Fly 8:2, 95–100; April/May/June 2014; © 2014 Landes Bioscience

Fruitless isoforms and target genes specify the sexually dimorphic nervous system underlying *Drosophila* reproductive behavior

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Courtship is pivotal to successful reproduction throughout the animal kingdom. Sexual differences in the nervous system are thought to underlie courtship behavior. Male courtship behavior in *Drosophila* is in large part regulated by the gene *fruitless* (*fru*). *fru* has been reported to encode at least three putative BTB-zinc-finger transcription factors predicted to have different DNA-binding specificities. Although a large number of previous studies have demonstrated that *fru* plays essential roles in male courtship behavior, we know little about the function of Fru isoforms at the molecular level. Our recent study revealed that male-specific Fru isoforms are expressed in highly overlapping subsets of neurons in the male brain and ventral nerve cord. Fru isoforms play both distinct and redundant roles in male courtship behavior. Importantly, we have identified for the first time, by means of the DamID technique, direct Fru transcriptional target genes. Fru target genes overwhelmingly represent genes previously reported to be involved in the nervous system development, such as *CadN*, *lola* and *pdm2*. Our study provides important insight into how the sexually dimorphic neural circuits underlying reproductive behavior are established.

Keywords: sexual behavior, courtship, sexual dimorphism, *fruitless*, transcription factor, DamID, nervous system development

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Submitted: 03/25/2014

Revised: 04/29/2014

Accepted: 05/06/2014

Published Online: 05/09/2014

http://dx.doi.org/10.4161/fly.29132

Extra View to: Neville MC, Nojima T, Ashley E, Parker DJ, Walker J, Southall T, Van de Sande B, Marques AC, Fischer B, Brand AH, et al. Male-specific fruitless isoforms target neurodevelopmental genes to specify a sexually dimorphic nervous system. Curr Biol 2014; 24:229-41; PMID:24440396; http://dx.doi.org/10.1016/j.cub.2013.11.035

fru is a Masculinization Factor Required for Male Sexual Behavior

Male fruit flies carrying mutations in the *fru* locus show little, if any, courtship toward virgin females, resulting in limited reproductive success. In addition, *fru* mutants can also display elevated levels of courtship toward males, suggesting they have lost their ability to discriminate between the sexes.[1-5] Importantly, when *fru* is artificially expressed in the female nervous system, these females are able to display a range of, although not all, male-typical courtship behaviors.[6,7] In addition to these behavioral phenotypes, *fru* also plays a role in the induction of the male-specific muscle of Lawrence (MOL), formed in the fifth segment of the adult abdomen.[2,8-12] *fru* is expressed in a subset of neurons exclusively in the male nervous system, where it has been shown to contribute to neuronal dimorphisms between the sexes.[10,13,14] One of the most striking examples of this dimorphism in the brain is seen in the male-specific P1 neural cluster[15] (also known as pMP-e[16] or pMP4[17]), which consists of approximately 20 posteriorly located neurons that extend their neurites toward the anterior region of the superior protocerebrum. The male-specific P1 neurons are established through the action of *fru*, along with another sex determination gene *doublesex* (*dsx*).[15] The P1 neurons are thought to be courtship-triggering neurons, because, when these 20 neurons are ectopically induced in females, these females display male-typical courtship behaviors, even if other cells throughout the nervous system and body maintain a female identity.[15] In addition, when the P1 neurons are artificially activated, males display courtship behavior even in the absence of a potential mate.[18,19] Another well-characterized *fru*-expressing neuron is the MOL-inducing (Mind) motoneuron. The Mind neuron is a single

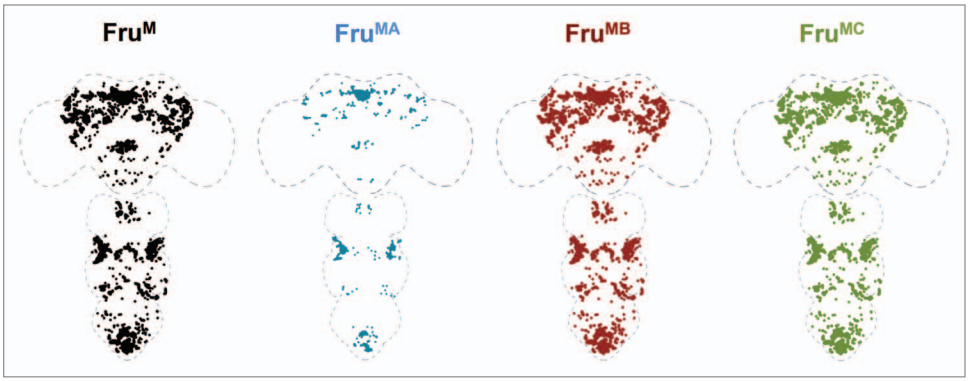

Figure 1. Schematic drawings showing the distributions of Fru^{M} isoforms in the CNS. All three Fru^{M} isoform distributions as detected with an anti-Fru^{M} antibody are shown in black. The distributions of Fru^{MA}, Fru^{MB} and Fru^{MC} as detected with isoform specific antibodies are shown in blue, red and green, respectively. Fru^{M} isoforms show a highly overlapping expression pattern.

glutamatergic motoneuron located in the abdominal ganglion. The Mind motoneuron innervates the MOL and supplies an inductive signal cell-non-autonomously by dynamin-dependent exocytosis during metamorphosis. The Mind motoneuron is inferred to be male-specific, and Fru may prevent this neuron from programmed cell death, because artificial expression of either *fru* or a cell death inhibitor, *p35*, in motoneurons ectopically induces the MOL in females, which otherwise lack it.[5,12]

The *fru* gene encodes multiple putative transcription factors.[2,3] The *fru* locus has at least four promoters, however only the proteins produced from the most distal promoter, P1, are male-specific (Fru^{M}).[3,10,13,20,21] Through alternative splicing, three protein isoforms are produced from the P1 promoter: Fru^{MA}, Fru^{MB}, and Fru$^{MC\ 3,\ 11}$ (also known as Fru^{AM}, Fru^{EM}, and Fru^{BM}, respectively[10]). Each isoform has a shared N-terminal BTB dimerization domain and distinct C-terminal zinc-finger motif containing domains.[2,3,10,11] What are the functional differences between the isoforms? A previous study began to unravel these diferences with the identification of a mutant specific to the Fru^{C} isoform (*fru*$^{\Delta C}$).[11] This study showed that the formation of the MOL depends solely on the expression of Fru^{MC} in the motoneurons. Fru^{MC} was also shown to play roles in male sexual behavior: males lacking the Fru^{MC} isoform show decreased mating success and fertility. Isoform complexity was further expanded by the analysis of male-specific serotonergic neurons in the abdominal ganglion, where both Fru^{MB} and Fru^{MC}, but not Fru^{MA}, were shown to be required for their sexually dimorphic innervations to the male reproductive system.[11,22,23]

Respective Roles of Fru Isoforms in Male Sexual Behavior

Why does *fru* encode multiple isoform variants and how do they individually contribute to the observed behavioral phenotypes in males? Other closely related BTB-zinc-finger genes also encode multiple isoforms, which often vary in the developmental and tissue specificity of their expression, suggesting variation in expression is fundamental to their individual functions. To determine if Fru^{M} isoforms display unique expression patterns in the central nervous system (CNS), contributing to their individual functions, we examined the distribution of the three Fru^{M} isoforms in the CNS and found, perhaps surprisingly, that Fru^{M} isoforms have highly overlapping patterns, with only the Fru^{A} distribution being in a much smaller subset of Fru^{M} neurons[24] (see schematic in **Figure 1**). These observations are consistent with a recently published complementary study that examined the distributions of the respective Fru isoforms in the CNS,[25] however this study found broader expression of the Fru^{A} isoform (a difference that could be a result of the unique antibodies used between studies, compounded by the apparent low level expression of Fru^{A} observed in both studies). Therefore broad differences in expression do not underlie Fru^{M} isoform-specific functions.

To directly compare the roles of all Fru male-specific isoforms, we set out to generate a full complement of Fru^{M} isoform-specific mutants for behavioral analyses. We established strains of flies carrying mutations in either Fru^{A}- or Fru^{B}-encoding exons, generating the novel mutants *fru*$^{\Delta A}$ and *fru*$^{\Delta B}$, respectively. We confirmed that *fru*$^{\Delta A}$ and *fru*$^{\Delta B}$ mutants specifically lack the Fru^{A} or Fru^{B} isoforms, respectively, in the adult CNS based on immunohistochemical staining with isoform-specific Fru antibodies. Consistent with previous studies,[5,10,12] we found that *fru*$^{\Delta C}$ mutant males lack the MOL, whereas neither *fru*$^{\Delta A}$ nor *fru*$^{\Delta B}$ mutant males do, again confirming that only the Fru^{MC} isoform is indispensable for the MOL formation.

Isoform-specific *fru* mutant males were examined in detail for their sexual behavior (summarized in **Table 1**). First, we performed single-pair mating assays, in which a male and a virgin female are put into a small observation chamber and the male's mating performance toward the female is recorded and analyzed afterwards. Under these experimental conditions, *fru*$^{\Delta B}$ mutant males less vigorously court females and show significantly decreased copulation success, as compared with control or other isoform-specific mutants. Even those that successfully copulate with females, take a significantly longer time getting there. *fru*$^{\Delta C}$ mutant males, in contrast, do not show any defects in courtship latency or courtship index. However, they never manage to copulate within the one-hour observation period, which may be, at least in part, due to significantly reduced levels of unilateral wing extension used to generate courtship song. We additionally conducted a behavioral assay in which multiple males are grouped together in the same observation chamber. It was previously reported that, when *fru* mutant males are grouped, they form so-called "courtship chains," in which a courting male is courted by another male which in turn is courted by another male, resulting in the long chain-like formations.[2] In our

assays, courtship chains were observed in both $fru^{\Delta B}$ and $fru^{\Delta C}$ mutant groups, suggesting both mutants have, at least partially, lost their ability to discriminate between the sexes. Interestingly, no detectable behavioral defects were found in our $fru^{\Delta A}$ mutant males under our experimental conditions, although another recently published study using an independently generated mutation in the Fru^{A}-encoding exon reported that Fru^{A} appears to play a role in copulation success (**Table 1**).[25]

During courtship, males extend their wings unilaterally to generate species-specific courtship song.[26] Courtship song consists of two discrete elements: alternating continuous oscillations called "sine song" and trains of pulses called "pulse song." The time between pulses (interpulse interval or IPI) varies among different species. The IPI of *D. melanogaster* is approximately 34 ms on average,[27] and that of a closely related species, *D. simulans*, is approximately 48 ms.[28] Courtship song contributes to species recognition and renders conspecific females sexually receptive.[27,29-32] We recorded and analyzed the sine and pulse songs generated by our isoform-specific *fru* mutants. $fru^{\Delta B}$ males show a slight but significantly longer IPI of nearly 40 ms, while $fru^{\Delta C}$ males showed an even longer IPI of 45 ms, which is much closer to the IPI of *D. simulans* than that of *D. melanogaster*.[28] Another striking song deficit observed in $fru^{\Delta C}$ males is the consistent and complete absence of sine song. Although we did not find any abnormalities in courtship song generated by males lacking Fru^{MA}, von Philipsborn et al., 2014[25] using their fru^{A} mutant, reported mild song defects including a longer IPI (**Table 1**). The differences in the behavioral profiles of the isoform-specific *fru* mutants between these studies may be at least in part due to the different techniques used to establish the mutant strains, the differing allelic combinations used, along with differing experimental conditions. Based on these observations, courtship song appears to be specified in large part by the Fru^{MC} isoform.

Collectively our phenotypic analyses using isoform-specific *fru* mutants show that the overall performance of courtship behavior can occur in the absence of any one isoform, although some phenotypes, such as the production of sine song, clearly depend on only one isoform. Clearly the Fru^{MB} and Fru^{MC} isoforms are the major players in most aspects of male sexual behavior, while the Fru^{MA} isoform plays a minor role, perhaps functioning more specifically in subtle phenotypes associated with the broad range of sensory inputs a male experiences in a natural setting. Interestingly, these findings may be supported by a previous study examining evolutionary changes and conservation of the *fru* locus[33] (**Fig. 2**). In *D. melanogaster*, *fru* has three C_2H_2 zinc-finger motif-encoding exons: A, B, and C; however in some non-drosophilid holometabolous insect species, two other exons are known to exist in the *fru* locus: F and G. Malaria mosquitoes, *Anopheles gambiae*, have the F exon in the *fru* locus, in addition to the

Table 1. Summary of the behavioral profiles of isoform-specific *fru* mutants

		Neville et al., 2014	von Philipsborn et al., 2014
Courtship latency	A	Normal	
	B	Increased *	
	C	Normal	
Courtship index	A	Normal	
	B	Decreased ***	
	C	Normal	
Wing extension index	A	Normal	
	B	Normal	
	C	Decreased ***	
Copulation success in a short experimental period	A	Normal	Decreased ***
	B	Decreased ***	Decreased ***
	C	Lost ****	Decreased ***
Copulation latency	A	Normal	
	B	Increased **	
	C	Not applicable	
Fertility	A	Normal	Normal
	B	Normal	Decreased
	C	Decreased ***	Decreased
Chaining index	A	None	
	B	Increased *	
	C	Increased ***	
Interpulse interval	A	Normal	Increased ***
	B	Increased **	Increased ***
	C	Increased ***	Increased ***
Pulse frequency	A	Normal	Decreased ***
	B	Normal	Normal
	C	Increased *	Increased **
Sine song	A	Normal	Normal
	B	Normal	Normal
	C	Lost ****	Lost ****
Sine frequency	A	Normal	Increased ***
	B	Normal	Normal
	C	Normal	Normal

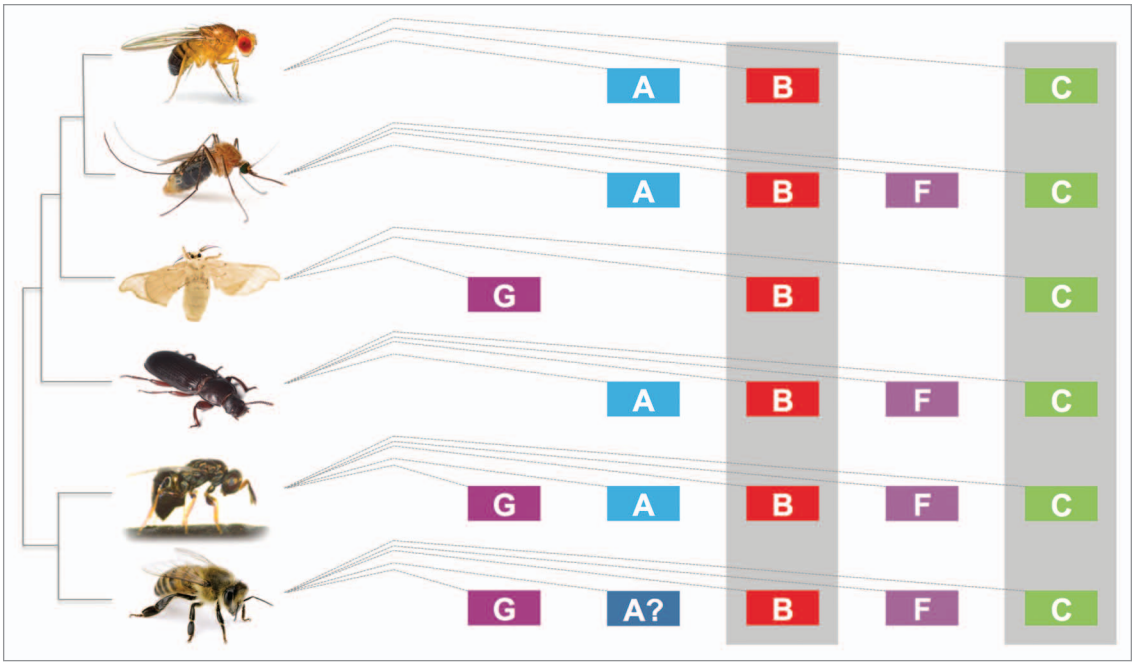

Figure 2. Comparison of Fru isoform-specific C_2H_2 zinc-finger containing exons between holometabolous insect species. From top to bottom, the Fru isoforms of *D. melanogaster*, *A. gambiae*, *B. mori*, *C. castaneum*, *N. vitripennis* and *A. melifera* are shown, respectively. In *A. melifera*, the A exon is either highly evolved or represents a novel exon. The conserved exons B and C are highlighted in gray. Figure modified from Bertossa et al., 2009.[33] ©Photographer name/Dreamstime.com for the images of *D. melanogaster*, *A. gambiae*, *B. mori*, *C. castaneum* and *A. melifera*. Image of *N. vitripennis* courtesy of Dr. Oliver Niehuis, University of Bonn.

A, B and C exons. In silkworm moths, *Bombyx mori*, the *fru* locus carries the G exon but lacks the A exon. In parasitoid wasps, *Nasonia vitripennis*, all five exons (A, B, C, F, and G) are included in the *fru* locus. Although the presence or absence of the respective C_2H_2 zinc-finger motif-encoding exons in the *fru* locus varies dramatically between species, only exons B and C are conserved in all species shown in **Figure 2**. Based on these observations, isoforms Fru^{MB} and Fru^{MC} likely play essential roles in various insect species, which is consistent with our findings that these isoforms are the major players in male-specific behaviors. The appearance and disappearance of *fru* C_2H_2 zinc-finger domains through presumably a combination of exon duplication and/or loss, enables a single gene to diversify its functions, while ensuring its essential functions are maintained. Interestingly, a recent finding by Parker et al., 2014[34] showed that the fru^A C_2H_2 zinc-finger containing exon is under strong positive selection within the *Drosophila* species, suggesting changes in this exon may contribute to speciation, while the other zinc-finger exons fru^B and fru^C are highly conserved.

fru Regulates the Transcription of Genes Required for the Development of the Nervous System, Thereby Specifying Sexually Dimorphic Neural Circuits

Although *fru* has long been postulated to encode transcription factors, its direct target genes were yet to be identified. Our recent study took advantage of the DNA adenine methyltransferase identification (DamID) technique to identify genes directly regulated by Fru.[35] We generated functional *Dam-fru* fusion constructs coding for all three Fru^M isoforms, as well as a control with a mutation in the C_2H_2 zinc-finger domain of Fru^{MB} rendering it unable to bind DNA. These *Dam-fru* fusions were expressed in flies and the CNSs were subsequently dissected out and then genomic DNA was isolated for analyses. As a result of the DamID experiments, we were able to show for the first time Fru^M isoform-specific interactions with the genome throughout development in the CNS. Interestingly, we found that many of the same genes were targeted by all three of the Fru^M isoforms throughout development, a highly significant proportion of which have previously been reported to play important roles in the development of the nervous system. This suggests potential cooperativity and/or redundancy in the targeting of these loci by multiple Fru^M isoforms, this complements our evolutionary understanding of Fru isoforms as well as the behavioral analysis of isoform-specific mutants.

We next looked for DNA motifs that were enriched in our Fru^M-genomic binding data and found that each isoform was associated with distinct motifs throughout development, suggesting unique DNA binding specificities. This complements the finding of Dalton et al., 2013[36] who used SELEX to show that Fru isoform-specific zinc-fingers indeed confer different DNA-binding specificities in vitro.[37] We found that the Fru^{MB}-enriched DNA motif was the most robust and consistent throughout development; in addition it closely resembles the in vitro binding site identified by Dalton et al., 2013.[36] As Fru^M is male-specific, we next tested whether Fru^{MB}-bound genomic regions containing the putative Fru^{MB}-binding sites exhibit sexually dimorphic expression patterns

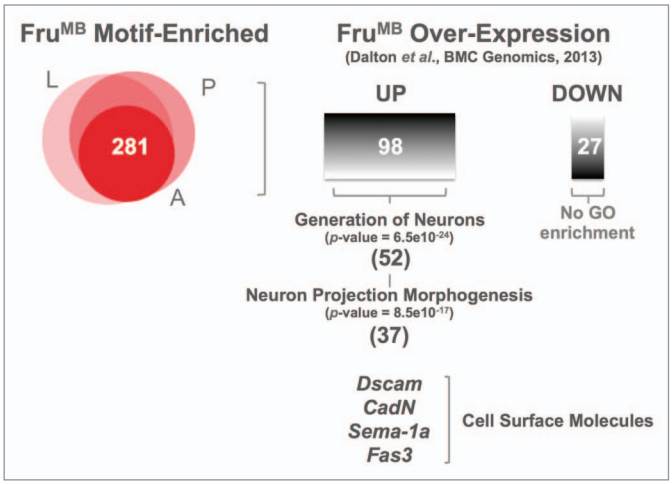

Figure 3. Fru^{MB} directly regulates key genes involved in sculpting the nervous system. Fru^{MB}-motif enriched genes found in the larvae (L), pupae (P) and adult (A) CNS were examined for expression changes in response to Fru^{MB} overexpression in *fruP1-GAL4* neurons.[36] A global gene ontological (GO) enrichment analysis of biological functions of up and downregulated genes revealed that genes known to play a role in the generation of neurons (GO:0048699) and more specifically in neuron projection morphogenesis (GO:0048812) were significantly enriched in those upregulated by Fru^{MB} overexpression, including key cell surface molecules.

in *fru*-expressing neurons. To do this, we made use of available FlyLight *Gal4* lines[38,39] carrying the genomic fragments of interest, in combination with *fru*^{FLP} to restrict GFP reporter expression (*UAS > stop > mCD8::GFP*) to *fru*-expressing neurons.[17] Of the 14 Gal4 lines where we observed expression in *fru*-expressing neurons, 10 showed sexually dimorphic expression patterns. Some lines, such as *lola-Gal4* (GMR44C03) and *stan-Gal4* (GMR32B11), show more intense expression in males than in females, and others, such as *pdm2-Gal4* (GMR11G05) and *Abl-Gal4* (GMR67B05), show female-biased expressions.

When examining the function of the genes associated with our identified Fru^{MB} motif we found a highly significant enrichment in genes associated with neuronal projection morphogenesis. Comparing these genes in relation to genes shown to be over- and under-expressed when the Fru^{MB} isoform is overexpressed in *fruP1-GAL4* neurons,[36,40] we found that the majority of the Fru^{MB} motif-enriched genes whose expression changes when Fru^{MB} is overexpressed appear to be directly upregulated by Fru^{MB} (**Fig. 3**). Interestingly, a functional gene ontology analysis showed that genes directly involved in the sculpting of the nervous system appear to be specifically upregulated by Fru^{MB}, including a number of key cell surface molecules, such as Dscam,[41] CadN,[42] Sema-1a,[43] and Fas3.[44] These results suggest that these cell surface molecules are likely indispensable for the establishment of the sexually dimorphic nervous system underlying sexual differences in behavior. Indeed, we found that a genomic enhancer associated with *CadN* (GMR32D06) shows sexually dimorphic expression patterns in the abdominal ganglion. In addition, when *CadN* expression was abolished in *fru*^{GAL4} neurons,[45] males display no courtship behavior toward females.[24] Cell surface molecules act as guidance cues in the nervous system, mediating the intricate connections between neuronal processes during development. Our data suggest that Fru acts to sculpt a sexually dimorphic nervous system, at least in part through the direct targeting of these cell surface molecules, where it likely changes the 'cocktail' of these molecules in a cell (or cluster)-specific manner, leading to the appropriate neuronal connections in males.

Future Investigation of Individual Fru Isoforms and Target Genes

Our recent study demonstrates that Fru isoforms play both specific and redundant roles in establishing the sexually dimorphic nervous system underlying male sexual behavior.[24] However, directly connecting Fru isoform function with the regulation of the specific target genes required for the sexual differentiation of certain subsets of neurons remains an open question. Future work will focus on first refining our studies of Fru-DNA interactions, unequivocally establishing binding-site specificity in vivo, in addition to examining the temporal dynamics of Fru^{M} binding at specific loci.

We would like to correlate occupancy with transcriptional control, however as Fru^{M} clearly plays key developmental roles in a large number of cell clusters throughout the nervous system, we will focus on small subsets of *fru*-expressing cells, establishing the specific developmental role Fru^{M} plays in establishing a transcriptional regulatory code leading to a male-specific nervous system.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work discussed here was supported in part by grants from the Wellcome Trust to S.F.G. (WT085521MA and WT082987MF) and the Natural Environment Research Council to S.F.G. (NE/J023647/1).

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