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Immunohistochemical colocalization of Yellow and male-specific Fruitless in *Drosophila melanogaster* neuroblasts

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Abstract

The *Drosophila melanogaster fruitless* gene encodes multiple male-specific transcription factors that are hypothesized to regulate a hierarchy of genes responsible for the development of male courtship behavior. Here we show that there are dramatically increased levels of the protein product of the male courtship behavior gene *yellow* associated with male-specific Fruitless protein in a subset of neuroblasts in third-instar larval male brains. We hypothesize that *yellow* is downstream of *fruitless* in a male courtship behavior developmental genetic pathway. © 2002 Elsevier Science (USA). All rights reserved.

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In *Drosophila melanogaster*, the BTB-ZF (*Bric-a-brac, Tramtrack and Broad Complex*-like Zinc-Finger)-family transcription factor Fruitless (FRU) is a member of the cell-autonomous somatic sex-determination hierarchy, is limited to the central nervous system (CNS), and is hypothesized to control a regulatory cascade responsible for proper development of adult male sexual behaviors [1,2]. These behaviors are a small group of separate but interrelated fixed-action patterns and include wing extension, courtship song (wing vibration following extension), and attempted copulation [1,3–5], whose developmental foci are in distinct regions of the CNS [6]. It is widely hypothesized that genes downstream of the three known male-specific FRU zinc-finger transcription factors (FRU^M) are necessary for the proper development and/or maintenance of each of these specific aspects of male courtship behavior [1,2,7]. Here we report that the protein product of the courtship behavior gene *yellow*, Yellow, is associated with FRU^M in male brains. To our knowledge, this is the first report of a protein associated with, and a gene possibly downstream of, male-specific FRU transcription factors.

Materials and methods

The wild-type standard laboratory strain Oregon-R and the *yellow*-null strain $Df(1)y\text{-}ac^{22}$ were used in this study. Third instar larvae were sexed and brains were dissected and processed as previously described [8]. Primary antibodies were rat anti-FRU^M (1:300) [5], rat anti-FRU^{COM} (1:300) [9], and Guinea pig anti-DLG (1:1000). Secondary Cy2, Cy3, or Cy5 conjugated antibodies were used (Jackson; 1:100). We raised a polyclonal rabbit anti-Yellow antibody against all but the first seven amino acids of the Yellow protein [10], and it was used at a dilution of 1:150. Images were taken on a BioRad MRC 1024 confocal microscope. Utilization of the *discs large* (DLG) neural cell membrane marker [8,11] allowed visualization of different cell types within the brain, and localization of Yellow within cells.

Results and discussion

We immunohistochemically localized the expression of FRU^M and Yellow in male and female larval brains.

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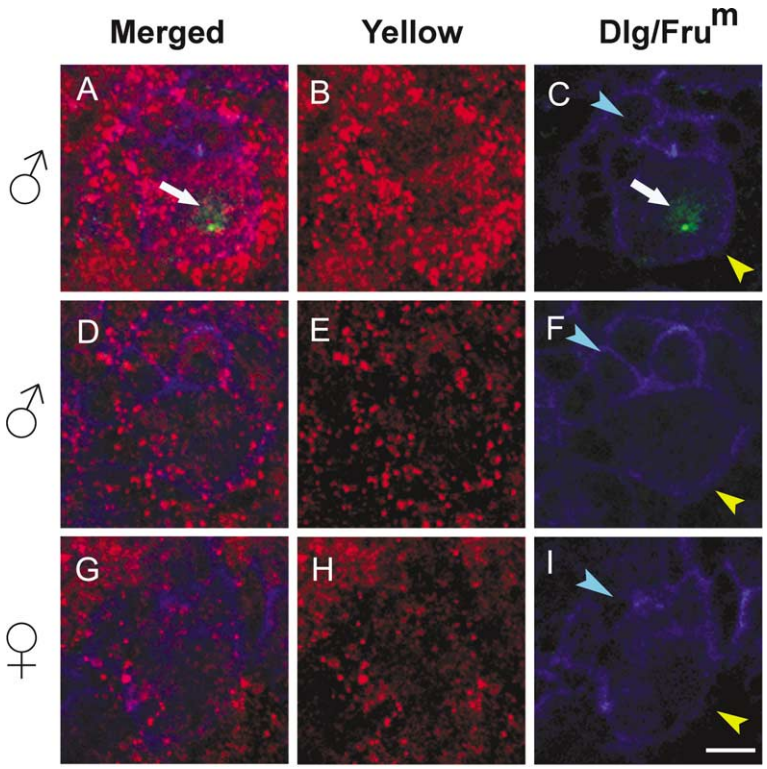

Fig. 1. Expression of male Fruitless in larval neuroblasts is correlated with higher levels of Yellow. Confocal images of neuroblasts from male (A–F) and female (G–I) larval brains labeled with anti-Yellow (red), anti-FRU^{M} (green), and anti-Dlg (blue). Anti-Dlg was used as a marker for the membrane outline of neuronal cells. Yellow-colored arrowheads indicate neuroblasts and blue arrowheads indicate ganglion mother cells. A neuroblast from a male brain that shows FRU^{M} expression in the nucleus (indicated by an arrow) (A–C) shows significantly higher levels of Yellow than neuroblasts from male brains with no FRU^{M} (D–F), and from female brains (G–I). Several cells were observed with higher Yellow levels and no significant FRU^{M} signal. We attribute this to the fact that those cells were undergoing mitosis, and FRU^{M} was dispersed throughout the cytoplasm. Bar: approximately 5 μm.

At the late-3rd-instar larval developmental stage, the start of the critical period for programming male-specific behavior [12], numerous male dorsal posterior neuroblasts which show anti-FRU^{M} staining in the nucleus also show a high level of anti-Yellow staining in both the cytoplasm and in the area surrounding the cell (Fig. 1A–C). The latter is perhaps a consequence of Yellow being secreted into and/or inherited by the progeny of the FRU^{M}-expressing neuroblast. Yellow is semi-non-autonomous and is known to be a secreted molecule in at least one cell type, the cuticle, where it plays a biochemical role in melanization [10,13]. FRU^{M} is only known to be present in a small subset of cells in the male brain [1,2,9], and wild-type male neuroblasts in the same brains not showing FRU^{M} expression did not show a correlated increase of Yellow levels (Fig. 1D–F). Since FRU^{M} is a predicted transcription factor (and Yellow appears not to be [13,14]), this result suggests that FRU^{M}, or a downstream target(s) of FRU^{M}, upregulates *yellow*. Wild-type 2nd-instar larval brains neither show reactivity with anti-FRU^{M} nor with anti-Yellow (results not shown), consistent with the timing of male-specific *fru* expression starting in the 3rd larval instar [9], and again suggesting a molecular association between FRU^{M} and Yellow. It is noteworthy that there was no correlated presence of Y in or near male or female brain cells associated with the staining pattern of an antibody against a portion of FRU common to male-, female-, and sex-non-specific proteins (FRU^{COM}; results not shown) [9].

Previous to this study, nothing was known about Yellow presence, distribution, or regulation in the CNS. Besides Yellow protein which is associated with FRU^{M} in male brains (Fig. 1A–C), we also noted Yellow protein distributed across both male and female brains that was not associated with FRU^{M}-expressing cells (Fig. 1D–I). Although at this time any role for non-FRU^{M} associated Yellow would be highly speculative, the anti-Yellow staining pattern is shown to be representative of the pattern of Yellow expression in male and female brains by the absence of anti-Yellow staining in *Df(1)y-ac*^{22} flies with a null *yellow* gene (results not shown). Hence, Yellow appears to be present in the brain through at least two different mechanisms: possible upregulation of *yellow* via FRU^{M}, and non-FRU mediated *yellow* function.

The current major question in the development of *D. melanogaster* male courtship behavior is, what genes are downstream of FRU^M, and how do they work together during development to build the neural circuitry underlying adult behavior [1,2]? Despite our demonstrated association of Y and FRU^M in the brain, the data reported here do not constitute solid evidence that *yellow* is downstream of FRU^M. However, a behavioral line of evidence suggests that this is the case. Lesions in both *fru* and *yellow* are known to cause defects in a specific aspect of the male courtship ritual, wing extension [7,15,16]. However, viable *fru* mutants generally affect multiple other aspects of the male ritual [7], while *yellow* lesions appear to specifically affect wing extension ([15,16]; M.D.D. and A.D.L., unpublished observations). Additionally, *fru* lesions affecting wing extension generally reduce it to roughly 1–3% of its normal level [7], while *yellow* null mutant males have roughly 45–50% the normal level of wing extension ([14,15]; M.D.D. and A.D.L., unpublished observations). These data are consistent with a model in which *fru*, a downstream member of the well-characterized sex-determination cascade [2], encodes multiple male-specific transcription factors which regulate downstream genes in pathways which control the development of multiple aspects of the male courtship ritual, and *yellow* is a downstream gene in a pathway branch only necessary for proper development of wing extension. However, only genetic experiments consisting of manipulation of male-specific *fru* expression will be able to determine if *yellow* is indeed part of the genetic hierarchy responsible for normal development of *D. melanogaster* male courtship behavior.

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