/UAS is sufficient for wild-type levels of wing extension during male courtship. Males carrying either a "ubiquitous" *Actin5c-GAL4* driver, a 3rd-instar CNS *c81-GAL4* driver, or a wild-type Ives genetic background with no GAL4 driver were crossed to *y; UAS-y* females to direct *y* expression in male progeny. Here we show the wing extension index (WEI) for each genotype, where WEI is the percentage of the courtship ritual during which a male's wing is extended and vibrating (singing). $n = 15$ males for each treatment. Plotted are the mean ± S.E.M. of the 15 males in each treatment, because we detected no "block" effects in our analysis (see Materials and Methods). There were significant differences among genotypes for WEI (ANOVA, $p \leq 0.00001$).

effect of genotype [$F(2, 36) = 15.40$; $p \leq 0.00001$]. There was no significant interaction between genotype and block [$F(4, 36) = 0.5804$; $p \leq 0.68$]. Analysis of arcsine-square root-transformed WEI data yielded identical results (not shown). This suggests that 3rd-instar CNS expression of *y* is sufficient to increase WEI levels to roughly that of wild-type males (43 ± 5 to 50 ± 4; see for example Villella et al., 1997, though exact genotypes and apparatus are different). These data also show that global overexpression of *y* does not have dominant negative effects on sexual behavior, nor is it generally toxic.

The effect of *y* CNS expression on wing extension appears to be relatively specific. For each genotype, we measured two additional behaviors, which are the two components of WEI. First, for each individual male, we measured the rate of wing extension, which is the number of wing extensions per second of the courtship ritual. Second, for each male, we measured the mean length of each individual wing extension bout. In each case we again found no significant effects of block or a genotype-by-block interaction (data not shown). However, we did find a significant increase of wing extension rate in *Actin5c* and *c81* flies compared to those with no GAL4 driver [$F(2, 36) = 6.5321$; $p \leq 0.004$]. In contrast, we found no effect of ectopic *y* expression on mean wing extension duration [$F(2, 36) = 2.2636$; $p \leq 0.12$].

These data imply that *y* plays a developmental role in the 3rd-instar CNS, leading to an increased rate of wing extension in adult males during courtship. This increased wing extension rate accounts for a greater percentage of time spent singing song, presumably leading to a greater/faster sexual stimulation of the female. This presumption is borne out by a preliminary experiment in which we competed either *Actin5c* or *c81* males against *y; +/UAS-y* males (one of each) for first copulations with single wild-type Oregon-R females in eight-dram food vials. We found that males carrying either GAL4 driver achieved the first copulation with the female approximately 95% of the time ($n \approx 50$ in each experiment), demonstrating the ultimate functional importance of *y* CNS expression.

DISCUSSION

A Putative Developmental Pathway Necessary for Normal Male Wing Extension during Courtship

In total, our genetic data demonstrate that the sexual behavior gene *y* is downstream of the gene that is hypothesized to "specify" all aspects of male sexual behavior, *fru* (Baker et al., 2001). We hypothesize that in the *D. melanogaster* developing CNS, *y* is a member of the *fru* pathway branch specifying wing extension, but not courtship song or attempted copulation. *y* null mutants do not appear to affect either of the latter two courtship behaviors (Burnet et al., 1973; Kyriacou and Hall, 1986; M.D. Drapeau and A.D. Long, unpublished observations). The $FRU^{M\{BM\}}$ zinc-finger transcription factor may head this "wing extension" branch (Fig. 8).

Because neuroblasts are the stem cells that are ultimately responsible for the development of the *D. melanogaster* adult nervous system, we speculate that the increased level of Yellow in specific male neuroblasts may play a role in those neuroblasts later giving rise to differentiated neurons subserving wing extension. In our cellular model for Yellow action in the CNS [Fig. 1(E)], we propose that Yellow is secreted/inherited from FRU^{M} neuroblasts into neuronal precursors and neurons, similar to how it is secreted from cuticle cells (Kornezos and Chia, 1992; Wittkopp et al., 2002a). It is noteworthy that in the cuticle, Yellow is nonautonomous over short distances (Hannah, 1953).

Few mutations have been identified (except for those severely affecting gross morphology of the wing or musculature) that specifically alter the amount of wing extension during the courtship ritual, except those in *fru* and *y*. Mutations in the *ebony* and *flamenco* genes may be other examples (Kyriacou et al., 1978; Romanova et al., 2000). Because wing extension phenotypes (i.e., WEI, WE rate, mean WE) are relatively difficult to measure, few mutations have been screened for this phenotype. As interest in the developmental genetics of instinctual *Drosophila* male courtship behavior rises, screens for such genes will become a major focus. Regarding the identification and understanding of such genes, it is of interest that the musculature that controls some aspects of flight and some aspects of wing movement during courtship is the same (Ewing, 1979). Specifically, a subset of the direct and axillary wing muscles, and perhaps just a single muscle, controls wing extension (Nachtigall and Wilson, 1967; Levine, 1973; Ewing, 1979; see below), and therefore mutants that are known to affect wing phenotypes besides courtship can be used to screen for WE mutants. This general strategy was employed by Barnes et al. (1998), who screened wing-beat frequency mutants for changes in a number of courtship song parameters.

Regardless of the method by which WE mutants are identified, we can form at least one specific hypothesis based on the data presented in this article. That is, genes harboring mutations that reduce wing

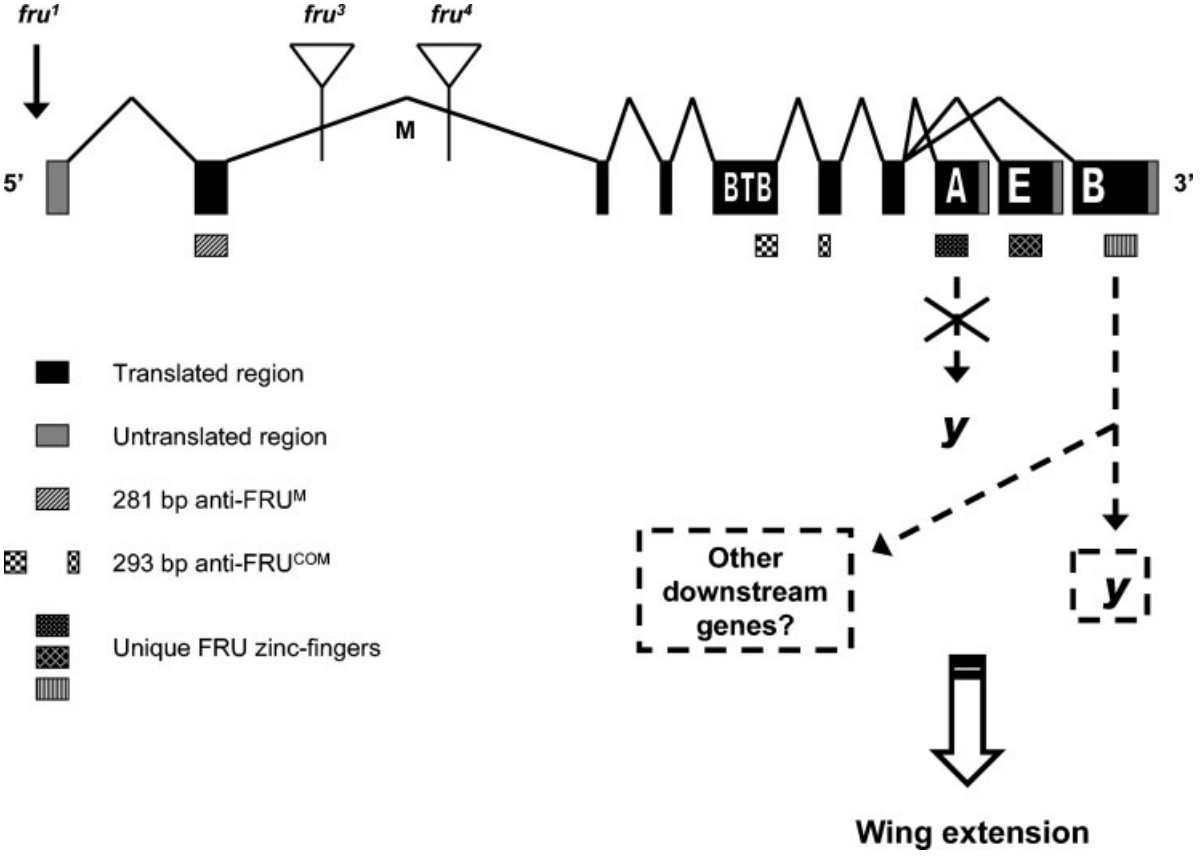


Figure 8 A model of *fruitless* (*fru*)-regulated aspects of sexual differentiation. Here, *yellow* (*y*) is in a branch of the *fruitless* set of pathways downstream of its DNA-binding zinc-finger domains, and Yellow up-regulation is dependent on $FRU^{M\{BM\}}$, but not $FRU^{M\{AM\}}$, zinc-finger function ("B" and "A" for Zn-fingers B and A, "M" for male 5′ splice variant). In *D. melanogaster* males, cells are XY, the X-to-autosome ratio is 0.5, and cells differentiate to be malelike. In a male somatic cell, *fru* produces male-specific transcripts ("M") that are presumably necessary for establishing numerous male-specific characteristics. (Details of alternatively spliced female-*fru* transcripts are not shown.) One of these male-specific *fru* transcripts (Type BM) ("B") is translated into a zinc-finger protein that is upstream of *y*. This $FRU^{M\{BM\}}$ protein could bind directly to *y* regulatory DNA or could act through one or more intermediaries. Another male-specific *fru* zinc-finger protein, $FRU^{M\{AM\}}$ ("A"), was found to not be upstream of *y*. *fru* transposable element insertion mutants fru^3 and fru^4, which do not have full-length transcripts or proteins, lack Yellow up-regulation ability, presumably because they lack zinc-finger DNA-binding properties. The fru^1 mutant is an inversion that leads to ectopic expression of the normal set of *fru* transcripts; fru^1 flies appear to have normal Yellow levels in the CNS. One fru^1 inversion breakpoint is shown, and the other occurs outside the *fru* locus. The biochemical role of the Yellow protein in the nervous system is unknown, but Yellow contains a putative signal peptide and is secreted from cuticle cells. It may be similarly secreted from neural stem cells into developing neurons. We hypothesize that *y* and other genes downstream of $FRU^{M\{BM\}}$ are necessary for the development of wing extension behavior in the courtship ritual of adult male flies. Abbreviations: BTB, *Bric-a-brac Tramtrack,* and *Broad-complex*-like conserved region.

extension are predicted to be downstream of $FRU^{M\{BM\}}$ along with *y*, and may molecularly interact with *y* or its protein product in some manner. The *ebony* gene is an ideal candidate for future work on the developmental genetics of wing extension. At least one *ebony* mutant appears to reduce wing extension during male courtship (Kyriacou et al., 1978), and *y* and *ebony* have interacting functions in determining adult cuticle pigmentation patterns (Wittkopp et al., 2002a). It is currently unclear what the precise developmental role of these genes is in the larval CNS that influences the frequency of WE during adult male courtship.

What Is the Biochemical Function of Yellow in the CNS?

The mechanism by which Yellow might act at a genetic, cellular, or biochemical level to create, maintain, or regulate a neural circuit subserving male wing extension behavior is not obvious, but circumstantial evidence allows us to speculate.

Little is known about the biochemical function of Yellow in general. We recently reported the presence of 12 Yellowlike proteins in *D. melanogaster* (Drapeau, 2001b; see also Maleszka and Kucharski, 2000). Unfortunately, this new information does not shed much light on the biochemical function of Yellow, as none of these proteins are related to proteins in other organisms that are not already related to Yellow. Additionally, the functions of the 12 new genes are mostly uncharacterized, although some are known to be expressed in the head (Maleszka and Kucharski, 2000), and the *yellow-f* and *yellow-f2* genes may act as dopachrome conversion enzymes in the melanization process (Han et al., 2002). The role of *yellow-f* in melanization may be related to the *Drosophila* immune response (DeGregorio et al., 2001).

Interestingly, Yellow is evolutionarily related to the Major Royal Jelly Proteins (MRJPs) of the honeybee *Apis mellifera* (Albert et al., 1999). The MRJPs are the major protein constituent of the Royal Jelly fed to larvae to determine development into a queen. In addition to this nutritional role, at least one MRJP, MRJP3, is expressed in a subset of the adult brain, the mushroom bodies, well known to be involved in various behaviors in numerous species (Kucharski and Maleszka, 1998). The function of MRJP3 in the brain is unknown, however. Future research on neural functions of the MRJP family may shed light on the neural role(s) of Yellow in *D. melanogaster* and presumably other *Drosophila* species.

Besides a putative function of Yellow in the CNS (Radovic et al., 2002; this article), Yellow has a well-known but mysterious pleiotropic role in cuticle melanization (Sturtevant, 1913; Nash and Yarkin, 1974; Walter et al., 1991, 1996; Kornezos and Chia, 1992; Wittkopp et al., 2002a,b), which may or may not be related to the CNS and/or behavior. Flies lacking *y* function have reduced "black" melanin (DOPA-melanin; see Wittkopp et al., 2002a) and lack dark pigmentation over much of their body cuticle, making the flies yellow/golden brown (hence the gene name "*yellow*"). Although it had earlier been hypothesized that Yellow was necessary for cross-linking indole-5,6 quinone during cuticle melanization (Geyer et al., 1986), recent experimental evidence supports the role of Yellow in melanization as enzymatic, although that remains to be directly shown (Wittkopp et al., 2002a; see also Johnson et al., 2001 for indirect support), and there is some empirical biochemical evidence against this theory (Han et al., 2002). It is unclear if the biochemical function of Yellow in the melanization process is related to its necessity for normal male wing extension, although it is tempting to speculate that *y* mutant behavioral defects are related to abnormal levels of dopamine (DA), because DA and melanin are synthesized from tyrosine in the same basic biochemical pathway and are similar molecules (Wright, 1987).

The relationship between melanin and DA has not been overlooked by behavior geneticists studying *y* mutant alleles and behavior, and it was long ago hypothesized that *y* effects on male courtship behavior are related to levels of DA (Burnet and Connolly, 1974). This is a reasonable hypothesis, because DA is a molecule of both developmental and neurobiological importance (see, for example, Neckameyer, 1996), and mutations in other genes causing both behavioral and melanization defects are known to have abnormal DA levels. For example, various *tan* mutants have abnormally low DA levels (Konopka, 1972), while the *ebony* (*e*) mutant e^{11} has a significantly increased DA level (Hodgetts and Konopka, 1973). Unlike Yellow, however, the Ebony and Tan proteins have well-characterized biochemical functions in the dopamine synthetic pathway (see Wright, 1987 for review).

Pharmacological evidence bears on the "dopamine theory" of the relationship between *y* and behavior. Nonspecific pharmacological suppression by α–dimethyltyrosine (α-DMT) of rate-limiting enzymes in the DA and serotonin (5-HT) biochemical synthesis pathways results in *y* behavior and pigmentation phenocopies (Burnet et al., 1973), and more recently these dual phenocopies have been created using 3-I-Y, a TH-specific inhibitor that affects the DA but not the 5-HT pathway (Neckameyer et al., 2001). Consistent with our data showing the Yellow protein in the 3rd-instar CNS, the critical period for induction of *y* phenocopies by α-DMT is the 3rd-instar (Newcomb and Lambert, 1988), and Neckameyer et al.'s (2001) experiment was performed at this same developmental stage. A specific biochemical prediction from the combined genetic and pharmacological data is that levels of enzymes in and products of the DA pathway will be altered during CNS development in *y* mutants with abnormal wing extension.

Classical *Drosophila* Pigment Mutant Pleiotropy

Because *y* was on the first genetic linkage map (Sturtevant, 1913), and because its necessity for normal male mating behavior has been known for over 87 years (Sturtevant, 1915), *y* is most likely the first gene whose function is necessary for behavior to be linkage mapped in any organism [with the exception of the mutant *white*^{1} (see below), mapped in the same study]. However, *y* was not initially identified because of its role in behavior, but rather because of its role in pigmentation—*y* null flies are yellow/golden-brown instead of the wild-type grey/black (Morgan, 1911; Lindsley and Zimm, 1992; Flybase, 1999). Is it highly unusual for "classic" mutations that affect relatively well-defined pathways, such as cuticle melanization, to be in genes that also have neurobiological/behavioral functions? Are the effects of such mutations on neurobiology or behavior any less significant than those that appear to have relatively specific effects on behavior?

Other loci besides *yellow* that were first implicated through mutation analysis in the pigmentation process have recently been shown through biochemical and genetic methods to play neurobiological/behavioral roles outside the cuticle (or eye). The *ebony* locus has a well-defined role in the cuticular melanization process (Wright, 1987), yet the gene has recently been found to have a well-defined expression pattern in the 3rd-instar CNS (Hovemann et al., 1998). Flies carrying *ebony* mutations are known to have poor male mating success and other courtship behavior defects (e.g., Rendel, 1951; Kyriacou et al., 1978; Hall, 1994a). Similarly, the *white* and *brown* loci, which have white-eyed and brown-eyed mutant phenotypes, respectively (Lindsley and Zimm, 1992; Flybase, 1999), have a defined biochemical role in pigment-producing cells of the fly, and flies carrying some alleles of these genes have visual defects (Kalmus, 1943). However, the *white* and *brown* genes were only recently discovered to have a function necessary for nonvisual olfactory-related behavior (Campbell and Nash, 2001), and these same researchers localized a *white* transcript to the neural tissues of the head. In general, such results suggest that there is a somewhat general pleiotropy of alleles causing both pigmentation and behavioral/neurobiological defects.

Hall (1994b) has noted that many genes that have functions necessary for behavior have been found to be pleiotropic. While there certainly are pleiotropic effects of the much-studied *y* allele *y*^{1}, the common delineation of *y* as a "classic pleiotropic gene" does not take into account the fact that behavior genetic experiments on *y*^{1} often failed to control for background genetic and/or environmental effects, or did so poorly, and in addition highly inbred flies were assayed without exception. Furthermore, few of these behaviors have been assayed in *y*^{1} flies more than once, except for cuticle pigmentation, wing extension, and male mating success. We have observed that when *y*^{1} and *y*$^{+}$ fully outbred flies with identical genetic and environmental backgrounds are contrasted, they are not significantly different for many of the behavioral phenotypes claimed in the literature (M.D. Drapeau and A.D. Long, unpublished results). Therefore, at least in the case of *y*, pleiotropy on both pigmentation and neurobiological/behavioral phenotypes cannot simply be explained as a property of "sick flies."

Three categories of pleiotropic behavioral genes are defined by Hall (1994b): "general metabolic mutants", "vital genes that influence behavior", and "nonvital genes identified mainly by behavioral variants". Can *y* easily be placed in one of these categories? Mutants in the *y* gene do not seem to fit the profile of "general metabolic mutants" in genes such as *couch potato* and *technical knockout*, the latter of which can disrupt courtship behavior (Toivonen et al., 2001). These are very "sick" flies, in the sense that they are generally "slothful" (Hall, 1994b) and can be overly sensitive to insults such as mechanical shock. Additionally, both "general metabolic mutants" and "vital genes that influence behavior" tend to be lethal mutable. It is known, however, that *y* nulls are not lethal, and in fact are quite healthy.

This leaves the category "nonvital genes identified mainly by behavioral variants". While a lengthy discussion of genes in this category is outside the scope of this article, in general *y* is similar to the genes Hall (1994b) describes, such as *apterous*, *sevenless*, *dunce*, *rutabaga*, and in particular, *period*. Genes in this category tend to have very specific behavioral defects, which in some cases have been related to expression of the gene in a certain tissue. These genes also have pleiotropic effects on diverse characters such as visual system physiology, wing development, and female fertility. Additionally, they often have expression patterns that appear to be unrelated to the gene's behavioral effects, which in many cases are currently unexplained. From this description, we may conclude that *y* is similar to many other "behavior genes" in the nonvital category, and hence that its initial identification by a pigmentation phenotype makes *yellow* no less interesting as a "behavior gene."

Evolutionary Genetics of Wing Extension: Courtship Developmental Genes as Candidate Speciation Genes

Drosophila species have greatly diverged wing extension behavior in male courtship rituals (Bastock, 1967). For example, a closely related sibling species of *D. melanogaster*, *D. simulans*, performs relatively less wing extension for the purpose of vibration (song), but rather "scissors" its wings while standing in front of the female. This species also has a number of other wing movements, including wing "rows" and wing extension with no vibration (Cobb and Ferveur, 1996; M.D. Drapeau and A.D. Long, unpublished results). Another more distantly related species, *D. subobscura* (roughly 35 million years diverged from *D. melanogaster*), as a rule displays no male wing vibration (Bastock, 1967), yet a species closely related to *D. subobscura*, *D. persimilis*, does sing courtship song (e.g., Bennet-Clark and Ewing, 1968).

A "candidate gene" approach to identifying genes involved in differences within and between species and populations has proven remarkably useful in both morphological (Lai et al., 1994; Long et al., 1998; Stern, 1998; Sucena and Stern, 2000; Beldade et al., 2002; Wittkopp et al., 2002b) and behavioral (Kyriacou and Hall, 1986; Campesan et al., 2001) studies. Genes such as *fru* and *y*, which are generally conserved at a nucleotide level throughout the genus *Drosophila* (e.g., Munte et al., 1997; Davis et al., 2000a,b), and other as-yet undiscovered genes influencing the development of wing extension, are excellent candidates for dissecting the molecular evolutionary genetics of species differences in sexual behavior (Orr, 2001). These same genes may also be involved in the speciation process, if sexual behaviors are causally involved in reproductive isolation between populations.

GENERAL CONCLUSIONS

The data we present in this article suggest that *y* expression in the developing CNS, regulated directly or indirectly by a male-specific FRU protein, is important for the normal development of male wing extension. It will be of interest in future experiments to understand the relationship between *fru*, *y*, and the development of wing extension more precisely. For example, in a *fru* mutant background that reduces the WEI to $\approx$3 (e.g., fru^3, fru^4), can ectopic expression of *y* in the CNS rescue WEI? If so, can WEI be rescued to wild-type levels, or to an increased but still "mutant" level?

Another general question regarding the relationship between *y* and wing extension is the following: What is the minimum expression pattern of *y* that is sufficient to rescue wing extension to wild-type levels in a y^1 background? The converse question is equally interesting: In what subset of the CNS is the Yellow protein necessary for normal wing extension? Finally, what is the temporal expression pattern of *y* that is necessary and sufficient for normal wing extension? The combination of increasingly narrowly expressed GAL4 drivers, inducible GAL4 drivers, and *UAS-y-IR* constructs that can interfere with *y* RNA to eliminate translation (i.e., RNAi; Piccin et al., 2001) will enable us to begin to understand how Yellow acts in the CNS to enable normal development of wing extension.

Finally, and more speculatively, it is tantalizing to note that muscle control of wing extension may be extremely simple. Though relatively little work on the relationship between specific wing muscles and specific portions of flight and courtship song has been performed, the consensus from the study of various Dipterans is that one, or very few, muscles control the extension of a wing (Nachtigall and Wilson, 1967; Levine, 1973). If the neural connections to this "wing opener muscle" (Levine, 1973) are also simple, this may be a promising system within which to understand the relationship between gene products in the nervous system during development and muscle control over behavior in an adult animal.

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REFERENCES

Albert S, Bhattacharya D, Klaudiny J, Schmitzová J, Simúth J. 1999. The family of Major Royal Jelly Proteins and its evolution. J Mol Evol 49:290–297.

Anand A, Villella A, Ryner LC, Carlo T, Goodwin SF, Song HJ, Gailey DA, Morales A, Hall JC, Baker BS, Taylor BJ. 2001. Molecular genetic dissection of the sex-specific and vital functions of the *Drosophila melanogaster* sex determination gene fruitless. Genetics 158:1569–1595

Arthur Jr. BI, Jallon J-M, Caflisch B, Choffat Y, Nöthiger R. 1998. Sexual behavior in *Drosophila* is irreversibly programmed during a critical period. Curr Biol 8:1187–1190.

Baker BS, Taylor BJ, Hall JC. 2001. Are complex behaviors specified by dedicated regulatory genes? Reasoning from *Drosophila.* Cell 105:13–24.

Barnes PT, Sullivan L, Villella A. 1998. Wing-beat frequency mutants and courtship behavior in *Drosophila melanogaster* males. Behav Genet 28:137–151.

Bastock M. 1956. A gene mutation which changes a behavior pattern. Evolution 10:421–439.

Bastock M. 1967. Courtship: An Ethological Study. Chicago, IL: Aldine Publishing Company. 220 p.

Bastock M, Manning A. 1955. The courtship of *Drosophila melanogaster.* Behaviour 8:85–111.

Beldade P, Brakefield PM, Long AD. 2002. Contribution of *Distal-less* to quantitative variation in butterfly eyespots. Nature 415:315–318.

Belote JM, Baker BS. 1987. Sexual behavior—its genetic control during development and adulthood in *Drosophila melanogaster*. PNAS USA 84:8026–8030.

Bennet-Clark HC, Ewing EW. 1968. The wing mechanism involved in the courtship of *Drosophila*. J Exp Biol 49:117–128.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Burnet B, Connolly K. 1974. Activity and sexual behaviour in *Drosophila melanogaster.* In: van Abeelen JHF, editor. The Genetics of Behaviour. New York: American Elsevier Publishing Company, Inc., p 201–258.

Burnet B, Connolly K, Harrison B. 1973. Phenocopies of pigmentary and behavioral effects of *yellow* mutant in *Drosophila* induced by alpha-dimethyltysosine. Science 181:1059–1060.

Calleja M, Moreno E, Pelaz S, Morata G. 1996. Visualization of gene expression in living adult *Drosophila*. Science 274:252–255.

Campbell JL, Nash HA. 2001. Volatile general anesthetics reveal a neurobiological role for the *white* and *brown* genes of *Drosophila melanogaster.* J Neurobiol 49:339–349.

Campesan S, Dubrova Y, Hall JC, Kyriacou CP. 2001. The *nonA* gene in *Drosophila* conveys species-specific behavioral characteristics. Genetics 158:1535–1543.

Cline TW, Meyer BJ. 1996. Vive la différence: Males vs. females in flies vs. worms. Annu Rev Genet 30:637–702.

Cobb M, Ferveur J-F. 1996. Evolution and genetic control of mate recognition and stimulation in *Drosophila.* Behav Process 35:35–54.

Davis T, Kurihara J, Yamamoto D. 2000a. Genomic organization and characterization of the neural sex-determination gene *fruitless* in the Hawaiian species *Drosophila heteroneura*. Gene 246:143–149.

Davis T, Ito H. 2001. Genomic structure of the sexual behaviour gene *fruitless*. Drosophila Information Service 84:65–66.

Davis T, Kurihara J, Yoshino E, Yamamoto D. 2000b. Genomic organization of the neural sex determination gene *fruitless* (*fru*) in the Hawaiian species *Drosophila silvestris* and the conservation of the *fru* BTB protein-protein-binding domain throughout evolution. Hereditas 132:67–78.

DeGregorio E, Spellman PT, Rubin GM, Lemaitre B. 2001. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. Proc Natl Acad Sci USA 98:12590–12595.

Dow MA. 1975. Environmental modification of a genetically based behaviour pattern in *D. melanogaster*. Nature 255:172.

Dow MA. 1977. Activity of *yellow* females. Drosophila Information Service 52:85.

Drapeau MD. 2001a. Beyond heritability: the future of behavioral genetics. Trends Genet 17:561–562.

Drapeau MD. 2001b. The family of Yellow-related *Drosophila melanogaster* proteins. Biochem Biophys Res Comm 281:611–613.

Drapeau MD, Long AD. 2000. The Copulatron, a multichamber apparatus for observing *Drosophila* courtship behaviors. Drosophila Information Service 83:194–196.

Ewing AW. 1979. The neuromuscular basis of courtship song in *Drosophila*: The role of the direct and axillary wing muscles. J Comp Physiol 130:87–93.

FlyBase. 1999. The FlyBase database of the *Drosophila* genome projects and community literature. Nucleic Acids Res 27:85–88. http://flybase.bio.indiana.edu/

Gaines P, Tompkins L, Woodard CT, Carlson JR. 2000. *quick-to-court*, a *Drosophila* mutant with elevated levels of sexual behavior, is defective in a predicted coiled-coil protein. Genetics 154:1627–1637.

Geyer PK, Spana C, Corces VG. 1986. On the molecular mechanism of *gypsy*-induced mutations at the *yellow* locus of *Drosophila melanogaster*. EMBO J 5:2657–2662.

Gill KS. 1965. A mutation causing abnormal mating behavior. Drosophila Information Service 38:33.

Goodwin SF. 1999. Molecular neurogenetics of sexual differentiation and behavior. Curr Opin Neurobiol 9:759–765.

Goodwin SF, Taylor BJ, Villella A, Foss M, Ryner LC, Baker BS, Hall JC. 2000. Aberrant splicing and altered

spatial expression patterns in *fruitless* mutants of *Drosophila*. Genetics 154:725–745.

Greenspan RJ. 1997. A kinder, gentler analysis of behavior: dissection gives way to modulation. Curr Opin Neurobiol 7:805–811.

Greenspan RJ, Ferveur J-F. 2000. Courtship in *Drosophila.* Annu Rev Genet 34:205–232.

Hall JC. 1978. Courtship among males due to a male-sterile mutation in *Drosophila melanogaster.* Behav Genet 8:125–141.

Hall JC. 1979. Control of male reproductive behavior by the central nervous system of *Drosophila*—dissection of a courtship pathway by genetic mosaics. Genetics 92:437–457.

Hall JC. 1994a. The mating of a fly. Science 264:1702–1714.

Hall JC. 1994b. Pleiotropy of behavioral genes. In: Greenspan RJ, Kyriacou CP, editors. Flexibility and Constraint in Behavioral Systems. New York: Wiley, p 15–27.

Hall JC, Greenspan RJ, Harris WA. 1982. Genetic Neurobiology. Cambridge, MA: The MIT Press. 284 p.

Han Q, Fang J, Ding H, Johnson JK, Christensen BM, Li J. 2002. Identification of *Drosophila melanogaster yellow-f* and *–f2* proteins as dopachrome conversion enzymes. Biochem J. Immediate Publication, doi:10.1042/BJ20020272.

Hannah A. 1953. Non-autonomy of *yellow* in gynandromorphs of *Drosophila melanogaster.* J Exp Zool 123: 523–560.

Heinrichs V, Ryner LC, Baker BS. 1998. Regulation of sex-specific selection of *fruitless* 5′ splice sites by *transformer* and *transformer-2*. Mol Cell Biol 18:450–458.

Hodgetts RN, Konopka RJ. 1973. Tyrosine and catecholamine metabolism in wild-type *Drosophila melanogaster* and a mutant, *ebony.* J Insect Physiol 19:1211–1220.

Hough CD, Woods DF, Park S, Bryant PJ. 1997. Organizing a functional junctional complex requires specific domains of the *Drosophila* MAGUK *Discs large*. Genes Dev 11:3242–3253.

Hovemann BT, Ryseck RP, Walldorf U, Stortkuhl KF, Dietzel ID, Dessen E. 1998. The *Drosophila ebony* gene is closely related to microbial peptide synthetases and shows specific cuticle and nervous system expression. Gene 221:1–9.

Ito H, Fujitani K, Usui K, Shimizu-Nishikawa K, Tanaka S, Yamamoto D. 1996. Sexual orientation in *Drosophila* is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes and zinc finger protein with a BTB domain. PNAS USA 93:9687–9692.

Ives PT. 1970. Further genetic studies of the South Amherst population of *Drosophila melanogaster*. Evolution 24: 507–518.

Johnson JK, Li J, Christensen BM. 2001. Cloning and characterization of a dopachrome conversion enzyme from the yellow fever mosquito, *Aedes aegypti*. Insect Biochem Mol Biol 31:1125–1135.

Kalmus H. 1943. The optomotor responses of some eye mutants in *Drosophila.* J Genet 45:206–213.

Kazantsev A, Walker HA, Slepko N, Bear JE, Preisinger E, Steffan JS, Zhu YZ, Gertler FB, Housman DE, Marsh JL, Thompson LM. 2002. A bivalent Huntington binding peptide suppressed polyglutamine aggregation and pathogenesis in *Drosophila.* Nat Genet 30: 367–376.

Konopka RJ. 1972. Abnormal concentrations of dopamine in a *Drosophila* mutant. Nature 239:281–282.

Kornezos A, Chia W. 1992. Apical secretion and association of the *Drosophila yellow* gene product with developing larval cuticular structures during embryogenesis. Mol Gen Genet 235:397–405.

Kucharski R, Maleszka R. 1998. A Royal Jelly Protein is expressed in a subset of Kenyon cells in the mushroom bodies of the honey bee brain. Naturwissenshaften 85: 343–346.

Kyriacou CP, Burnet B, Connolly K. 1978. Behavioral basis of overdominance in competitive mating success at the *ebony* locus of *Drosophila melanogaster.* Animal Behav 26:1195–1206.

Kyriacou CP, Hall JC. 1986. Interspecific genetic control of courtship song production and reception in *Drosophila*. Science 232:494–497.

Lai CG, Lyman RF, Long AD, Langley CH, Mackay TFC. 1994. Naturally-occurring variation in bristle number and DNA polymorphisms at the *scabrous* locus of *Drosophila melanogaster*. Science 266:1697–1702.

Lee G, Foss M, Goodwin SF, Carlo T, Taylor BJ, Hall JC. 2000. Spatial, temporal, and sexually dimorphic expression patterns of the *fruitless* gene in the *Drosophila* central nervous system. J Neurobiol 43:404–426.

Lee G, Hall JC. 2000. A newly uncovered phenotype associated with the *fruitless* gene of *Drosophila melanogaster*: Aggression-like head interactions between mutant males. Behav Genet 30:263–275.

Lee G, Hall JC. 2001. Abnormalities of male-specific FRU protein and serotonin expression in the CNS of *fruitless* mutants. J Neurosci 21:513–526.

Levine J. 1973. Properties of the nervous system controlling flight in *Drosophila melanogaster*. J Comp Physiol 84: 129–166.

Lindsley DL, Zimm GG. 1992. The Genome of *Drosophila melanogaster.* San Diego, CA: Academic Press, Inc. 1133 p.

Long AD, Lyman RF, Langley CH, Mackay TFC. 1998. Two sites in the Delta gene region contribute to naturally occurring variation in bristle number in *Drosophila melanogaster*. Genetics 149:999–1017.

Maleszka R, Kucharski R. 2000. Analysis of *Drosophila yellow-b* cDNA reveals a new family of proteins related to the Royal Jelly proteins in the honeybee and to an orphan protein in an unusual bacterium *Deinococcus radiodurans.* Biochem Biophys Res Commun 270:773–776.

Manseau L, Baradaran A, Brower D, Budhu A, Elefant F, Phan H, Philp AV, Yang M, Glover D, Kaiser K, et al.

1997. GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of *Drosophila.* Dev Dyn 209:310–322.

Morgan TH. 1911. The origin of nine-wing mutations in *Drosophila*. Science 33:496–499.

Munte A, Aguade M, Segarra C. 1997. Divergence of the *yellow* gene between *Drosophila melanogaster* and *D. subobscura*: Recombination rate, codon bias, and synonymous substitutions. Genetics 147:165–175.

Nachtigall W, Wilson DM. 1967. Neuro-muscular control of Dipteran flight. J Exp Biol 47:77–97.

Nash WG, Yarkin RJ. 1974. Genetic regulation and pattern formation – study of *yellow* locus in *Drosophila melanogaster*. Genet Res 24:19–26.

Neckameyer W, O'Donnell J, Huang ZN, Stark W. 2001. Dopamine and sensory tissue development in *Drosophila melanogaster.* J Neurobiol 47:280–294.

Neckameyer WS. 1996. Multiple roles for dopamine in *Drosophila* development. Dev Biol 176:209–219.

Newcomb RD, Lambert DM. 1988. The sensitive period for *yellow* phenocopy induction in *Drosophila melanogaster.* Experentia 44:618–621.

Orgad S, Rosenfeld G, Greenspan RJ, Segal D. 2000. *Courtless*, the *Drosophila* UBC7 homolog, is involved in male courtship behavior and spermatogenesis. Genetics 155: 1267–1280.

Orr HA. 2001. The genetics of species differences. Trends Ecol Evol 16:343–350.

Parmentier M-L, Woods D, Greig S, Phan PG, Radovic A, Bryant P, O'Kane CJ. 2000. Rapsynoid/Partner of Inscutable controls asymmetric division of larval neuroblasts in *Drosophila*. J Neurosci 20:RC84 (1–5).

Piccin A, Salameh A, Benna C, Sandrelli F, Mazzotta G, Zordan M, Rosato E, Kyriacou CP, Costa R. 2001. Efficient and heritable functional knock-out of an adult phenotype in *Drosophila* using a GAL4-driven hairpin RNA incorporating a heterologous spacer. Nucleic Acids Res 29:e55.

Radovic A, Wittkopp PJ, Long AD, Drapeau MD. 2002. Immunohistochemical colocalization of Yellow and male-specific Fruitless in *Drosophila melanogaster* neuroblasts. Biochem Biophys Res Commun 293:1262–1264.

Rendel JM. 1951. Mating of *ebony vestigal* and wild type *Drosophila melanogaster* in light and dark. Evolution 5:226–230.

Robinow S, White K. 1991. Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. J Neurobiol 22:443–461.

Romanova LG, Romanova NI, Subocheva EA, Kim AI. 2000. [Mating success and courtship ritual in strains of *Drosophila melanogaster* carrying mutation *flamenco*] (in Russian). Genetika 36:500–504.

Ryner LC, Goodwin SF, Castrillon DH, Anand A, Villella A, Baker BS, Hall JC, Taylor BJ, Wasserman SA. 1996. Control of male sexual behavior and sexual orientation in *Drosophila* by the *fruitless* gene. Cell 87:1079–1089.

Sokolowski MB. 2001. *Drosophila*: Genetics meets behavior. Nat Rev Genet 2:879–890.

Stern DL. 1998. A role of *Ultrabithorax* in morphological differences between *Drosophila* species. Nature 396:463–466.

Sturtevant AH. 1913. The linear arrangement of six sex-linked factors in *Drosophila*, as shown by their mode of association. J Exp Zool 14:43–59.

Sturtevant AH. 1915. Experiments on sex recognition and the problem of sexual selection in *Drosophila.* J Anim Behav 5:351–366.

Sucena E, Stern DL. 2000. Divergence of larval morphology between *Drosophila sechellia* and its sibling species caused by *cis*-regulatory evolution of *ovo/shaven-baby*. PNAS USA 97:4530–4534.

Threlkeld SFH, Procwat RA, Abbott KS, Yeung AD. 1974. Genetically based behaviour patterns in *Drosophila melanogaster*. Nature 247:232–233.

Toivonen JM, O'Dell KMC, Petit N, Irvine SC, Knight GK, Lehtonen M, Longmuir M, Luoto K, Touraille S, Wang ZS, et al. 2001. *technical knockout*, a *Drosophila* model of mitochondrial deafness. Genetics 159: 241–254.

Tully T, Gergen JP. 1986. Deletion mapping of the *Drosophila* memory mutant *amnesiac.* J Neurogenet 3:33–47.

Usui-Aoki K, Ito H, Ui-Tei K, Takahashi K, Lukacsovich T, Awano W, Nakata H, Piao ZF, Nilsson EE, Tomida J, et al. 2000. Formation of the male-specific muscle in female *Drosophila* by ectopic *fruitless* expression. Nat Cell Biol 2:500–506.

Villella A, Gailey DA, Berwald B, Oshima S, Barnes PT, Hall JC. 1997. Extended reproductive roles of the *fruitless* gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. Genetics 147:1107–1130.

von Schlicher F, Hall JC. 1979. Neural topography of courtship song in sex mosaics of *Drosophila melanogaster.* J Comp Physiol 129:85–95.

Walter MF, Black BC, Afshar G, Kermabon AY, Wright TRF, Biessmann H. 1991. Temporal and spatial expression of the *yellow* gene in correlation with cuticle formation and Dopa decarboxylase activity in *Drosophila* development. Dev Biol 147:32–45.

Walter MF, Zeineh LL, Black BC, McIvor WE, Wright TRF, Biessmann H. 1996. Catecholamine metabolism and *in vitro* induction of premature cuticle melanization in wild type and pigmentation mutants of *Drosophila melanogaster*. Arch Insect Biochem Physiol 31:219–233.

Wilson R, Burnet B, Eastwood L, Connolly K. 1976. Behavioral pleiotropy of *yellow* gene in *Drosophila melanogaster.* Genet Res 28:75–88.

Wittkopp PJ, True JR, Carroll SB. 2002a. Reciprocal functions of the *Drosophila* Yellow and Ebony proteins in the

development and evolution of pigment patterns. Development 129:1849–1858.

Wittkopp PJ, Vaccaro K, Carroll SB. 2002b. Evolution of *yellow* gene regulation and pigmentation in *Drosophila.* Curr Biol 12:1547–1556.

Woods D, Bryant PJ. 1991. The *discs-large* tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. Cell 66:451–464.

Wright TRF. 1987. The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster.* Adv Genet 24:127–222.

Yamamoto D, Fujitani K, Usui K, Ito H, Nakano Y. 1998. From behavior to development: genes for sexual behavior define the neuronal sexual switch in *Drosophila.* Mech Dev 73:135–146.

Yamamoto D, Jallon JM, Komatsu A. 1997. Genetic dissection of sexual behavior in *Drosophila melanogaster.* Annu Rev Ent 42:551–585.

Yamamoto D, Nakano Y. 1998. Genes for sexual behavior. Biochem Biophys Res Commun 246:1–6.

Yamamoto D, Nakano Y. 1999. Sexual behavior mutants revisited: molecular and cellular basis of *Drosophila* mating. Cell Mol Life Sci 56:634–646.