fruitless tunes functional flexibility of courtship circuitry during development

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ABSTRACT

Drosophila male courtship is controlled by the male-specific products of the *fruitless* (*fru*^{M}) gene and its expressing neuronal circuitry. *fru*^{M} is considered a master gene that controls all aspects of male courtship. By temporally and spatially manipulating *fru*^{M} expression, we found that *fru*^{M} is required during a critical developmental period for innate courtship towards females, and its function during adulthood is relatively trivial. By altering or eliminating *fru*^{M} expression, we generated males that are innately heterosexual, homosexual, bisexual, or without innate courtship but could acquire such behavior in an experience-dependent manner. These findings show that *fru*^{M} is not absolutely necessary for courtship but is critical during development to build a sex circuitry with reduced flexibility and enhanced efficiency and provide a new view about how *fru*^{M} tunes functional flexibility of a sex circuitry.

Keywords:

Drosophila; courtship; innate behavior; fruitless; circuit flexibility; sexual orientation

Introduction

Drosophila male courtship is one of the best understood innate behaviors in terms of genetic and neuronal mechanisms (Dickson, 2008; Yamamoto and Koganezawa, 2013). It has been well established that the *fruitless* (*fru*) gene and its expressing neurons control most aspects of such innate behavior (Ito et al., 1996; Manoli et al., 2005; Ryner et al., 1996; Stockinger et al., 2005). The male-specific products of the P1 promoter of the *fru* gene (fru^M) are expressed in ~2000 neurons, which are inter-connected to form a sex circuitry from sensory neurons to motor neurons (Cachero et al., 2010; Lee et al., 2000; Manoli et al., 2005; Stockinger et al., 2005; Usui-Aoki et al., 2000; Yu et al., 2010). fru^M function is necessary for the innate courtship behavior and sufficient for at least some aspects of courtship (Baker et al., 2001; Demir and Dickson, 2005; Manoli et al., 2005). Thus, the study of fru^M function in controlling male courtship serves as an ideal model to understand how innate complex behaviors are built into the nervous system by regulatory genes (Baker et al., 2001).

Although fru^M serves as a master gene controlling *Drosophila* male courtship, we recently found that males without fru^M function, although do not court if raised in isolation, were able to acquire at least some courtship behaviors if raised in groups (Pan and Baker, 2014). Such fru^M-independent but experience-dependent courtship acquisition requires another gene in the sex determination pathway, the *doublesex* (*dsx*) gene (Pan and Baker, 2014). *dsx* encodes male- and female-specific DSX

proteins (DSX^M and DSX^F, respectively) (Burtis and Baker, 1989), and DSX^M is expressed in ~700 CNS neurons, the majority of which also express fru^M (Rideout et al., 2010; Robinett et al., 2010). It has been found that the fru^M and dsx^M co-expressing neurons are required for courtship in the absence of fru^M function (Pan and Baker, 2014). Thus fru^M-expressing neurons, especially those co-expressing dsx^M, control the expression of courtship behaviors even in the absence of FRU^M function. Indeed, although the gross neuroanatomical features of the fru^M-expressing circuitry are largely unaffected by the loss of fru^M, detailed analysis revealed morphological changes of many fru^M-expressing neurons (Kimura et al., 2005; Kimura et al., 2008; Mellert et al., 2010). Recent studies further reveal that FRU^M specifies neuronal development by recruiting chromatin factors and changing chromatin states, and also by turning on and off the activity of the transcription repressor complex (Ito et al., 2012; Ito et al., 2016; Sato et al., 2019a; Sato et al., 2019b; Sato and Yamamoto, 2020).

That FRU^M functions as a transcription factor to specify development and/or physiological roles of certain fru^M-expressing neurons, and perhaps the interconnection of different fru^M-expressing neurons to form a sex circuitry raises important questions regarding when fru^M functions and how it contributes to the sex circuity (*e.g.*, how the sex circuitry functions differently with different levels of FRU^M), especially in the background that fru^M is not absolutely necessary for male courtship (Pan and Baker, 2014). To at least partially answer these questions, we

72 temporally or spatially knocked down *fru*^{M} expression, and compared courtship
73 behavior in these males with that in wild-type males or *fru*^{M} null males and revealed
74 crucial roles of *fru*^{M} during a narrow developmental window for the innate courtship
75 towards females. We also found that the sex circuitry with different *fru*^{M} expression
76 has distinct function such that males could be innately heterosexual, homosexual,
77 bisexual, or without innate courtship but could acquire such behavior in an
78 experience-dependent manner. Thus, *fru*^{M} tunes functional flexibility of the sex
79 circuitry instead of switching on its function as conventionally viewed.
80
81
82 **Results**
83
84 To specifically knock down *fru*^{M} expression, we used a microRNA targeting *fru*^{M}
85 (*UAS-fruMi*) and a scrambled version as a control (*UAS-fruMiScr*) as previously used
86 (Chen et al., 2017). We firstly tested male courtship without food in the behavioral
87 chamber. Knocking down *fru*^{M} in all the *fru*^{GAL4} labeled neurons eliminated male
88 courtship towards females (courtship index [CI], which is the percentage of
89 observational time that males displayed courtship, is nearly 0) (Figure 1A), consistent
90 with previous findings that *fru*^{M} is required for innate male-female courtship (Demir
91 and Dickson, 2005; Pan and Baker, 2014). We then added a temperature dependent
92 *tub-GAL80*^{ts} transgene to restrict *UAS-fruMi* expression (*e.g.*, at 30˚C) at different

93 developmental stages. We raised *tub-GAL80^{ts}/+; fru^{GAL4}/UAS-fruMi* flies at 18˚C
94 (permissive for GAL80^{ts} that inhibits GAL4 activity) and transferred these flies to
95 fresh food vials every two days. In this way we generated *tub-GAL80^{ts}/+;*
96 *fru^{GAL4}/UAS-fruMi* flies at 9 different stages from embryos to adults and incubated all
97 flies at 30˚C to allow *fru^{M}* knock-down for 2 days, then placed all flies back to 18˚C
98 until courtship test (Figure 1B). We found that males with *fru^{M}* knocked down from
99 stage 5 to 6, matching the pupation phase, rarely courted (CI < 10%) and none
100 successfully mated, while males with *fru^{M}* knocked down near this period showed a
101 partial courtship deficit (Figure 1C, D). Knocking down *fru^{M}* specifically during
102 adulthood for 2 days did not affect male courtship (CI > 80%) and mating success.
103 These results reveal a critical developmental period where *fru^{M}* is required for adult
104 male courtship towards females.

105 As we did not see an obvious courtship deficit in males with *fru^{M}* knocked down
106 in adulthood for 2 days, we further tested the role of *fru^{M}* in adulthood using different
107 approaches. We set out to express the female-specific *transformer* (*traF*) gene (Baker
108 and Ridge, 1980; McKeown et al., 1988) to feminize all *fru^{GAL4}* labeled neurons, in
109 addition to the *fru^{M}* RNAi experiments. We express *UAS-traF* or *UAS-fruMi* in all the
110 *fru^{GAL4}* labeled neurons specifically during adulthood for 4 days before test (see
111 procedure above each figure) for single-pair male-female, male-male, and male
112 chaining (in groups of 8 males) behaviors. We found that overexpression of *traF* in all
113 *fru^{GAL4}* labeled neurons during adulthood for 4 days did not affect male-female

courtship (Figure 1E), but slightly increased male-male (Figure 1F) and male chaining behaviors (Figure 1G). Furthermore, knocking down fru^{M} in all fru^{GAL4} labeled neurons during adulthood for 4 days did not affect male-female (Figure 1E) or male-male courtship (Figure 1F), but slightly increased male chaining behaviors (Figure 1G). Together these results indicate that fru^{M} function during pupation is crucial for adult courtship towards females, while its function during adulthood is dispensable for female-directed courtship, though it plays a minor role in inhibiting male-male courtship behaviors.

To further reveal the role of fru^{M} in male courtship, we tried to spatially knock down fru^{M} expression using a simple way: fru^{M} in brain and fru^{M} outside brain. We used *Otd-Flp* expressing FLP specifically in the central brain (Asahina et al., 2014) to divide fru^{GAL4} expression (Figure 2A) into two parts: fru^{M}- and *Otd*-positive neurons (specifically in brain) in *Otd-Flp*/*UAS-myrGFP*; fru^{GAL4}/*tub>GAL80>* males (Figure 2B), and fru^{M}-positive but *Otd*-negative neurons (theoretically outside brain, but still with few in brain) in *Otd-Flp*/*UAS-myrGFP*; fru^{GAL4}/*tub>stop>GAL80* males (Figure 2C). We also checked GFP expression in peripheral nervous system in these males, and found a few GFP-positive cells in antennae and forelegs in *Otd-Flp/UAS-myrGFP;* fru^{GAL4}*/tub>stop>GAL80* males, but no expression in *Otd-Flp/UAS-myrGFP;* fru^{GAL4}*/tub>GAL80>* or wild-type males (Figure 2D-G). Thus, we successfully divided fru^{GAL4} expression into two categories, one with *GAL4* expressed

in $fru^{+}Otd^{+}$ neurons in brain, and the other with *GAL4* expressed in $fru^{+}Otd^{-}$ neurons outside brain.

We then used the above intersectional strategy to knock down fru^{M} expression in all fru^{GAL4} neurons or those neurons in or outside brain, and compared male courtship with that in wild-type males and fru^{M} null males. We tested one-time single-pair male-female and male-male courtship (single-housed before test) as well as male chaining in groups of 8 males over 3 days on food for better comparison of these courtship assays, as courtship by fru^{M} null males largely depends on food presence (Pan and Baker, 2014). We found that male-male courtship in fru^{M} knocked down males is higher if tested on food, consistent with a courtship promoting role by food (Grosjean et al., 2011; Pan and Baker, 2014), while courtship in wild-type males on food or without food is not changed in our assays (Figure 3—figure supplement 1). We found that wild-type males performed intensive courtship behavior towards virgin females (CI > 80%) and rarely courted males (CI ~0) (Figure 3A). Furthermore, these control males did not show any chaining behavior after grouping from 3 hours to 3 days (ChI = 0) (Figure 3B). In striking contrast, fru^{M} null mutant (fru^{LexA}/fru^{4-40}) males rarely courted either females or males (Figure 3C); however, these males developed intensive chaining behavior after grouping for 1-3 days (Figure 3D). These observations replicated previous findings that there exists a fru^{M}-independent experience- and dsx^{M}-dependent courtship pathway (Pan and Baker, 2014) (Figure 3E). To compare behavioral differences by fru^{M} null males and fru^{M} RNAi knocked

down males, we quantified to how much extent the microRNA against *fru*^{M} worked. We found that the *fru*^{M} mRNA level was reduced to ~40% of that in control males (Figure 3F). Interestingly, while males with *fru*^{M} knocked down in all *fru*^{M} neurons rarely courted females (CI~5%, Figure 3G), they displayed a high level of male-male courtship behavior (CI > 50%, Figure 3G) and constantly high level of male chaining (Figure 3H), dramatically different from *fru*^{M} null males. These results reveal distinct roles of low *fru*^{M} (RNAi) and high *fru*^{M} (wild-type) in regulating male-male and male-female courtship (Figure 3I). To further dissect the role of *fru*^{M} in male courtship, we knocked down *fru*^{M} specifically in brain, and found that such males had a reduced level of courtship towards females (CI = 56.61 ± 5.86%), but their sexual orientation was not changed as they courted males in a much lower level (CI = 15.94 ± 3.26%, Figure 3J). Furthermore, males with *fru*^{M} knocked down in brain showed low male chaining behavior initially but increasing levels of chaining behavior over 1-3 days (ChI[3h] = 9.35 ± 5.40%, ChI[3d] = 68.82 ± 5.53%, Figure 3K). These results indicate that *fru*^{M} function in brain promotes male-female courtship and inhibits acquisition or progression of the experience-dependent chaining behavior (Figure 3L). In contrast, males with *fru*^{M} knocked down outside brain showed equally intensive male-female and male-male courtship (CI[male-female] = 85.62 ± 1.42%, CI[male-male] = 82.89 ± 2.76%, Figure 3M), indicating an inhibitory role of *fru*^{M} in these neurons for male-male courtship (Figure 3O). These males performed a high level of male chaining behavior initially (ChI[3h] =92.90 ± 3.08%), but decreased levels of

chaining behavior over 1-3 days (ChI[3d] = 20.01 ± 3.75%, Figure 3N), consistent with the above finding that *fru*^{M} function in the brain which is intact in these males inhibits acquisition or progression of male chaining behavior (Figure 3L).

Taken together, the above results indicate crucial role of *fru*^{M} expression level during a critical developmental period for the manifestation of courtship behaviors and reveal functional flexibility of the *fru*^{M}-expressing sex circuitry (Figure 4).

Discussion

Previous findings show that *fru*^{M} expression commences at the wandering third instar larval stage, peaks at the pupal stage, and thereafter declines but does not disappear after eclosion (Lee et al., 2000), which suggests that *fru*^{M} may function mainly during development for adult courtship behavior despite of no direct evidence. Here we temporally knocked down *fru*^{M} expression in different developmental stages for 2 days and found that males with *fru*^{M} knocked down during pupation rarely courted, while males with *fru*^{M} knocked down during adulthood courted normally towards females. This is the first direct evidence that *fru*^{M} is required during development but not adulthood for courtship behavior. However, we also found a minor role of *fru*^{M} during adulthood in suppressing male-male courtship, as males with *fru*^{M} knocked down or *tra* overexpressed during adulthood displayed slightly enhanced male-male

197 courtship or male chaining behaviors. Note that a previous study found that removal
198 of *transformer 2* (*tra2*) specifically during adulthood using a temperature sensitive
199 *tra2* allele induced 8 out of 96 females to show male-type courtship behaviors (Belote
200 and Baker, 1987), which suggests that expression of FRU^M and DSX^M (by removal of
201 *tra2* function in females) during adulthood is sufficient to masculinize CNS to some
202 extent and induce a small fraction of females to display courtship behaviors. A recent
203 study also found that fru^M expression in the *Or47b*-expressing olfactory receptor
204 neurons depends on the activity of these neurons during adulthood (Hueston et al.,
205 2016). Based on all these findings, we propose that fru^M expression during pupation is
206 crucial for specifying a sex circuitry that allows innate courtship towards females, and
207 its expression during adulthood may be activity-dependent in at least some neurons
208 and modulates some aspects of courtship (*e.g.*, inhibits male-male courtship). Thus,
209 there are at least two separate mechanisms that fru^M contributes to the sex circuitry,
210 one during a critical developmental period to build the female-directed innate
211 courtship into that circuity, and the other during adulthood to modulate neuronal
212 physiology in an experience-dependent manner.

213 Most importantly, we revealed striking flexibility of the fly sex circuitry by
214 manipulating fru^M expression. We listed four cases with fru^M manipulation here for
215 comparison: (1) males with a sex circuitry having wild-type fru^M function have innate
216 heterosexual courtship, as they court readily towards females, but do not court males
217 no matter how long they meet; (2) males with a sex circuitry having no fru^M function

lose the innate courtship ability, but have the potential to acquire courtship towards males, females, and even other species in an experience-dependent manner; (3) males with a sex circuitry having limited fru^M expression (*e.g.*, 40%) have innate homosexual courtship, as they court readily towards other males, but rarely court females; (4) males with a sex circuitry having limited fru^M expression outside brain (but intact fru^M expression in brain) are innately bisexual, as they court equally towards females or males. Although previous studies found that different fru^M alleles (*e.g.*, deletions, inversions or insertions related to *fru*) showed very different courtship abnormalities (Anand et al., 2001; Villella et al., 1997), it was very hard to link fru^M function to the flexibility of sex circuitry, and often seen as allele-specific or background-dependent phenotypes. Our study using relatively simple genetic manipulations that generate dramatical different courtship behaviors promoted us to speculate a different view about the role of fru^M: instead of simply being a master gene that controls all aspects of male courtship, fru^M is not absolutely necessary for courtship, but changes the wiring of the sex circuitry during development such that the sex circuitry may function in very different ways, ranging from innately heterosexual, homosexual, bisexual to largely experience-dependent acquisition of the behavior. Such flexibility of the sex circuitry is tuned by different fru^M expression, such that changes of fru^M regulatory regions during evolution would easily select a suitable functional mode of the sex circuitry.

239

240 **Materials and methods**

241

242 **Fly Stocks**

243 Flies were maintained at 22 or 25˚C in a 12 h:12 h light:dark cycle. Canton-S flies

244 were used as the wild-type strain. Other stocks used in this study include the

245 following: fru^{GAL4} (Stockinger et al., 2005), *UAS-fruMi* and *UAS-fruMiScr* (Meissner

246 et al., 2016), fru^{LexA} and fru^{4-40} (Pan and Baker, 2014) and *Otd-Flp* (Asahina et al.,

247 2014). *UAS-traF* (BL#4590), *tub-*$GAL80^{ts}$ (BL#7019), *tub>GAL80>* (BL#38881)

248 and *tub>stop>GAL80* (BL#39213) were from Bloomington Drosophila Stock

249 Center.

250

251 **Courtship and Chaining Assays**

252 For the single-pair courtship assay, the tester males and target flies (4-8 days old)

253 were gently aspirated into round 2-layer chambers (diameter: 1 cm; height: 3 mm per

254 layer) and were separated by a plastic transparent barrier that was removed ~30 min

255 later to allow courtship test. Courtship index (CI), which is the percentage of

256 observation time a fly performs any courtship step, was used to measure courtship to

257 female targets or between 2 males. Paired male-male courtship used 2 males of the

258 same genotype but focused on the male fly that first initiated courtship (courtship of

the initiator to the other). All tester flies were single housed if not otherwise mentioned. Each test was performed for 10 min.

For male chaining assay, tester males (4-8 days old) were loaded into large round chambers (diameter: 4 cm; height: 3 mm) by cold anesthesia. Tests were performed daily for 4 consecutive days (3 hours after grouping as day 0, then days 1–3). Chaining index (ChI), which is the percentage of observation time at least 3 flies engaged in courtship together, was used to measure courtship in groups of 8 males.

Immunohistochemistry

We dissected brains and ventral nerve cords of 5-7 days old males in Schneider's insect medium (Thermo Fisher Scientific, Waltham, MA) and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 min at room temperature. After washing four times in PAT (0.5% Triton X-100 and 0.5% bovine serum albumin in PBS), tissues were blocked in 3% normal goat serum (NGS) for 60 min, then incubated in primary antibodies diluted in 3% NGS for ~24 hr at 4˚C, washed (4 × 15-min) in PAT at room temperature, and incubated in secondary antibodies diluted in 3% NGS for ~24 hr at 4˚C. Tissues were then washed (4 × 15-min) in PAT and mounted in Vectorshield (Vector Laboratories, Burlingame, CA) for imaging. Primary antibodies used were rabbit anti-GFP (1:1000; A11122, Invitrogen, Waltham, MA) and mouse anti-Bruchpilot (1:50; nc82, Developmental Studies Hybridoma Bank, Iowa City, IA). Secondary antibodies used were goat anti-mouse

IgG conjugated to Alexa 555 (1:500, A28180, Invitrogen) and goat anti-rabbit IgG conjugated to Alexa 488 (1:500, A11008, Invitrogen). Samples were imaged at 10× or 20× magnification on a Zeiss 700 confocal microscope and processed with ImageJ.

Statistics

Experimental flies and genetic controls were tested at the same condition, and data are collected from at least two independent experiments. Statistical analysis is performed using GraphPad Prism and indicated inside each figure legend. Data presented in this study were first verified for normal distribution by D'Agostino–Pearson normality test. If normally distributed, Student's *t* test is used for pairwise comparisons, and one-way ANOVA is used for comparisons among multiple groups, followed by Tukey's multiple comparisons. If not normally distributed, Mann-Whitney U test is used for pairwise comparisons, and Kruskal-Wallis test is used for comparisons among multiple groups, followed by Dunn's multiple comparisons.

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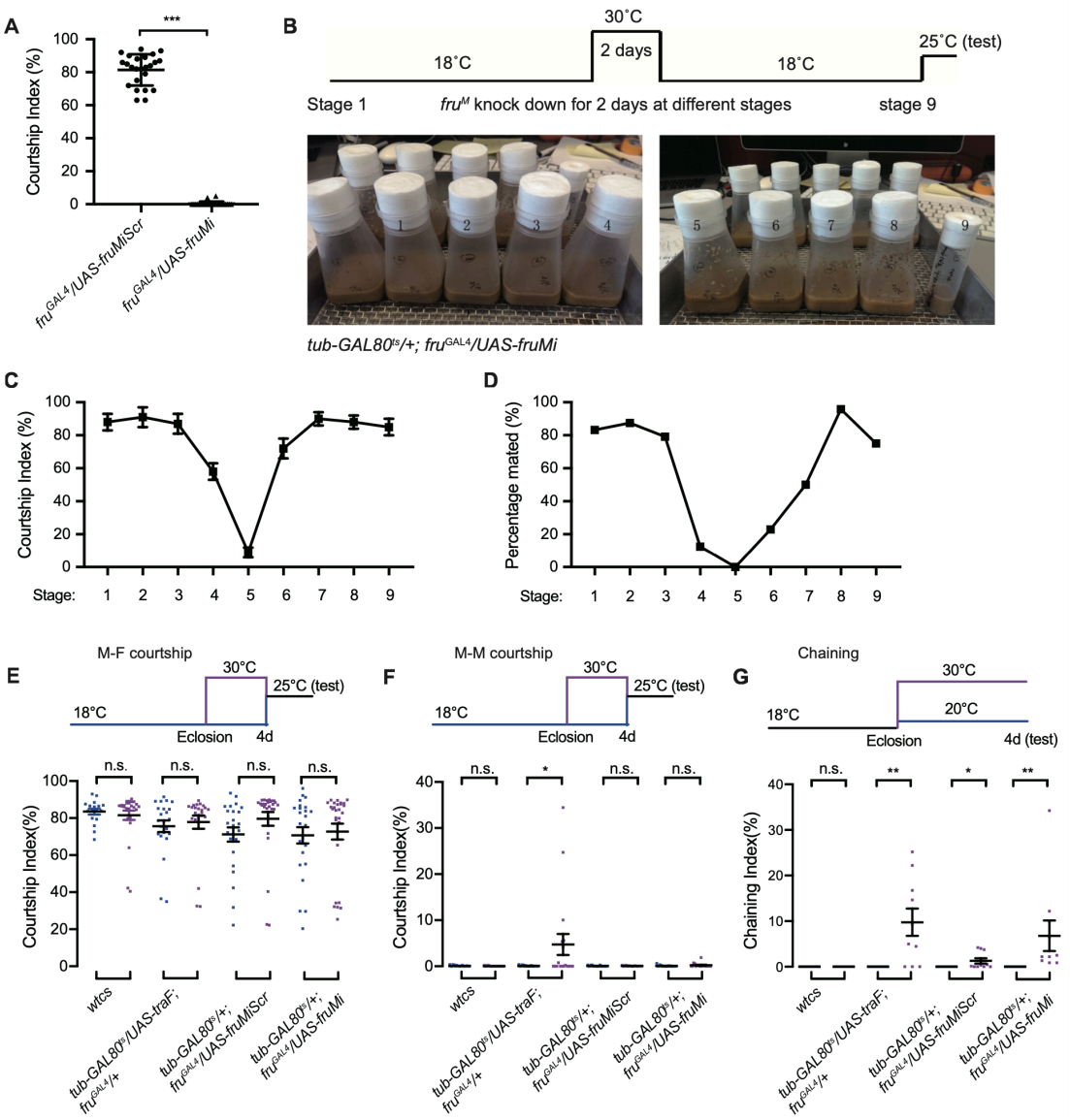
402

403 **Figure legends**

404



405

406 **Figure 1.** Distinct roles of *fru*^{M} during development and adulthood in regulating male

407 courtship. **A:** Knocking down *fru*^{M} using RNAi throughout development and

408 adulthood eliminated male courtship towards virgin females. $n = 24$ for each. ***$p <$

409 0.001, unpaired t-test. **B:** A schematic of genetic strategy to knock down *fru*^{M} at

410 different developmental stages for 2 days. Stages 1 to 9 refer to specific

411 developmental stages from embryos to adults with interval of 2 days. **C** and **D**:

412 Courtship indices of males with *fru*^{M} knocked down at specific developmental stages
413 as indicated above towards virgin females. Males with *fru*^{M} knocked down at stage 5
414 for 2 days (a period of pupation from stage 5 to 6, see above picture) rarely courted
415 virgin females (**C**), and none successfully mated (**D**). Knocking down *fru*^{M} at stages
416 near 5 (*e.g.*, stage 4 or 6) also partially impairs courtship and mating success.
417 Knocking down *fru*^{M} at stage 9 (adult) has no obvious effect on courtship and mating.
418 $n = 24$ for each. **E-G**: Knocking down *fru*^{M} specifically during adulthood slightly
419 increased male-male courtship behaviors. For male-female courtship (**E**), $n = 17$, 26,
420 23, 23, 24, 27, 24 and 28 respectively (from left to right), n.s., not significant,
421 unpaired t-test. For single-pair male-male courtship (**F**), $n = 18$ for each. n.s., not
422 significant, $*p < 0.05$, unpaired t-test. For male chaining among 8 males as a group
423 (**G**), $n = 8, 8, 8, 10, 8, 9, 8$ and 9 respectively (from left to right). n.s., not significant,
424 $*p < 0.05$, $**p < 0.01$, Mann-Whitney test. Error bars indicate SEM.
425

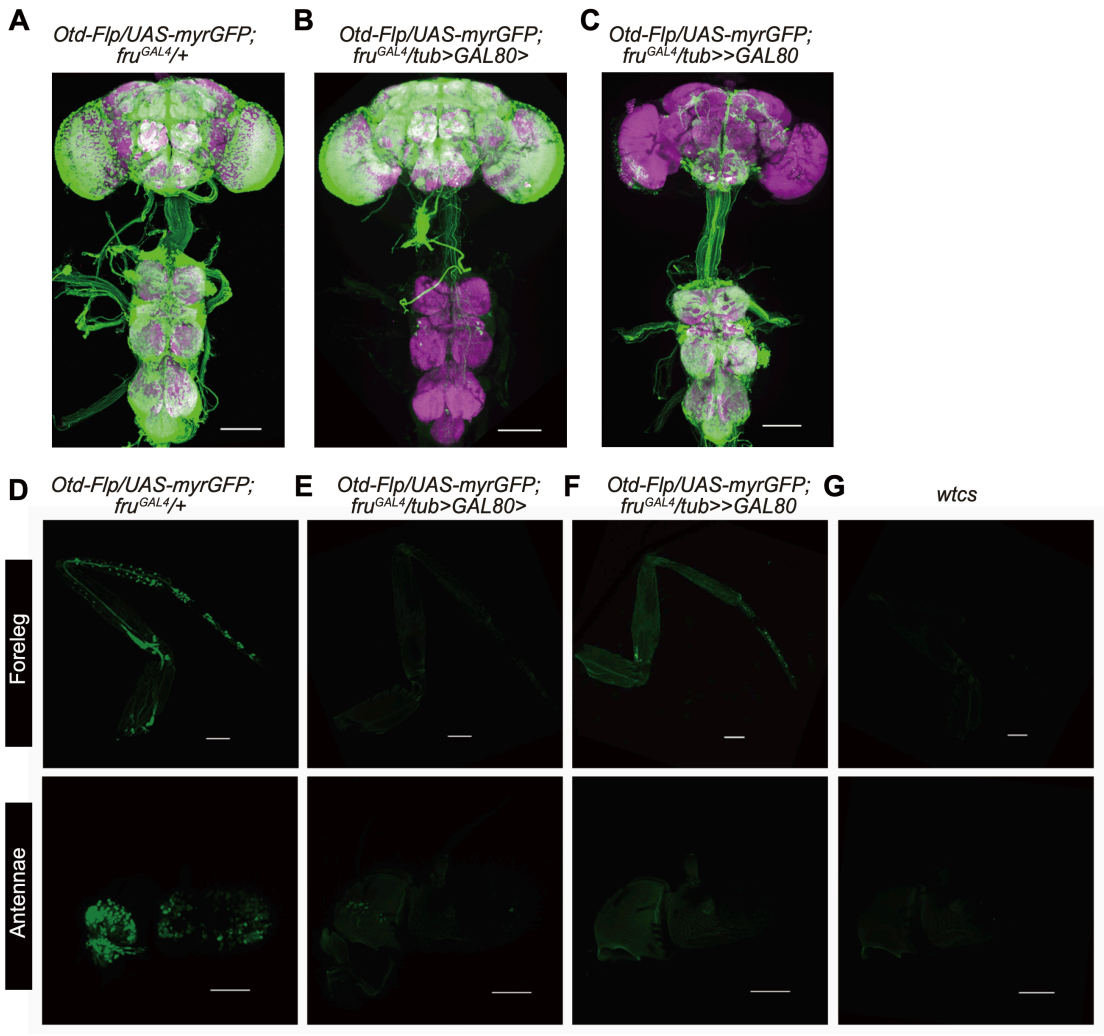


Figure 2. Dividing *fru*^{M} expression into two complementary parts. **A**: Expression pattern of the *fru*^{M} circuitry revealed by *fru*^{GAL4} driving *UAS-myrGFP* (green), co-stained with nc82 (magenta). **B** and **C**: Genetic strategy to divide *fru*^{M} neurons (green) into two parts. Scale bars above, 100μm. **D-G**: Expression pattern of *fru*^{GAL4} driving *UAS-myrGFP* (green) in forelegs (scale bars, 100 mm) and antennae (scale bars, 50 mm) in males with indicated genotypes. Representative of 5 samples each.

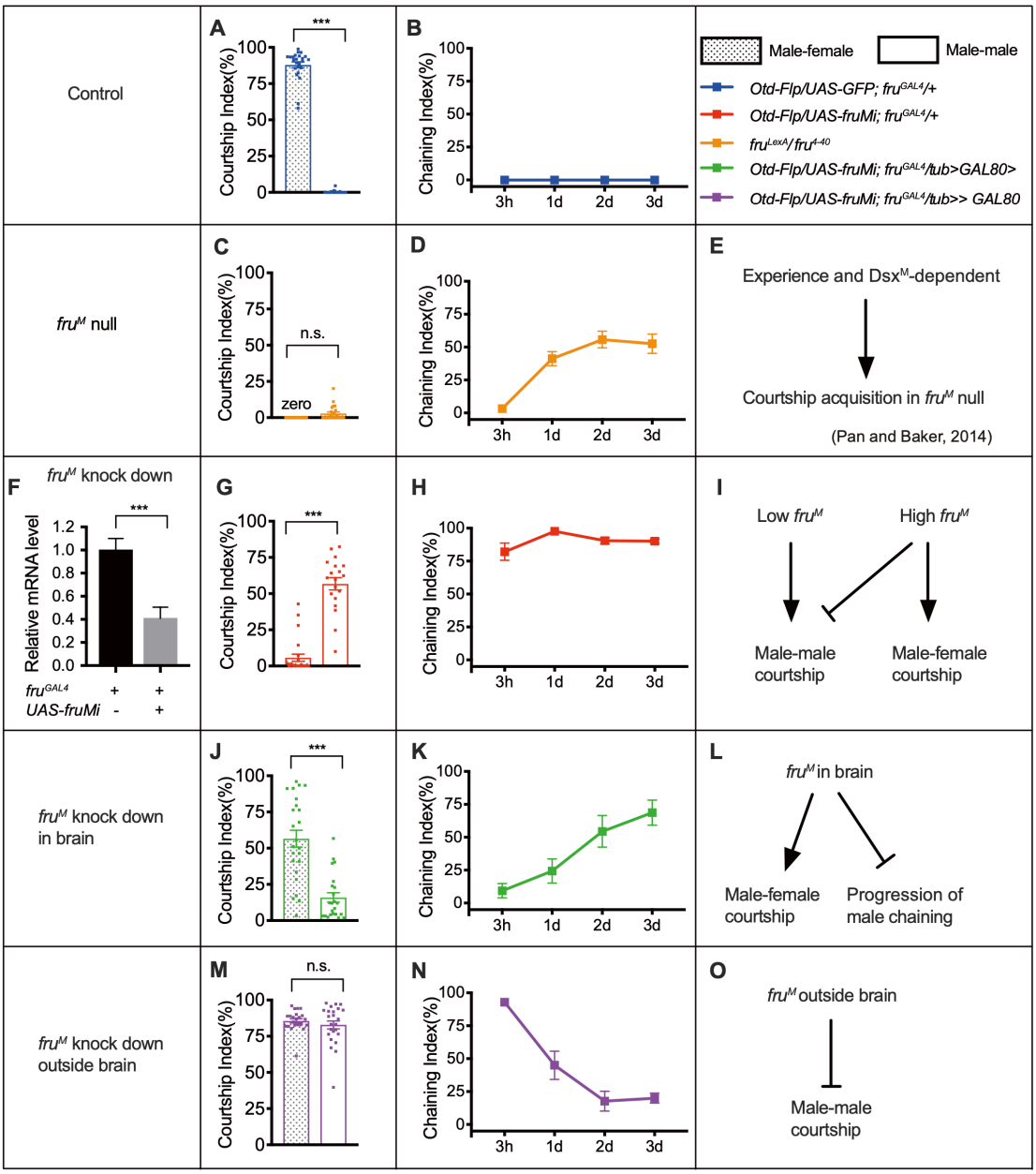


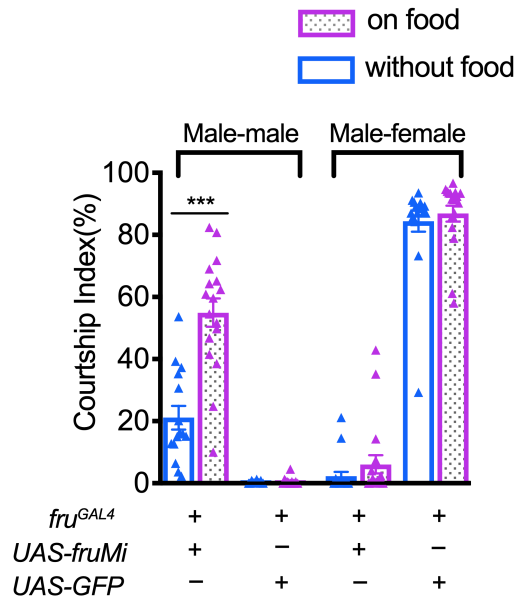
Figure 3. *fru*^{M} tunes functional flexibility of the *fru*^{M} circuitry. **A** and **B**: Wild-type males courted intensively towards virgin females (**A**, left bar), but rarely courted males (**A**, right bar) or displayed chaining behavior in groups of 8 males (**B**). $n = 24$, 24, 8 respectively. ***$p < 0.001$, unpaired t-test. **C**: *fru*^{LexA}/*fru*$^{4\text{-}40}$ (*fru*^{M} null) males rarely courted either females or males. $n = 24$ for each. n.s., not significant, unpaired t-test. **D**: *fru*^{LexA}/*fru*$^{4\text{-}40}$ males did not show chaining behavior after 3-hr group-housing, but developed intensive chaining behavior after1-3 days. $n = 8$. **E**: A

444 summary of courtship acquisition independent of fru^M. **F**: RNAi against fru^M
445 efficiently decreased but not fully eliminated fru^M expression. $n = 3$. ***$p < 0.001$,
446 Mann-Whitney U test. **G**: Knocking down fru^M in all fru^{GAL4} neurons generated males
447 that have reversed sexual orientation such that they rarely courted females but
448 intensively courted males. $n = 24$ and 19 respectively. ***$p < 0.001$, unpaired t-test.
449 **H**: Males with fru^M knocked down in all fru^{GAL4} neurons showed intensive chaining
450 behavior at all time points (from 3 hours to 3 days upon group-housing). $n = 7$. **I**:
451 Distinct roles of low fru^M (RNAi) and high fru^M (wild-type) in regulating male-male
452 and male-female courtship. **J**: Males with fru^M knocked down in fru^{GAL4} neurons in
453 the brain had a lower level of courtship towards females, but their sexual orientation
454 was not changed. $n = 24$ and 23 respectively. ***$p < 0.001$, unpaired t-test. **K**: Males
455 with fru^M knocked down in fru^{GAL4} neurons in brain showed low male chaining
456 behavior initially but increasing levels of chaining behavior over 1-3 days. $n = 6$. **L**: A
457 summary of the role of fru^M in brain in promoting male-female courtship and
458 suppressing the experience-dependent acquisition or progression of male chaining
459 behavior. **M**: Males with fru^M knocked down in fru^{GAL4} neurons outside brain
460 generated bisexual males that have intensive male-female and male-male courtship. n
461 = 24 for each. n.s., not significant, unpaired t-test. **N**: Males with fru^M knocked down
462 in fru^{GAL4} neurons outside brain showed high male chaining behavior initially, but
463 decreased levels of chaining behavior over 1-3 days. $n = 8$. **O**: A summary of the role

464 of *fru*^{M} outside brain in suppressing male-male courtship behavior. Error bars indicate

465 SEM.

466



467

468 **Figure 3—figure supplement 1.** Comparison of male courtship with or without food.

469 Male-male courtship in *fru*^{M} knocked down males is higher in the presence of food. *n*

470 = 15, 17, 15, 18, 18, 18, 18 and 18 from left to right respectively. ***$p < 0.001$,

471 unpaired t-test. Error bars indicate SEM.

472

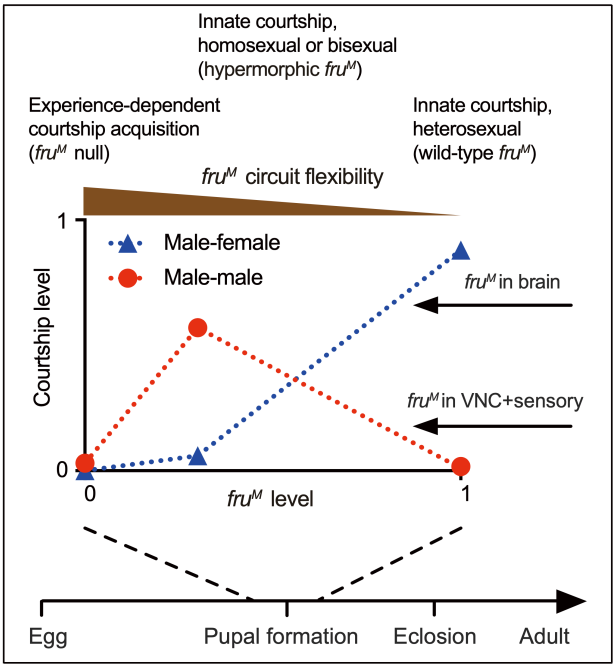


Figure 4. A summary of *fru*^{M} function in male courtship. Firstly, *fru*^{M} is largely required during a specific developmental period for courtship, and plays a minor role during adulthood; secondly, the sex circuitry without *fru*^{M} or with different levels of *fru*^{M} has different properties such that males would have experience-dependent courtship acquisition, or innate courtship but with different sexual orientation. Such flexibility of the sex circuitry is tuned by different *fru*^{M} expression. Red circles and blue triangles represent corresponding *fru*^{M} levels and courtship levels (red: male-male courtship; blue: male-female courtship).