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RESEARCH ARTICLE

# *Drosophila* female-specific Ilp7 motoneurons are generated by Fruitless-dependent cell death in males and by a double-assurance survival role for Transformer in females

**Sarah Rose C. Garner, Monica C. Castellanos, Katherine E. Baillie, Tianshun Lian and Douglas W. Allan***

## ABSTRACT

Female-specific Ilp7 neuropeptide-expressing motoneurons (FS-Ilp7 motoneurons) are required in *Drosophila* for oviduct function in egg laying. Here, we uncover cellular and genetic mechanisms underlying their female-specific generation. We demonstrate that programmed cell death (PCD) eliminates FS-Ilp7 motoneurons in males, and that this requires male-specific splicing of the sex-determination gene *fruitless* (*fru*) into the $Fru^{MC}$ isoform. However, in females, *fru* alleles that only generate $Fru^{M}$ isoforms failed to kill FS-Ilp7 motoneurons. This blockade of $Fru^{M}$-dependent PCD was not attributable to *doublesex* gene function but to a non-canonical role for *transformer* (*tra*), a gene encoding the RNA splicing activator that regulates female-specific splicing of *fru* and *dsx* transcripts. In both sexes, we show that Tra prevents PCD even when the $Fru^{M}$ isoform is expressed. In addition, we found that $Fru^{MC}$ eliminated FS-Ilp7 motoneurons in both sexes, but only when Tra was absent. Thus, $Fru^{MC}$-dependent PCD eliminates female-specific neurons in males, and Tra plays a double-assurance function in females to establish and reinforce the decision to generate female-specific neurons.

**KEY WORDS: Sexual dimorphism, Motoneuron development, Programmed cell death, Sex determination, Insulin-like peptide 7**

## INTRODUCTION

Males and females of most species exhibit distinct behavioral repertoires that are genetically hardwired within dimorphic neuronal circuits. Studies in *Drosophila* have provided important insight into the genetic pathways that underlie the construction of sexually dimorphic neural circuits (Manoli et al., 2013; Villella and Hall, 2008). *Drosophila* sexual identity arises from sensing the number of X chromosomes (Erickson and Quintero, 2007). In females, this leads to female-specific expression of functional RNA splicing factor proteins, Sex lethal (Sxl) and Transformer (Tra) (Salz and Erickson, 2010). Tra drives alternative pre-mRNA splicing of transcripts for two transcription factor genes, *fruitless* (*fru*) and *doublesex* (*dsx*), leading to the expression of the $Dsx^{F}$ isoform and a $fru^{F}$ transcript encoding a premature stop codon. The absence of Tra protein in males allows for default male-specific splicing of these targets, leading to male-specific expression of functional $Fru^{M}$ and $Dsx^{M}$ isoforms (Burtis and Baker, 1989; Ryner et al., 1996). These male and female isoforms are expressed in partially overlapping neuronal lineages and postmitotic neurons, and function as effectors that direct dimorphic neuronal development in the male and female nervous systems (Burtis and Baker, 1989; Heinrichs et al., 1998; Hoshijima et al., 1991; Nagoshi et al., 1988; Ryner et al., 1996; Villella and Hall, 2008).

How $Fru^{M}$ and $Dsx^{M}$ isoforms construct male-specific circuitry has been intensely studied. $Fru^{M}$ and $Dsx^{M}$ have mostly non-overlapping roles in generating male-specific neurons, and in neuronal function, morphology and connectivity (Billeter et al., 2006; Cachero et al., 2010; Kimura et al., 2008; Rideout et al., 2007, 2010; Sanders and Arbeitman, 2008; Yu et al., 2010). The specific activities of $Fru^{M}$ are determined by which of the four isoforms ($Fru^{MA\text{-}MD}$) are expressed, which differ only in their C-terminal DNA-binding zinc-finger domains. $Fru^{MA}$, $Fru^{MB}$ and $Fru^{MC}$ are expressed in partially overlapping domains in the nervous system, and have unique activities in shaping male nervous system development, whereas $Fru^{MD}$ is not thought to be expressed in the CNS (Billeter et al., 2006; Dalton et al., 2013; Ito et al., 1996, 2016; Meissner et al., 2016; Neville et al., 2014; Nojima et al., 2014; Ryner et al., 1996; Soller et al., 2006; Usui-Aoki et al., 2000; von Philipsborn et al., 2014).

Recently, attention has increasingly turned to the study of female-specific behaviors and physiology. Numerous underlying circuits have now been identified (Bussell et al., 2014; Castellanos et al., 2013; Feng et al., 2014; Ferveur, 2010; Gligorov et al., 2013; Kapelnikov et al., 2008; Kimura et al., 2015; Laturney and Billeter, 2014; Rezával et al., 2014, 2012; Yang et al., 2009; Zhou et al., 2014). Yet, an understanding of how female-specific neurons and circuits are generated lags behind that of males. For example, female-specific neurons have been identified (Castellanos et al., 2013; Feng et al., 2014; Kimura et al., 2015; Rezával et al., 2014; Zhou et al., 2014), but the mechanisms leading to the generation of female-specific neuronal populations are only starting to be defined (Kimura et al., 2015). Here, we undertake a genetic analysis to determine the cellular and genetic mechanisms that generate female-specific Insulin-like peptide 7-expressing (FS-Ilp7) oviduct motoneurons, which are required for egg-laying and female fertility (Fig. 1) (Castellanos et al., 2013).

Pertinent lessons in how female-specific neurons may arise can be drawn from studies in males. First, differences in marker expression can be perceived as differences in neuronal number. For example, *Lgr3* is directly downregulated by $Fru^{M}$, and differentially regulated by $Dsx^{F}$ in discrete neuronal populations resulting in sex-specific marker expression (Meissner et al., 2016). Second, male-specific neurons have been shown to be generated by virtue of female-specific programmed cell death (PCD) or enhanced proliferation of neuronal progenitors in males. For example, in certain neuronal lineages, $Dsx^{F}$ can trigger PCD in females and $Dsx^{M}$ can trigger progenitor proliferation in males (Birkholz et al.,

Department of Cellular and Physiological Sciences, University of British Columbia, 2420 Life Sciences Institute, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada.

*Author for correspondence (doug.allan@ubc.ca)

S.R.C.G., 0000-0001-7790-820X; M.C.C., 0000-0001-7417-3674; K.E.B., 0000-0002-6341-0600; T.L., 0000-0001-6359-7244; D.W.A., 0000-0002-3488-8365

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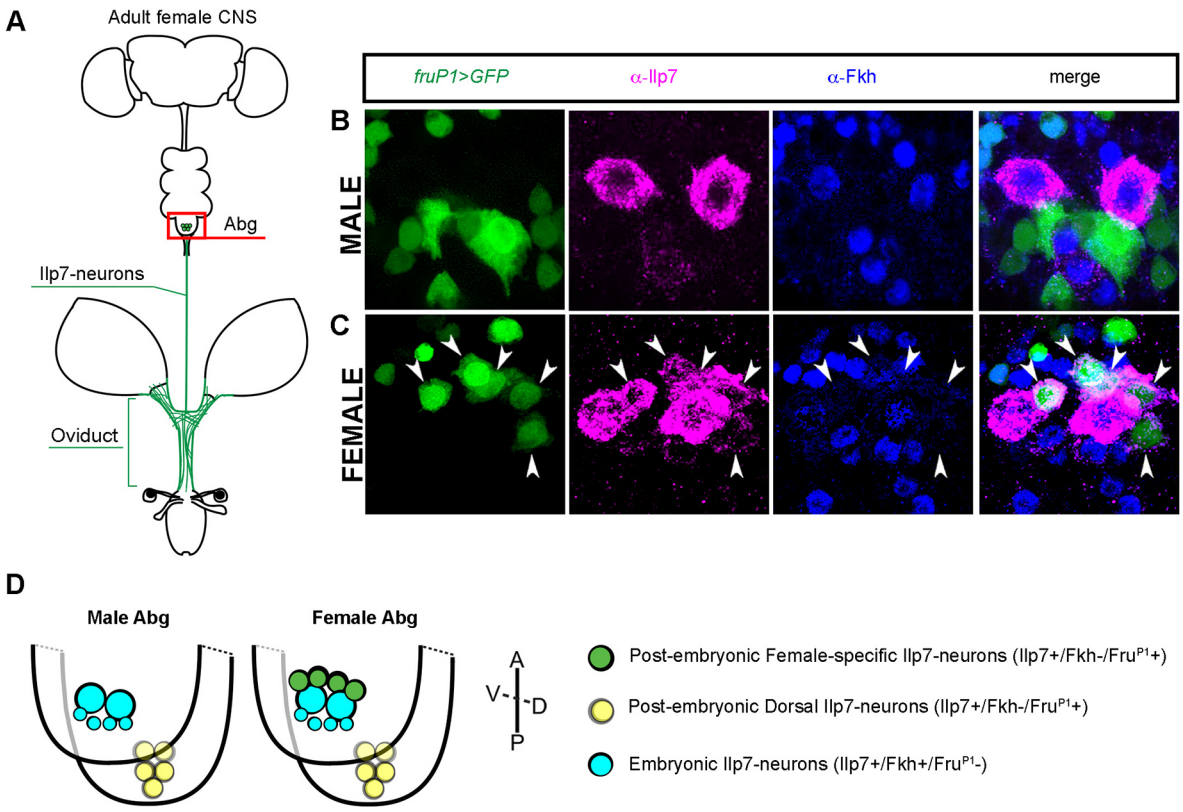


**Fig. 1. A subset of Insulin-like peptide 7 (Ilp7)-expressing motoneurons is female specific.** (A) Cartoon of the adult *Drosophila* central nervous system (CNS), showing the abdominal ganglion (Abg) (highlighted, red box) and the female reproductive tract. The female-specific Ilp7 motoneurons (FS-Ilp7 motoneurons) reside in the Abg and provide motor input to the oviduct (green). (B,C) FS-Ilp7 motoneurons are present in females (C, arrowheads) but not in males (B). FS-Ilp7 motoneurons are uniquely identifiable in females by the coincidence of Ilp7 immunoreactivity (α-Ilp7; magenta) and *fru*$^{P1\text{-}GAL4}$, *UAS-GFP* (*fruP1>GFP*; green) reporter activity. Embryonic Ilp7 neurons are marked by α-Ilp7 and α-Fork head immunoreactivity (α-Fkh, blue). (D) Cartoon summary and marker profile of embryonic (blue) and post-embryonic Ilp7 neurons (green, FS-Ilp7 motoneurons; yellow, dorsal Ilp7-motoneurons) in males and females. V, ventral; D, dorsal; A, anterior; P, posterior.

2013; Sanders and Arbeitman, 2008; Taylor and Truman, 1992). In addition, Dsx$^{M}$ and Fru$^{M}$ can both block PCD in certain neuronal lineages in males (Kimura et al., 2005; Sanders and Arbeitman, 2008). Applying such lessons, we postulate that either Dsx$^{F}$ promotes female-specific progenitor proliferation or that Dsx$^{M}$ might trigger male-specific PCD. Indeed, a recent report shows that Dsx$^{M}$ triggers PCD to reduce the number of pMN2 neurons in males relative to that in females (Kimura et al., 2015). However, roles for Tra and Fru have not been explored in relation to the generation of female-specific neurons.

We reported that Tra protein expression is necessary in females and sufficient in males for the presence of FS-Ilp7 motoneurons (Castellanos et al., 2013). Examining sex determination gene expression in these motoneurons, we found that they do not express Dsx$^{F}$ protein; using *dsx-GAL4* to immortalize lineage marker expression, we found that FS-Ilp7 motoneurons never became marked by this lineage marker. Thus, the *dsx* gene was postulated to not contribute to FS-Ilp7 motoneuron generation (Castellanos et al., 2013). In contrast, the sex-specifically spliced *fru* transcript (from the P1 promoter) is expressed in FS-Ilp7 motoneurons. In testing a role for Fru$^{M}$, we found that Fru$^{M}$ protein expression was required for the absence of FS-Ilp7 motoneuron in males. However, in females expressing Fru$^{M}$, generated from the *fru*$^{M}$ allele that only generates Fru$^{M}$ isoforms, we did not observe any loss of FS-Ilp7 motoneurons. This left the precise function of *fru* unresolved and suggested that an additional factor is required. Moreover, it was unclear whether the apparent absence of FS-Ilp7 motoneurons in males was merely an artifact of absent Ilp7 protein expression in those neurons, or indeed whether these neurons are unique to females by virtue of enhanced neuroblast proliferation in females or programmed cell death in males.

Here, we have performed cellular and genetic analyses to define roles for *tra* and *fru* in female-specific FS-Ilp7 motoneuron generation. We find that the dimorphic emergence of FS-Ilp7 motoneurons in females is due to selective PCD in males. Moreover, we find that the Fru$^{MC}$ isoform is required for PCD. By examining the emergence of FS-Ilp7 motoneurons in a *fru* allelic series in both males and females, we confirm that Fru$^{M}$ only triggers FS-Ilp7 motoneuron elimination in males. In exploring this insufficiency in females, we found that Tra can prevent PCD of FS-Ilp7 motoneurons, even in the presence of *fru* alleles that can only generate Fru$^{M}$ isoforms. Further genetic analysis indicated that this function is not likely mediated through Tra-dependent Dsx$^{F}$ activity. These data provide evidence that Tra antagonizes the function of Fru$^{M}$ in a pathway running parallel or downstream to its role in *fru* splicing, in an apparent double-assurance function to ensure the absence of Fru$^{M}$ activity in subsets of female neurons.

## RESULTS

### Male-specific PCD of FS-Ilp7 motoneurons

Previously, we identified a set of female-specific (FS) Ilp7 motoneurons that are generated by post-embryonic neurogenesis. These motoneurons cluster around a set of embryonically born Ilp7-expressing neurons that innervate the gut (Castellanos et al.,

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2013) (Fig. 1A-D). These two neuronal populations can be discriminated by their differential expression of *fork head* (*fkh*) and *fru*. FS-Ilp7 motoneurons do not express the transcription factor, Fkh, but do express $fru^{P1\text{-}GAL4}$ (referred to as *fruP1>*), a reporter for the sex-specifically spliced *fru* transcript transcribed from the P1 promoter (Stockinger et al., 2005). In contrast, embryonic Ilp7- neurons express Fkh but not *fruP1>GFP* (Castellanos et al., 2013) (Fig. 1B,C).

We examined whether male-specific programmed cell death (PCD) explains the absence of FS-Ilp7 motoneurons in males, as opposed to an absence of anti-Ilp7 staining in the male counterparts of the motoneurons or enhanced proliferation in females. We expressed the anti-apoptotic caspase inhibitor, baculovirus p35 (*UAS-p35*) (Hay et al., 1994) in *fruP1*-expressing cells. In females, *fruP1>+* controls had one to five FS-Ilp7 motoneurons per fly (3.0±0.4 motoneurons per fly) and this was not significantly changed in *fruP1>*p35 females (3.4±0.3 motoneurons per fly) (Fig. 2B,D). In contrast, male *fruP1>+* controls had zero FS-Ilp7 motoneurons per fly (Fig. 2A,C), but in *fruP1>p35* males we observed frequent rescue of one to five FS-Ilp7 motoneurons per VNC (2.7±0.4 per fly) (Fig. 2A,C). Thus, male-specific PCD within *fruP1*-expressing cells accounts for the absence of FS-Ilp7 motoneurons in males and that their generation is female specific.

We then examined whether PCD occurs prior to the onset of *Ilp7* expression. We have not identified other discriminatory markers for FS-Ilp7 motoneurons or their progenitors; therefore, the onset of *Ilp7* expression provides the earliest unambiguous marker for these motoneurons. We generated a transgenic reporter for *Ilp7* expression, *Ilp7-nls.tdTomato* (see Materials and Methods), that provided the earliest robust marker for *Ilp7* expression. We verified that *Ilp7-nls.tdTomato* is expressed in all abdominal Ilp7 motoneurons, using a published anti-Ilp7 antibody (Yang et al., 2008) and a newly generated anti-Ilp7 antibody (see Materials and Methods) (Fig. S1). Using *Ilp7-nls.tdTomato*, we first detected FS-Ilp7 motoneurons between 41 and 46 h after puparium formation (APF), in cells that express *fruP1>GFP* at a high level (Fig. S2A-C′). In males, *Ilp7-nls.tdTomato* was only seen in the embryonically born Ilp7 neurons, and was never seen in any adjacent cells that could represent FS-Ilp7 motoneurons prior to PCD. In *fruP1>p35* males, *Ilp7-nls.tdTomato* expression in surviving FS-Ilp7 motoneurons cells was first observed at 50-55 h APF (Fig. S2D,E). Thus, PCD occurs prior to the onset of *Ilp7* expression in FS-Ilp7 motoneurons. In agreement, we found that driving *UAS-p35* from

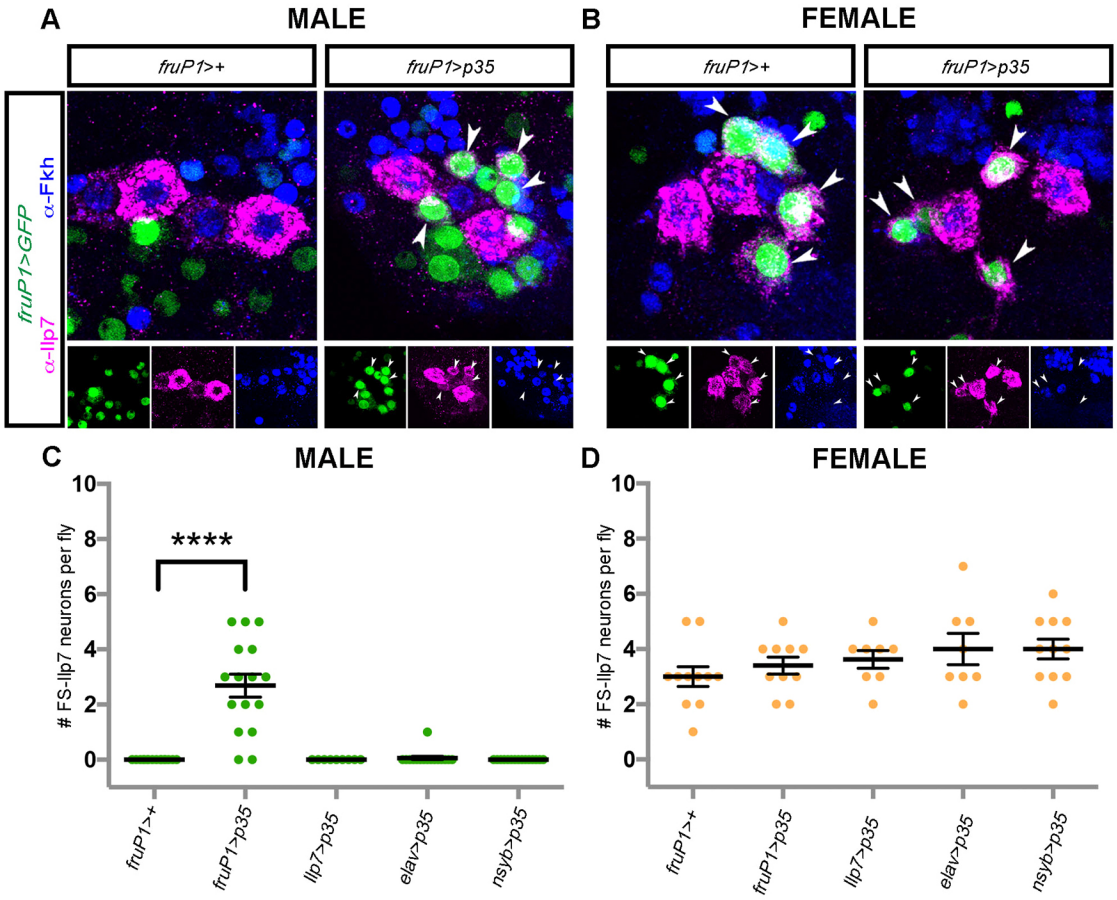


**Fig. 2. FS-Ilp7 motoneurons are eliminated by programmed cell death in males.** (A-D) We expressed the baculovirus *p35* caspase blocker (*UAS-p35*), which blocks programmed cell death (PCD), in cells expressing $fru^{P1\text{-}GAL4}$, *Ilp7-GAL4* or the pan-neuronal GAL4 drivers $elav^{C155\text{-}GAL4}$ and *nsyb-GAL4*. We quantified FS-Ilp7 motoneuron numbers in both sexes. (A,B) Representative *z*-projections of FS-Ilp7 motoneurons in males (A) and females (B), in control (*fruP1>+*, left panels) and *fruP1>p35* (right panels). FS-Ilp7 motoneurons were not observed in wild-type males (*fruP1>+*) but were always observed in wild-type females (*fruP1>+*) (arrows, Ilp7+/Fkh-/*fruP1+*). In the *fruP1>p35* genotype, FS-Ilp7 motoneurons were generated in males (A, right panel, arrowheads), but were not produced at increased numbers in females (B, right panel, arrowheads). (C,D) Quantification of FS-Ilp7 motoneuron numbers per fly (each point in scatter plot) of each sex and of each genotype (shown along the *x*-axis). FS-Ilp7 motoneurons were generated only in males of the *fruP1>p35* genotype. No difference in the number of FS-Ilp7 motoneurons was observed in females of the genotypes tested. Data are mean±s.e.m. ****$P$<0.0001 compared with *fruP1>+* control.

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*Ilp7-GAL4* failed to prevent the elimination of FS-Ilp7 motoneurons in males (Fig. 2C). We also tested whether FS-Ilp7 motoneurons could be rescued from PCD by expressing *UAS-p35* from early postmitotic pan-neuronal drivers $elav^{C155\text{-}GAL4}$ (Berger et al., 2007; Lin and Goodman, 1994) and *nsyb-GAL4* (Pauli et al., 2008) (Fig. 2C). In both cases, PCD was not blocked, indicating that PCD must occur before these GAL4 drivers are expressed or before they can drive sufficient levels of p35 to rescue cells from PCD. No significant difference in the number of FS-Ilp7 motoneurons was observed in any of these genotypes in females. We note that one FS-Ilp7 motoneuron survived in one *elav>p35* male. This may provide some evidence that PCD occurs at a time of low level $elav^{C155\text{-}GAL4}$ expression; however, this GAL4 line is expressed in subsets of neuronal and glial progenitors and then robustly in postmitotic motoneurons, so this does not fully resolve whether PCD occurs in postmitotic motoneurons (Berger et al., 2007). The identification of markers that allow us to image lineage progression will be required to precisely determine the developmental stage of PCD.

## $Fru^{MC}$ is necessary for cell death of FS-Ilp7 motoneurons in males

We previously reported that Tra was necessary in females and sufficient in males for FS-Ilp7 motoneuron survival. We also found that $Fru^{M}$ was necessary for FS-Ilp7 motoneuron elimination in males, but that $Fru^{M}$ in females (generated from a hemizygous $fru^{M}$ allele that constitutively splices *fru* in the male mode at the endogenous locus in *fruP1*-expressing cells) failed to eliminate FS-Ilp7 motoneurons in females (Castellanos et al., 2013). This discrepancy was intriguing, because in females the constitutive male-splicing $fru^{M}$ allele often masculinizes neuronal morphology and function (Cachero et al., 2010; Demir and Dickson, 2005).

As our previous conclusions regarding *fru* function in PCD came from the analysis of single *fru* heteroallelic genotypes in either sex, we wished to extend the genetic analysis of *fru* in FS-Ilp7 generation to a more extensive *fru* allelic series. We generated heteroallelic combinations of *fru* by combining one of four *fru* alleles that each reduces or eliminates $Fru^{M}$ protein expression ($fru^{3}$, $fru^{F}$, $fru^{P1\text{-}GAL4}$, $fru^{Sat15}$, see Materials and Methods for details), with either wild-type *fru* (+) or an engineered *fru* allele that only generates either functional male $Fru^{M}$ isoforms (the $fru^{M}$ allele) or female-spliced transcripts that do not generate functional Fru protein (the $fru^{F}$ allele) (Demir and Dickson, 2005). The nature of these alleles is depicted in Fig. 3A. In males, we found that any genotype in which $Fru^{M}$ is severely reduced or eliminated resulted in significant survival of FS-Ilp7 motoneurons; this averaged between 4.2 and 6.5 FS-Ilp7 motoneurons per fly (Fig. 3B), with the $fru^{F}/fru^{3}$ genotype being the highest at 6.5±0.5. In

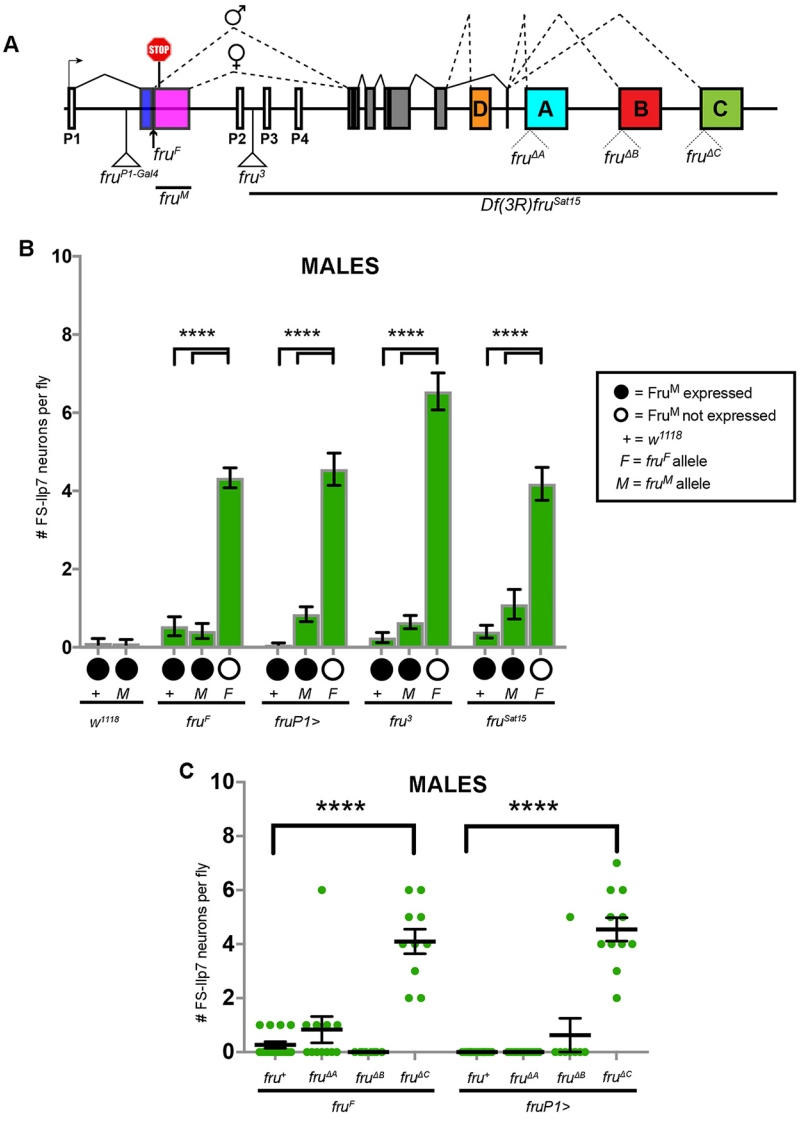


**Fig. 3. $Fru^{MC}$ is necessary to eliminate FS-Ilp7 motoneurons in males.** (A) Schematic of the *fru* locus (not to scale) showing the *fru* alleles used (see also Materials and Methods), the *fru* promoters (white boxes), the exons (colored boxes), as well as splicing (solid lines) and alternate splicing (broken lines). P1 transcripts are sex-specifically spliced, as shown, so that male isoforms do not include an early stop codon, but female isoforms do include the stop codon. Four $Fru^{M}$ protein isoforms are generated ($Fru^{MA}$, $Fru^{MB}$, $Fru^{MC}$ and $Fru^{MD}$) by alternate use of exons A-D (colored boxes). (B,C) We tested a role for $Fru^{M}$ and its isoforms in male FS-Ilp7 motoneuron PCD. (B) We placed alleles that generate only male-specific splicing (*M*, $fru^{M}$), female-specific splicing (*F*, $fru^{F}$) or a control chromosome (+, $w^{1118}$) over a series of alleles that prevent/reduce $Fru^{M}$ protein expression ($fru^{F}$, *fruP1>*, $fru^{3}$ and $fru^{Sat15}$). In these genotypes (shown along the *x* axis), we counted FS-Ilp7 motoneurons per fly and present these as mean±s.e.m. FS-Ilp7 motoneurons were rarely observed in males with one or more $Fru^{M}$-expressing alleles (filled circles). However, four to six FS-Ilp7 motoneurons were observed in genotypes that had no $Fru^{M}$ protein expression (unfilled circles). (C) We tested which $Fru^{M}$ isoforms are required for PCD. We placed nonsense $Fru^{M}$ isoform mutants ($fru^{\Delta A}$, $fru^{\Delta B}$ and $fru^{\Delta C}$) over either $fru^{F}$ or *fruP1>* alleles, and counted the number of FS-Ilp7 motoneurons, represented as scatter plots and shown as mean ±s.e.m. FS-Ilp7 motoneurons survived in $fru^{\Delta C}$ heteroallelic combinations in comparable numbers to wild-type females, showing that only $Fru^{MC}$ is required for PCD. Significant differences are shown compared with pertinent controls (+); ****$P$<0.0001.

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contrast, genotypes with a single copy of either $fru^{+}$ or the $fru^{M}$ allele all resulted in a significant reduction in FS-Ilp7 motoneuron number (averaging 0.06 to 1.1 FS-Ilp7 motoneurons per fly) (Fig. 3B). Thus, by testing numerous *fru* heteroallelic combinations, we confirmed that $Fru^{M}$ is required for PCD of FS-Ilp7 motoneurons in males. In a subset of the genotypes where only a single allele generates $fru^{+}$ or $fru^{M}$, we did observe occasional survival of FS-Ilp7 motoneurons in a few animals, and often slightly higher numbers in $fru^{M}$ than in $fru^{+}$. We interpret this as being due to the escape of some cells from PCD when $Fru^{M}$ levels are lowered.

Three $Fru^{M}$ isoforms, $Fru^{MA}$, $Fru^{MB}$ and $Fru^{MC}$, are expressed in the male nervous system. These differ in their $C_2H_2$ zinc-finger DNA-binding domains (Billeter et al., 2006; Dalton et al., 2013; Ito et al., 1996; Neville et al., 2014; Nojima et al., 2014; Ryner et al., 1996; Usui-Aoki et al., 2000; von Philipsborn et al., 2014). We wished to test which isoform(s) were necessary for male-specific PCD of FS-Ilp7 motoneurons. To first test which isoforms may be required for PCD, we blocked PCD with *fruP1>p35* and assayed which isoforms are expressed in 'undead' FS-Ilp7 motoneurons. In these animals, we took advantage of the $fru^{Amyc}$, $fru^{Bmyc}$ and $fru^{Cmyc}$ alleles, which express a functional Myc-tagged version of each $Fru^{M}$ isoform (von Philipsborn et al., 2014). We found that 'undead' male FS-Ilp7 motoneurons expressed $Fru^{MB}$ and $Fru^{MC}$, but not $Fru^{MA}$ (Fig. S3).

We next tested which isoform is necessary for male PCD using isoform-specific mutants that contain a premature stop codon within one of the distinct 3′ exons, referred to as $fru^{\Delta A}$, $fru^{\Delta B}$ and $fru^{\Delta C}$ (Fig. 3A,C) (Billeter et al., 2006; Neville et al., 2014). We combined these with the $fru^{F}$ or $fru^{P1\text{-}GAL4}$ alleles that severely reduce $Fru^{M}$ expression. In males, FS-Ilp7 motoneurons were mostly absent in $fru^{\Delta A}$ or $fru^{\Delta B}$ heteroallelic genotypes, confirming that $Fru^{MA}$ and $Fru^{MB}$ are not necessary for PCD. In $fru^{\Delta C}$ heteroallelic genotypes, however, we observed robust survival of FS-Ilp7 motoneurons (4.1±0.5 and 4.5±0.4 FS-Ilp7 motoneurons for $fru^{\Delta C}/fru^{F}$ and $fru^{\Delta C}/fru^{P1\text{-}GAL4}$, respectively) (Fig. 3C). Therefore, the $Fru^{MC}$ isoform is necessary for PCD.

### $Fru^{M}$ in females is insufficient to kill Ilp7 motoneurons

In numerous cases where $Fru^{M}$ is necessary for a male-specific neuronal property or function, its expression in females often masculinizes corresponding neurons in females (Cachero et al., 2010; Demir and Dickson, 2005; Rideout et al., 2010; von Philipsborn et al., 2014). In contrast, we had shown that females hemizygous for the $fru^{M}$ allele failed to eliminate FS-Ilp7 motoneurons (Castellanos et al., 2013). Here, we re-examined this conclusion by testing an expanded *fru* allelic series. Importantly, we found that FS-Ilp7 motoneurons were not eliminated in any genotype that generates $Fru^{M}$ protein in the female. Even in $fru^{M}/fru^{\Delta tra}$ females where both alleles produce only $Fru^{M}$ protein, irrespective of Tra activity (Demir and Dickson, 2005), we observed 3.0±0.3 FS-Ilp7 motoneurons, which was not significantly different from $w^{1118}$ controls (Fig. 4A). Only in one case did we find a modest but significant $Fru^{M}$-driven reduction in FS-Ilp7 motoneurons, when comparing $fru^{M}/fru^{F}$ (3.2 ±0.4 motoneurons per fly) with $fru^{F}/fru^{F}$ (5.0±0.3 motoneurons per fly) (Fig. 4A). This could support the notion that the presence of $Fru^{M}$ may subtly but incompletely increase the likelihood of PCD in females. Nevertheless, the weight of evidence demonstrates that $Fru^{M}$ expression in females is not sufficient to eliminate FS-Ilp7 motoneurons in females, as it is in males.

### Tra prevents $Fru^{M}$-dependent PCD to promote FS-Ilp7 motoneuron survival

We have previously reported that Tra knockdown in females recapitulates male-like elimination of FS-Ilp7 motoneurons (Castellanos et al., 2013). Therefore, Tra must have a function that is independent of *fru* splicing into female-specific transcripts and explains its necessity and sufficiency for FS-Ilp7 motoneuron survival. We revisited a possible role for *dsx* downstream of Tra in playing an antagonistic role to $Fru^{M}$ activity in females, despite evidence suggesting that Dsx is not expressed in FS-Ilp7 motoneurons or their lineage (Castellanos et al., 2013). We tested *dsx* nulls and observed no significant change in the number of FS-Ilp7 motoneurons in either males or females (Fig. S4A,B). Thus, *dsx* is not required for either FS-Ilp7 motoneuron elimination in males (via $Dsx^{M}$ expression) or survival in females (via $Dsx^{F}$ expression). However, $Dsx^{F}$ expression in females has been postulated to antagonistically repress the masculinizing effect of the $fru^{M}$ and $fru^{\Delta tra}$ alleles on courtship behavior (Shirangi et al., 2006). To test the hypothesis that $Dsx^{F}$ may be required to antagonize $Fru^{M}$ function in PCD leading to FS-Ilp7 motoneuron survival, we overexpressed $UAS\text{-}Dsx^{F}$ in male and female $fru^{P1\text{-}GAL4}$-expressing cells (Fig. S4C,D). This did not prevent FS-Ilp7 motoneuron elimination in the majority of males and did not significantly increase FS-Ilp7 motoneuron numbers in females. In one male, overexpressed $Dsx^{F}$ did rescue the survival of two FS-Ilp7 motoneurons, which may suggest that excess $Dsx^{F}$ has limited capacity to reduce $Fru^{M}$-dependent PCD, or may represent a rare case of survival in a *fru* heterozygotic genotype as observed in *fruP1>+* in Fig. 3B. Regardless, our results do not support an essential role for DsxF as the crucial factor downstream of Tra that mediates Tra-dependent antagonism of $Fru^{M}$-dependent PCD of FS-Ilp7 motoneurons.

We tested a role for Tra itself as a modifier of $Fru^{M}$ function, uncoupled from its role in *fru* transcript splicing. We ectopically expressed $UAS\text{-}tra^{F}$ from the $fru^{P1\text{-}GAL4}$ driver in males and observed significant survival of FS-Ilp7 motoneurons (2.1±0.4 motoneurons per fly) (Fig. 4B; $fruP1>tra^{F}$). This survival was expected, under the assumption that $Fru^{M}$ expression is prevented or reduced in these males. We note that these males did have fewer FS-Ilp7 motoneurons compared with wild-type females (Fig. 4A) and with males that lack $Fru^{M}$ expression (Fig. 3B). This could be due to residual $Fru^{M}$ expression resulting from insufficient Tra overexpression around the time of PCD. We then tested whether the ability of Tra to prevent PCD in males was mediated through *fru* transcript splicing, preventing generation of functional $Fru^{M}$ protein. We ectopically expressed $UAS\text{-}Tra^{F}$ in the presence of constitutive $Fru^{M}$ generation caused by the $fru^{M}$ allele. Notably, expression of $Fru^{M}$ failed to restore PCD in the presence of Tra, as we observed the survival of 2.7±0.3 FS-Ilp7 motoneurons (Fig. 4B; $fruP1>tra^{F}/fru^{M}$). To ensure that ectopic expression of Tra in males did not inadvertently rescue FS-Ilp7 motoneurons through the expression of $Dsx^{F}$, we co-expressed $UAS\text{-}tra^{F}$ with an effective $UAS\text{-}dsx^{RNAi}$ transgene (Hudry et al., 2016) in males and counted the number of FS-Ilp7 motoneurons by their co-expression of *Ilp7-nls.tdTomato*, anti-Ilp7 and *fruP1>nGFP* (Fig. S4). We found that co-expressing *UAS-Dcr2* with $UAS\text{-}tra^{F}$ and $UAS\text{-}dsx^{RNAi}$ in males generated the same numbers of FS-Ilp7 motoneurons as did expression of *UAS-Dcr2* with $UAS\text{-}tra^{F}$ alone in males (average of 1.4±-0.2 and 1.5±0.4 FS-Ilp7 motoneurons per fly, respectively). These data suggest that Tra can override $Fru^{M}$-dependent PCD in a mechanism unrelated to *fru* and *dsx* splicing.

To determine whether the $Fru^{M}$-modifying activity of Tra in males is an artifact of Tra overexpression, we tested the same epistatic relationship in females. We repeated and confirmed previous results (Castellanos et al., 2013) that RNAi-mediated knockdown of Tra in neurons eliminated all FS-Ilp7 motoneurons, using $elav^{GAL4\text{-}C155}$ to

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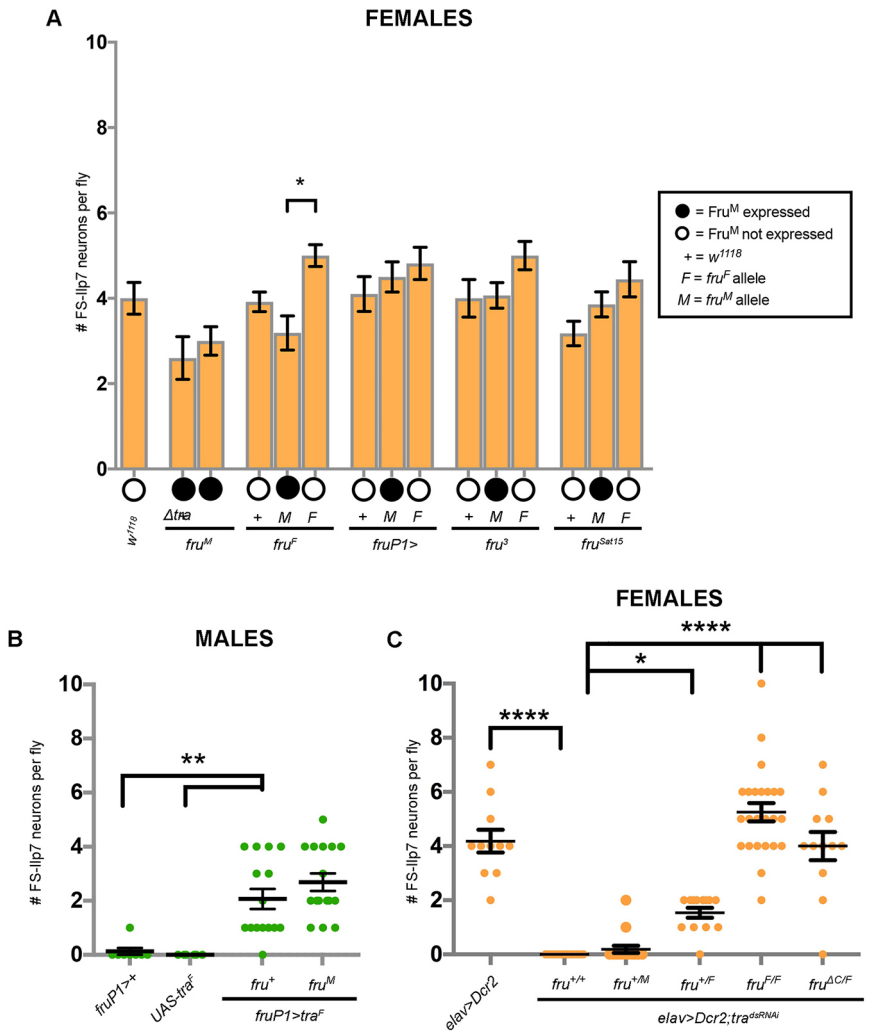


**Fig. 4. Tra blocks PCD of FS-Ilp7 motoneurons genetically downstream of *fru* splicing.** (A-C) We tested whether constitutive male-type of *fru* male mode is sufficient for PCD of FS-Ilp7 motoneurons in females, and tested genetic interactions between *tra* and *fru* that lead to FS-Ilp7 motoneuron survival in males and females. (A) We quantified FS-Ilp7 motoneurons per female in a *fru* allelic series (similar to Fig. 3) showing mean±s.e.m. per genotype (filled circles, $Fru^M$ protein-expressing genotypes; unfilled circles, genotypes that cannot express $Fru^M$). There was no loss of FS-Ilp7 motoneurons in any genotype tested. (B) In males, ectopic Tra expression (*fruP1>tra*$^F$) led to survival of FS-Ilp7 motoneurons in most animals, whereas survival was rare in controls (*fruP1>+* or *UAS-tra*$^F$). Introduction of a constitutive male-splicing allele *fru*$^M$ in this background (*fru*$^M$/*fruP1>tra*$^F$) did not decrease FS-Ilp7 motoneuron number in comparison with *fruP1>tra*$^F$. (C) We knocked down Tra using RNAi (*elav>Dcr2;tra*$^{dsRNAi}$*;fru*$^{+/+}$). This eliminated all FS-Ilp7 motoneurons. We then tested whether $Fru^M$ protein expression is specifically required for PCD in the absence of Tra. Therefore, we prevented male splicing of *fru* in the *tra* RNAi background (*elav>Dcr2;tra*$^{dsRNAi}$) and observed a dose response of *fru* in killing FS-Ilp7 motoneurons. Expressing two copies of *fru* (*fru*$^{+/M}$) that can each generate $Fru^M$ protein expression eliminated FS-Ilp7 motoneurons in most animals, with only two animals retaining one or two FS-Ilp7 motoneurons, suggesting that the *fru*$^M$ allele may generate less $Fru^M$ protein than the *fru+* wild-type allele. One copy of *fru* available for $Fru^M$ protein expression led to partial survival (*fru*$^{+/F}$); no copies of *fru* available for $Fru^M$ protein expression (*fru*$^{F/F}$) led to full survival of FS-Ilp7 motoneurons. Preventing expression of the $Fru^{MC}$ protein isoform (*fru*$^{\Delta C/F}$) also led to full FS-Ilp7 motoneuron rescue. This demonstrated that $Fru^{MC}$ protein expression alone is sufficient to elicit PCD in females when Tra is knocked down. Data are FS-Ilp7 motoneurons per fly in scatter plots (mean±s.e.m.). Significant differences within each experimental group are shown compared with the appropriate controls (+); **P*<0.05, ***P*<0.01, *****P*<0.0001.

drive *UAS-Dcr2* and *UAS-tra*$^{dsRNAi}$ (Fig. 4C). We also tested *tra* null mutant females (*tra*$^{KO}$/*tra*$^{KO}$) and found that these also have zero FS-Ilp7 motoneurons ($n$=7), whereas *tra*$^{KO}$/*TM6B* females have 5.3±0.4 FS-Ilp7 motoneurons ($n$=8) ($P$<0.0001, unpaired student $t$-test). To confirm this effect is due to Tra-dependent splicing, we tested whether the obligatory splicing co-factor of Tra, Tra2, is necessary to eliminate FS-Ilp7 motoneurons in females. In *tra2* mutant females and males [*tra2*$^{[B]}$/*Df(2L)trix*], there were 0±0 FS-Ilp7 motoneurons ($n$=13 and $n$=14, respectively). These results demonstrate that Tra and its splicing co-factor Tra2 are both necessary for FS-Ilp7 motoneuron survival in females.

Next, we tested whether elimination of FS-Ilp7 motoneurons in *elav>tra*$^{dsRNAi}$ females is due to the generation of $Fru^M$. To achieve this, we examined the number of FS-Ilp7 motoneurons in genotypes that progressively reduced a dose of $Fru^M$ in a *tra* RNAi knockdown background. In *elav>tra*$^{dsRNAi}$ females, 0±0 FS-Ilp7 motoneurons were generated in the presence of two copies of wild-type *fru*, and 0.2±0.1 FS-Ilp7 motoneurons were generated in the presence of a *fru*$^{+/M}$ genotype. Next, we found that, in *elav>tra*$^{dsRNAi}$ females, a *fru*$^{+/F}$ genotype resulted in 1.3±0.2 FS-Ilp7 motoneurons and that a *fru*$^{F/F}$ genotype resulted in survival of 5.3±0.3 FS-Ilp7 motoneurons (Fig. 4C). These data show that $Fru^M$ protein is indeed required for

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the elimination of FS-Ilp7 motoneurons in females, but it can function only in the absence of Tra.

Finally, we tested whether the $Fru^{MC}$ isoform is necessary in females to trigger PCD in Tra-deficient females, as in males. In confirmation, we found that in *elav >tra$^{dsRNAi}$* females, the presence of the *fru$^{\Delta C}$* mutant allele (*fru$^{\Delta C/F}$*) led to survival of 4.0±0.5 FS-Ilp7 motoneurons (Fig. 4C). This demonstrates that $Fru^{MC}$ is required for FS-Ilp7 motoneuron PCD in females when Tra is absent. Our results provide evidence for the ability of Tra to function downstream of *fru* splicing to block the function of $Fru^{M}$ once it is generated.

## DISCUSSION

Analysis of *fru*, *dsx* and *tra* function in the *Drosophila* nervous system has transformed our understanding of the construction of sexually dimorphic neuronal circuits (Yamamoto and Koganezawa, 2013). Owing to the elaborate stereotyped behaviors of males, studies have historically focused on construction of the male nervous system. In contrast, female nervous system construction is less well characterized. Here, we explore the cellular and genetic mechanisms that generate a population of female-specific motoneurons in *Drosophila*. In addressing this issue, we uncover functions for *fru* and *tra* that are of general significance to understanding the development of dimorphic nervous systems, and also the interpretation of genetic studies of these factors.

### $Fru^{MC}$ removes female-specific neurons from the male nervous system by PCD

Surveys of *fruP1*-expressing motoneurons have identified many male-specific populations (Cachero et al., 2010; Yu et al., 2010). The increased numbers of *fruP1*-positive motoneurons in males is largely attributed to sex-specific isoforms of *dsx*, through their control of PCD or proliferation in neuronal lineages (Kimura, 2011; Kimura et al., 2008; Rideout et al., 2007). $Fru^{M}$ is generally considered to be a regulator of the neuronal morphology and function of these motoneurons after periods of PCD or proliferation have established neuronal numbers (Yamamoto and Koganezawa, 2013). However, there is now evidence to implicate specific $Fru^{M}$ isoforms in the control of neuronal numbers leading to additional *fru*P1-expressing neurons in males (von Philipsborn et al., 2014). A mechanistic explanation comes from a study showing that $Fru^{M}$ prevents PCD of mAL neurons selectively in males (Kimura et al., 2005). Our results now provide an expanded view of $Fru^{M}$ function, showing that the $Fru^{MC}$ isoform removes female neuronal components from the male nervous system via PCD (Fig. 5). Typically, between two and six FS-Ilp7 motoneurons are generated per wild-type female or mutant male although we observed genotypes that can generate 8 to 10 FS-Ilp7 motoneurons. The earliest marker available to unambiguously identify these neurons is the Ilp7 reporter itself, which we used to identify these neurons at 41-46 h APF in segments A6 and A7, as predicted by our previous analysis of Hox gene expression in FS-Ilp7 motoneurons (Castellanos et al., 2013). The identification of discriminatory markers earlier than Ilp7 expression to image earlier lineage and postmitotic stages will be important to determine the developmental stage at which *fruP1* transcripts are first transcribed and when PCD occurs. In addition, it will be interesting to discover the mechanisms that give rise to the natural diversity of FS-Ilp7 neuronal numbers, and precisely how *fru* and *tra* genotypes affect this process. Our data are relevant in light of a recent examination of *fruP1*-expressing brain neuroblast lineages, showing that blockade of PCD using *UAS-p35* increased neuronal number in both sexes; thus, PCD was also proposed to restrict neuronal number in male lineages (Ren et al., 2016). We believe that our observation that $Fru^{MC}$ eliminates neurons in males through PCD provides a mechanism to account for neuronal loss in a number of those cases. These findings provide a novel framework that accounts for targeted elimination of neurons in males and the corresponding generation of female-specific neurons and circuitry.

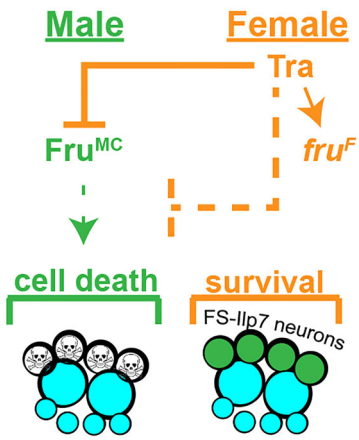


**Fig. 5. Fru and Tra have novel opposing roles in constructing sexually dimorphic FS-Ilp7 motoneurons.** In the male nervous system, $Fru^{M}$ isoforms A-C are expressed and direct most male-specific differences in behavior and neuronal morphology, whereas in the female nervous system, Tra prevents $Fru^{M}$ protein production via alternative splicing. (Left) In males, we found that $Fru^{MC}$ is necessary for PCD of FS-Ilp7 motoneurons. (Right) In females, we found that Tra not only prevents *fru$^{M}$* splicing, by generating *fru$^{F}$*, but genetically acts in parallel or downstream of $Fru^{MC}$ to prevent PCD of FS-Ilp7 motoneurons. We propose that this additional role for Tra outside of *fru* splicing acts as a failsafe to ensure the survival of FS-Ilp7 motoneurons, which are required for egg laying.

### A novel failsafe mechanism for *tra* that builds the female nervous system

Our genetic manipulation studies provide evidence that Tra can antagonize $Fru^{MC}$-dependent cell death in both males and females (Fig. 5). This provides a novel perspective for understanding the development of the female nervous system, and for studies in which forced male-specific splicing of *fru* is used to test the sufficiency of $Fru^{M}$ protein activity in females. For example, there have been numerous reports that $Fru^{M}$ expression in females is insufficient for full masculinization (Demir and Dickson, 2005; Kimura et al., 2008; Rideout et al., 2007). Males require $Fru^{M}$ for the enlargement of numerous brain regions relative to females, but these are only partially enlarged in females with the *fru$^{M}$* allele. In contrast, these regions are enlarged in *tra* mutant females to match males (Cachero et al., 2010). In addition, females with the *fru$^{M}$* allele do not exhibit the full $Fru^{M}$-dependent behavioral repertoire of males (Demir and Dickson, 2005; Kimura et al., 2008; Rideout et al., 2007). In contrast, *tra* mutant females have near full behavioral masculinization in all these behaviors (Kimura et al., 2008; Kyriacou and Hall, 1980; Rideout et al., 2007; Sturtevant, 1945). In both cases, *tra* mutant females more closely resemble a fully masculinized phenotype than do *fru$^{M}$* females. The other arm of the sex-determination cascade, which is regulated by *dsx*, can account for this discrepancy in numerous cases (Kimura et al., 2008; Rideout et al., 2007). However, our demonstration that Tra also antagonizes $Fru^{M}$ functional activity in females independently of *dsx* gene function in certain cellular contexts offers a novel perspective for studies in which $Fru^{M}$ is expressed in females.

What function might such a failsafe role for Tra play? Possibly, it may serve as a safeguard against incomplete splicing of *fru* (and

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perhaps also *dsx*) sex-specific transcripts. Indeed, RNA sequencing has shown that $fru^M$ RNA transcripts are in fact generated in wild-type females at a low level, and it is also possible that this may be exacerbated in stressful environments such as high temperature or hypoxia (Ferri et al., 2008; Graveley et al., 2011; Mohr and Hartmann, 2014; Usui-Aoki et al., 2000; Yamamoto, 2007; Yamamoto and Koganezawa, 2013). Tra and Tra2 binding have also been shown to repress reporter translation in S2 cells, suggesting a role for Tra in not only preventing $Dsx^M$ and $Fru^M$ expression by activating alternative splicing, but also in directly repressing their translation (Usui-Aoki et al., 2000). The use of such a failsafe in the sex-determination pathway is also seen in males, whereby *mir-124* targets *tra* transcripts for degradation in the male nervous system to ensure the total elimination of Tra protein in males (Weng et al., 2013).

*tra* has been shown to act independently of *fru/dsx* to promote female-specific properties of tissues outside of the CNS (Evans and Cline, 2013; Hudry et al., 2016; Rideout et al., 2015). In the fat body, *tra* is necessary for the non-cell-autonomous increase in growth and body size of females relative to males, in a mechanism that is insensitive to *dsx* and *fru* (Rideout et al., 2015). In intestinal stem cells that do not express *dsx* or *fru*, *tra* acts to enhance cellular proliferation to expand tissue size (Hudry et al., 2016). Thus, these studies provide evidence for non-canonical roles of *tra* outside of the CNS. In this report, we show that *tra* plays a double assurance role to antagonize $Fru^M$ isoform function in the female CNS. Thus, our findings add to a growing literature supporting a more expansive role for *tra* than had long been postulated. Overall, our results lend additional support to an emerging literature that *tra* and also *sxl* direct certain sexually dimorphic properties outside of a strictly linear sex-determination cascade that uses *fru* and *dsx* as sole effectors (Evans and Cline, 2013; Hudry et al., 2016; Rideout et al., 2015). It will be interesting to explore the mechanisms of action of such additional functions for *tra* and *sxl*, and determine the benefits that drove divergence from a strictly linear sex-determination cascade.

## MATERIALS AND METHODS

### Fly stocks

Flies were maintained on standard cornmeal food at 70% humidity at 18°C or 25°C. *Ilp7-GAL4* (Castellanos et al., 2013). Strains from Bloomington *Drosophila* Stock Center were: *P{10xUAS-IVS-Syn21-GFP-p10}* (referred to as *UAS-GFP*) (Pfeiffer et al., 2012); *P{UAS-p35.H}BH1* (Hay et al., 1994); $P\{GawB\}elav^{Gal\text{-}C155}$ (referred to as *elav>* and $elav^{C155\text{-}GAL4}$) (Lin and Goodman, 1994); *UAS-Dicer2* (Dietzl et al., 2007); $dsx^1$ (amorphic allele) (Ota et al., 1981); $Dp(1;Y)B^S;cn\ tra2^Bbw^1$ (amorphic *tra2* allele); *Df(2L)trix* (*tra2* deletion) (Goralski et al., 1989); $y^1v^1;P\{TRiP.JF02256\}attP2$ (referred to as $UAS\text{-}dsx^{RNAi}$); and $w^{1118}$ (referred to as '+'). The following alleles were obtained as generous gifts: *nsyb-GAL4* (Pauli et al., 2008); $UAS\text{-}dsx^F$ (Lee et al., 2002); and *Df(3R)dsx15* (*dsx* deficiency) (Baker et al., 1991). *tra* alleles used were: $UAS\text{-}tra^F$ (Ferveur et al., 1995); $UAS\text{-}tra^{dsRNAi}$ (Chan and Kravitz, 2007); and $tra^{KO}$ (amorphic allele) (Hudry et al., 2016). Putative or reported severe hypomorphs or nulls of *fru P1* transcripts include: $fru^{P1\text{-}GAL4}$ (Stockinger et al., 2005), $P\{PZ\}fru^3$ and $Df(3R)fru^{Sat15}$. The engineered *fru* alleles that constitutively splice into female- or male-specific isoforms include $fru^F$, $fru^{\Delta tra}$ and $fru^M$ (Demir and Dickson, 2005). The following are $Fru^M$ isoform-specific nonsense mutants: $fru^{\Delta A}$ and $fru^{\Delta B}$ (Neville et al., 2014), and $fru^{\Delta C}$ (Billeter et al., 2006). The following are Myc-tagged $Fru^M$ isoform-specific alleles: $fru^{Amyc}$, $fru^{Bmyc}$ and $fru^{Cmyc}$ (von Philipsborn et al., 2014).

### Generating the Ilp7-nls.tdTomato reporter

To generate the *Ilp7-nls.tdTomato* reporter, we PCR amplified −2964 to +424 (*Ilp7* start codon) relative to the transcriptional start site of the *Ilp7* gene. We overlapped the *Ilp7* translational start site with a *tdTomato* ORF (Han et al., 2011) fused at the C-terminus to the Tra nuclear localization signal and SV40-pA sequences from the pHstinger vector (Barolo et al., 2000). This construct was inserted into the psD7-001 vector. Fly transformation by P-element insertion was performed by Best Gene. P-element insertions on the second chromosome were recovered and established as stable fly strains.

### Tissue processing and immunohistochemistry

Verification of correct genotypes in adults was determined by loss of balancer chromosomes and/or by evidence of re-sexualization in appropriate genotypes (e.g. chaining behavior and/or changes in abdominal pigmentation and genitalia). Male and female adult VNCs were dissected within 24 h of eclosion. Standard protocols for immunohistochemistry were used (Eade and Allan, 2009). Primary antibodies used were rabbit anti-Ilp7 (1:1000; a gift from E. Hafen, ETH, Zurich, Switzerland); rabbit anti-Ilp7 (1:2000; this study, see below for details); guinea pig anti-Fork head (1:1000; a gift from H. Jäckle, Max Planck Institute, Göttingen, Germany); rat anti-Myc (1:1000; Abcam, JAC6); mouse anti-Elav (1:100; Developmental Studies Hybridoma Bank, University of Iowa). Secondary antibodies used were: anti-rabbit, anti-guinea pig, anti-mouse and anti-rat IgG (H+L) conjugated to DyLight 488, Cy3 or Cy5 (1:400, Jackson ImmunoResearch).

### Ilp7 antibody generation

We generated a rabbit polyclonal antibody to the Ilp7 neuropeptide using the antibody generation services of GenScript USA. They synthesized the KLH-conjugated peptide CRSQSDWENVWHQETHS. This peptide sequence was chosen based on the sequence from Yang and colleagues (Yang et al., 2008) (NH2-RSQSDWENVWHQETHS-CONH2), but we added an N-terminal cysteine for KLH conjugation and did not block the C terminus from amidation. GenScript tested the antigen purity by mass spectrometry, immunized rabbits, affinity purified the polyclonal antibody against antigen and tested the antibody titer using ELISA. We tested the polyclonal antibody on *Drosophila* VNCs and found that it generated immunoreactivity consistent with the anti-Ilp7 antibody generated by Yang and colleagues, localizing to the cytosol of all nuclei labeled using the *Ilp7-nls.tdTomato* reporter (Fig. S1).

### Image and statistical analysis

All images were acquired using an Olympus FV1000 confocal microscope. FS-Ilp7 motoneurons were manually counted using Fluoview Software (FV10-ASW). All representative images in figures were processed using Adobe Photoshop CS6 (identically for all images being compared), and figures were made in Adobe Illustrator CS6. For images collected from *fru>GFP* genotypes, we lowered the brightness of the green channel for the readers to easily observe the other channels; for *Ilp7-Tdtomato* genotypes we increased the brightness of the red channel to easily observe expression in FS-Ilp7 motoneurons. Where appropriate, images were false-colored for clarity, and colors were chosen for color-blind readers. All statistical analysis and graphing were performed using Prism 6 software (GraphPad Software). A minimum $n$=8 flies was used for each genotype studied. All data underwent D'Agostino and Pearson normality testing; data within graphs were compared using one-way ANOVA followed by Tukey post hoc analysis. Statistical differences are shown if $P<0.05$.

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**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
Conceptualization: S.R.C.G., M.C.C., D.W.A.; Methodology: S.R.C.G., T.L., D.W.A.; Validation: D.W.A.; Formal analysis: S.R.C.G., M.C.C., K.E.B., D.W.A.; Investigation: S.R.C.G., M.C.C., K.E.B., T.L., D.W.A.; Resources: D.W.A.; Data curation: S.R.C.G., M.C.C., K.E.B., D.W.A.; Writing - original draft: S.R.C.G., M.C.C., D.W.A.; Writing - review & editing: S.R.C.G., M.C.C., D.W.A.; Visualization: S.R.C.G., D.W.A.; Supervision: D.W.A.; Project administration: D.W.A.; Funding acquisition: S.R.C.G., M.C.C., D.W.A.

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**Supplementary information**
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.150821.supplemental

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