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

Tetsuya Nojima*, Megan C Neville, and Stephen F Goodwin
Department of Physiology, Anatomy and Genetics; University of Oxford; Oxford, UK

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.

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 cluster15 (also known as pMP-e16 or pMP417), 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 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.5 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
glutamergic motoneuron located in the abdominal ganglion. The Mind motoneuron innervates the MOL and supplies an inductive 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 the 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 (FruM).3,10,12 Through alternative splicing, three protein isoforms are produced from the P1 promoter: FruMA, FruMB, and FruMC 3,11 (also known as FruAM, FruBM, and FruMC, respectively). 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 differences with the identification of a mutant specific to the Fru isoform (fruMC).11 This study showed that the formation of the MOL depends solely on the expression of FruMC in the motoneurons. FruMC was also shown to play roles in male sexual behavior: males lacking the FruMC 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 FruMB and FruMC, but not FruMA, 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 FruM isoforms display unique expression patterns in the central nervous system (CNS), we examined the expression of the three FruM isoforms in the CNS and found, perhaps surprisingly, that FruM isoforms have highly overlapping patterns, with only the FruA distribution being in a much smaller subset of FruA neurons (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 FruA isoform (a difference that could be a result of the unique antibodies used between studies, compounded by the apparent low level expression of FruA observed in both studies). Therefore broad differences in expression do not underlie FruM isoform-specific functions.

To directly compare the roles of all Fru male-specific isoforms, we set out to generate a full complement of FruM isoform-specific mutants for behavioral analyses. We established strains of flies carrying mutations in either FruA- or FruB-encoding exons, generating the novel mutants fruAΔ and fruBΔ, respectively. We confirmed that fruAΔ and fruBΔ mutants specifically lack the FruA or FruB isoforms, respectively, in the adult CNS based on immunohistochemical staining with isoform-specific Fru antibodies. Consistent with previous studies,3,10,11 we found that fruMC mutant males lack the MOL, whereas neither fruAΔ nor fruBΔ mutant males do, again confirming that only the FruMC 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 the small observation chamber and the male’s mating performance toward the female is recorded and analyzed afterwards. Under these experimental conditions, fruAΔ 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. fruBΔ 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.3 In our

Figure 1. Schematic drawings showing the distributions of FruM isoforms in the CNS. All three FruM isoform distributions as detected with an anti-FruM antibody are shown in black. The distributions of FruMA, FruMB and FruMC as detected with isoform specific antibodies are shown in blue, red and green, respectively. FruM isoforms show a highly overlapping expression pattern.
assays, courtship chains were observed in both fruAΔ and fruBCΔ 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 fruAΔ mutant males under our experimental conditions, although another recently published study using an independently generated mutation in the FruA-encoding exon reported that FruA appears to play a role in copulation success (Table 1).  

During courtship, males extend their wings unilaterally to generate species-specific courtship song.  

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, and that of a closely related species, D. simulans, is approximately 48 ms. Courtship song contributes to species recognition and renders conspecific females sexually receptive. We recorded and analyzed the sine and pulse songs generated by our isoform-specific fru mutants. fruAΔ males show a slight but significantly longer IPI of nearly 40 ms, while fruBCΔ males showed an even longer IPI of 45 ms, which is much closer to the IPI of D. simulans than that of D. melanogaster. Another striking song deficit observed in fruBCΔ males is the consistent and complete absence of sine song. Although we did not find any abnormalities in courtship song generated by males lacking FruAΔ, von Philipsborn et al., 2014  

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                      |
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 C2H2 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* C2H2 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 showed that the *fru* C2H2 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 *fruB* and *fruC* are highly conserved.

**Figure 2.** Comparison of Fru isoform-specific C2H2 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. ©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.

**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. We generated functional Dam-fru fusion constructs coding for all three FruM isoforms, as well as a control with a mutation in C2H2 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 FruM 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 FruM 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 FruM 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 FruM-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 who used SELEX to show that Fru isoform-specific zinc-fingers indeed confer different DNA-binding specificities in vitro. 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. As FruM is male-specific, we next tested whether Fru MB-bound genomic regions containing the putative Fru MB-binding sites exhibit sexually dimorphic expression patterns.
in fru-expressing neurons. To do this, we made use of available FlyLight Gal4 lines\textsuperscript{38,39} carrying the genomic fragments of interest, in combination with fru\textsuperscript{\textit{P1}}-GAL4 to restrict GFP reporter expression (UAS $\rightarrow$ \\textit{stop} $\rightarrow$ mCD8::GFP) to fru-expressing neurons.\textsuperscript{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\textsuperscript{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\textsuperscript{MB} isoform is overexpressed in fruP1-GAL4 neurons,\textsuperscript{36,40} we found that the majority of the Fru\textsuperscript{MB} motif-enriched genes whose expression changes when Fru\textsuperscript{MB} is overexpressed appear to be directly upregulated by Fru\textsuperscript{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\textsuperscript{MB}, including a number of key cell surface molecules, such as Dscam,\textsuperscript{41} CadN,\textsuperscript{42} Sema-1a,\textsuperscript{43} and Fas3.\textsuperscript{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\textsuperscript{GAL4} neurons,\textsuperscript{45} males display no courtship behavior toward females.\textsuperscript{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, establishing the specific developmental roles Fru\textsuperscript{MB} plays in establishing a transcriptional regulatory code leading to a male-specific nervous system.

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.\textsuperscript{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\textsuperscript{MB} binding at specific loci.

We would like to correlate occupancy with transcriptional control, however as Fru\textsuperscript{MB} 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\textsuperscript{MB} 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).

References

1. Hall JC. Courtship among males due to a male-sterile mutation in Drosophila melanogaster. Behav Genet 1978; 8:125-41; PMID:99136; http://dx.doi.org/10.1007/BF01068870
2. Ito H, Fujitani K, Usui K, Shimizu-Nishikawa K, Tanaka S, Yamamoto D. Sexual orientation in Drosophila is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. Proc Natl Acad Sci U S A 1996; 93:9687-92; PMID:8790392; http://dx.doi.org/10.1073/pnas.93.18.9687
3. Ryner LC, Goodwin SF, Castrillon DH, Anand A, Villella A, Baker BS, Hall JC, Taylor BJ, Wasserman SA. Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene. Cell 1996; 87:1079-89; PMID:8978612; http://dx.doi.org/10.1016/S0092-8674(00)81802-4
4. Anand A, Villella A, Ryner LC, Carlo T, Goodwin SF, Song HJ, Gailey DA, Morales A, Hall JC, Baker BS, et al. Molecular genetic dissection of the sex-specific and vital functions of the Drosophila melanogaster sex determination gene fruitless. Genetics 2001; 158:1569-95; PMID:1154448
5. Billerter J-C, Rideout EJ, Dornan AJ, Goodwin SF. Control of male sexual behavior in Drosophila by the sex determination pathway. Curr Biol 2006; 16:R766-76; PMID:16950103; http://dx.doi.org/10.1016/j.cub.2006.08.025
15. Riederer DJ, Gardner A, Neville MC, Ritchie MG, Goodwin SF. The evolution of novelty in conserved genes: evidence of positive selection in the Drosophila fruitless gene is localized to alternatively spliced exons. Heredity (Edinb) 2014; 112:308-6; PMID:24249653; http://dx.doi.org/10.1038/hdy.2013.106

16. van Steensel B, Henikoff S. Identification of in vivo DNA targets of chromatin proteins using tethered dam methyltransferase. Nat Biotechnol 2000; 18:424-8; PMID:10748524; http://dx.doi.org/10.1038/74487

17. Dalton JE, Fear JM, Knott S, Baker BS, McIntyre LM, Armitage MN. Male-specific Fruitless isoforms have different regulatory roles conferred by distinct zinc finger DNA binding domains. BMC Genomics 2013; 14:659; PMID:23407428; http://dx.doi.org/10.1186/1471-2164-14-659

18. Kling SJ, Fumulok M. All you wanted to know about SELEX. Mol Rep Biom Rep 1994; 20:97-107; PMID:7736299; http://dx.doi.org/10.1007/BF00996358

19. Pfeiffer BD, Jenett A, Hammonds AS, Ngo T-TB, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Lavery TR, et al. Tools for neuroanatomy and neurogenetics in Drosophila. Proc Natl Acad Sci U S A 2008; 105:9715-20; PMID:18621688; http://dx.doi.org/10.1073/pnas.0803697105

20. Jenett A, Rubin GM, Ngo TT, Shepherd D, Murphy C, Dionne H, Pfeiffer BD, Cavallaro A, Hall D, Jerz J, et al. A GAL4-driver line resource for Drosophila neurobiology. Cell Rep 2012; 2:991-1001; PMID:23636546; http://dx.doi.org/10.1016/j.celrep.2012.09.011

21. Manoli DS, Foss M, Villella A, Taylor BJ, Hall JC, Baker BS. Male-specific Fruitless specifies the neural substrates of Drosophila courtship behaviour. Nature 2005; 436:395-400; PMID:15939648

22. Schmucker D, Clemens JC, Shu H, Worby CA, Xiao J, Muda M, Dixon JE, Zipursky SL. Drosophila Discans is an axon guidance receptor exhibiting extraordinary molecular diversity. Cell 2001; 101:671-84; PMID:10892653; http://dx.doi.org/10.1016/S0092-8674(00)00298-4

23. Hummel T, Zipursky SL. Affector induction of olfactory glomeruli requires N-calothrin. Neuroreport 2004; 15:257-62; PMID:15106266; http://dx.doi.org/10.1097/01.wnr.0000120292.00234.ee

24. Yu HH, Huang AS, Kolodkin AL. Semaphorin-1a acts in concert with the cell adhesion molecules fasciclin II and connectin to regulate axon fasciculation in Drosophila. Genetics 2000; 156:723-31; PMID:11014880

25. Patel NF, Snow PM, Goodman CS. Characterization and cloning of fascin in III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. Cell 1987; 48:975-88; PMID:3548998; http://dx.doi.org/10.1016/0092-8674(87)90706-9

26. Stockinger P, Kiviranta D, Rotkopf S, Tiririn L, Dickson BJ. Neural circuitry that governs Drosophila male courtship behavior. Cell 2005; 121:795-807; PMID:15935765; http://dx.doi.org/10.1016/j.cell.2005.04.026