LETTERS

Male-specific *fruitless* specifies the neural substrates of *Drosophila* courtship behaviour

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Robust innate behaviours are attractive systems for genetically dissecting how environmental cues are perceived and integrated to generate complex behaviours. During courtship, *Drosophila* males engage in a series of innate, stereotyped behaviours that are coordinated by specific sensory cues. However, little is known about the specific neural substrates mediating this complex behavioural programme[1]. Genetic, developmental and behavioural studies have shown that the *fruitless* (*fru*) gene encodes a set of male-specific transcription factors (Fru^M) that act to establish the potential for courtship in *Drosophila*[2]. Fru^M proteins are expressed in ~2% of central nervous system neurons, at least one subset of which coordinates the component behaviours of courtship[3,4]. Here we have inserted the yeast *GAL4* gene into the *fru* locus by homologous recombination and show that (1) Fru^M is expressed in subsets of all peripheral sensory systems previously implicated in courtship, (2) inhibition of Fru^M function in olfactory system components reduces olfactory-dependent changes in courtship behaviour, (3) transient inactivation of all Fru^M-expressing neurons abolishes courtship behaviour, with no other gross changes in general behaviour, and (4) 'masculinization' of Fru^M-expressing neurons in females is largely sufficient to confer male courtship behaviour. Together, these data demonstrate that Fru^M proteins specify the neural substrates of male courtship.

The Fru^M proteins are generated from sex-specifically spliced transcripts from the P1-*fru* promoter[2,5] (Fig. 1a, b). Using homologous recombination, we introduced the yeast *GAL4* coding sequence, including start and stop codons, into the *fru^M* coding sequence[6] (Fig. 1b) and simultaneously deleted the first two codons (ATGATG) of the *fru^M* open reading frame to prevent its translation. Proper integration into *fru* was verified using genomic polymerase chain reaction (PCR). This modified *fru* gene, *fruP1-GAL4*, is null for P1-*fru* function. Staining the central nervous system (CNS) of *fruP1-GAL4* homozygotes revealed no Fru^M protein (data not shown). These homozygotes do not show courtship behaviour but appear otherwise normal (Supplementary Fig. S1).

To determine whether *fruP1-GAL4* accurately reflects P1-*fru* expression, we compared the CNS expression patterns of Fru^M and a nuclear green fluorescent protein (GFP) marker (UAS-*GFPnls*) driven by *fruP1-GAL4*. Approximately 48 h after puparium formation, when Fru^M expression is maximal (~1,500–1,700 cells)[3], GFP and Fru^M signals are coincident (Fig. 1c, d). The number of Fru^M-expressing cells declines to ~1,200–1,300 cells in pharate adults, and remains relatively constant into young adulthood[3] (Supplementary Fig. S2). Whether this decrease reflects cell death or transient Fru^M expression is unknown. We also compared Fru^M expression and *fruP1-GAL4*-driven expression of GFP at later times (72–84 h after puparium formation), as GFP should remain in cells that transiently expressed *fruP1-GAL4*. We simultaneously drove the expression of UAS-*GFPnls* and UAS-*mCD8GFP*, which encodes a relatively stable membrane-bound form of GFP. Comparison of GFP and Fru^M signals revealed that most cells stained positively for GFP at the membrane, and for both Fru^M staining and GFPnls signal in the nucleus. In ~10% of cells there was neither Fru^M staining nor nuclear GFP, but GFP was present at the cell membrane (arrowheads in Fig. 2a; Supplementary Fig. S2), suggesting that in these neurons P1-*fru* expression was transient and the nuclear GFP and Fru^M proteins were depleted by turnover, while the more stable mCD8GFP persisted.

The site of *GAL4* insertion in *fruP1-GAL4* is common to P1-derived transcripts in both sexes, allowing us to determine sex-specific differences in the principal features of neurons expressing these transcripts. mCD8GFP expression driven by *fruP1-GAL4* revealed a complex pattern of neuronal projections with many prominently labelled nerve bundles and neuropil structures (Fig. 2b, c). No marked differences were seen between the principal features of the projections of P1-*fru* neurons in males and females, suggesting that Fru^M proteins do not specify distinct neural structures or function at the level of pathfinding and early development in the neurons in which they are expressed, but more likely specify their fine connectivity and/or physiology.

We next examined the expression of *fruP1-GAL4* throughout the body to determine whether technical limitations had previously prevented detection of Fru^M in other tissues. In all peripheral sensory systems implicated in courtship, we found substantial *fruP1-GAL4* expression in subsets of sensory neurons, but not their associated, non-neuronal support cells (Fig. 3 and Supplementary Fig. S3). *fruP1-GAL4* is expressed in ~100–150 olfactory receptor neurons (ORNs) in each antenna. On the basis of their distribution and CNS glomerular projection patterns (see below), these neurons are mostly from trichoid sensilla, which have been implicated in pheromone detection in other species[7] (arrow in Fig. 3a). *fruP1-GAL4* is also expressed in about four olfactory receptor neurons within each maxillary palp (Fig. 3c, inset). In the auditory system, *fruP1-GAL4* is expressed in most, if not all, neurons in Johnston's organ, a chordotonal organ found in the second antennal segment[8] (arrowhead in Fig. 3a), as well as in two small chordotonal organs at the base of the wing (Fig. 3e). This is consistent with the observation that proprioceptive feedback is necessary for proper courtship song[9,10]. The taste (gustatory) neurons of *Drosophila* innervate sensory bristles on the legs, proboscis and the oral tract[11], and *fruP1-GAL4* is expressed in ~20–23 gustatory neurons in the foreleg (Fig. 3f) as well as in ~20–30 gustatory neurons in the proboscis (Fig. 3c). In the visual system, we detect transient pupal *fruP1-GAL4* expression in the retina. Expression is seen in corresponding regions in the periphery of both sexes (data not shown).

The only mechanosensory neurons in which we detect *fruP1-GAL4* expression are the neurons innervating (1) the sex comb bristles on

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the male foreleg (Fig. 3f, inset, and Supplementary Fig. S3), (2) the genital clasper bristles, (3) the genital lateral plate bristles, (4) bristles on the ventral analia and (5) the hypandrial bristles associated with the penis apparatus (Fig. 3i and Supplementary Fig. S3). Notably, these are the only places where male-specific morphological specializations of mechanosensory bristles are found. Sex combs are used in grasping the female and spreading her wings during copulation in other species, although their function in *D. melanogaster* is unknown[12]. Mechanosensory information transduced through genital claspers and genital lateral plates bristles mediates species-specificity and positioning of the genitalia during attempted copulation[13]. Hypandrial bristles may be involved in the detection of sensory cues that elicit the sequential transfer of seminal fluids and sperm[14].

To determine whether peripheral *fruP1-GAL4* expression represented ectopic GAL4 expression, as has been found with *fru* transgenes[15], we used antibodies against Fru^{M} and *in situ* hybridization to *fru* transcripts to re-examine peripheral *fru* expression in males and females. We found Fru^{M} protein and *fru* transcript expression in peripheral neurons, consistent with the *fruP1-GAL4* expression pattern (Fig. 3 and Supplementary Fig. S3).

That Fru^{M} is expressed in subsets of sensory neurons suggests that males and females may detect distinct sensory stimuli at the level of sensory neurons themselves, or that they might process and perceive such sensory information in different ways. Moreover, these findings strongly suggest that sexual sensory cues are initially recognized in the Fru^{M}-expressing sensory neurons, and thus that these neurons are entry points for following the flow of specific visual, gustatory, olfactory, auditory and tactile information governing courtship.

We also examined whether Fru^{M} was expressed in higher-order visual and olfactory neurons. We found limited Fru^{M} expression in optic lobes[3], and *fruP1-GAL4* expression in medullary neurons as well as 4–5 clusters of neurons in the lobula, regions where integration and processing of visual information occurs (Supplementary Fig. S4). In addition, using a UAS-*synaptotagmin–HA* (UAS-*syt–HA*) marker to label presynaptic termini, *fruP1-GAL4* expression is seen in distinct tracts leaving the lobulae, including a major tract projecting to the anterior optical tubercle and superior medial protocerebrum (Supplementary Fig. S4).

The axons of olfactory receptor neurons terminate in antennal lobe glomeruli. *fruP1-GAL4*-directed reporter expression showed processes of *fruP1-GAL4* olfactory receptor neurons projecting primarily to 3–4 glomeruli (DA1, VA1l, VA1m and VL2), with much weaker labelling of other glomeruli (Fig. 2d and Supplementary Fig. S4). We observed dendritic projections to these glomeruli from *fruP1-GAL4* labelled projection neurons adjacent to the antennal lobes (Fig. 2d and Supplementary Fig. S4). Notably, others have shown that the DA1 glomerulus is sexually dimorphic in Hawaiian Drosophilids, and to a lesser extent in *D. melanogaster*[16].

Naive male *Drosophila* typically court other males upon first encountering them, but then sustainably habituate to all males[17]. To determine whether Fru^{M} function in primary and/or secondary olfactory neurons was involved in male–male habituation, we analysed males in which Fru^{M} was inhibited in the majority of olfactory receptor neurons. This inhibition was achieved by expression of an RNA-mediated interference transgene (UAS-*fru^{M}IR*) targeting the male-specific amino terminus of Fru^{M} isoforms[4]. Inhibition of Fru^{M} in most olfactory receptor neurons (through the *Or83b-GAL4*

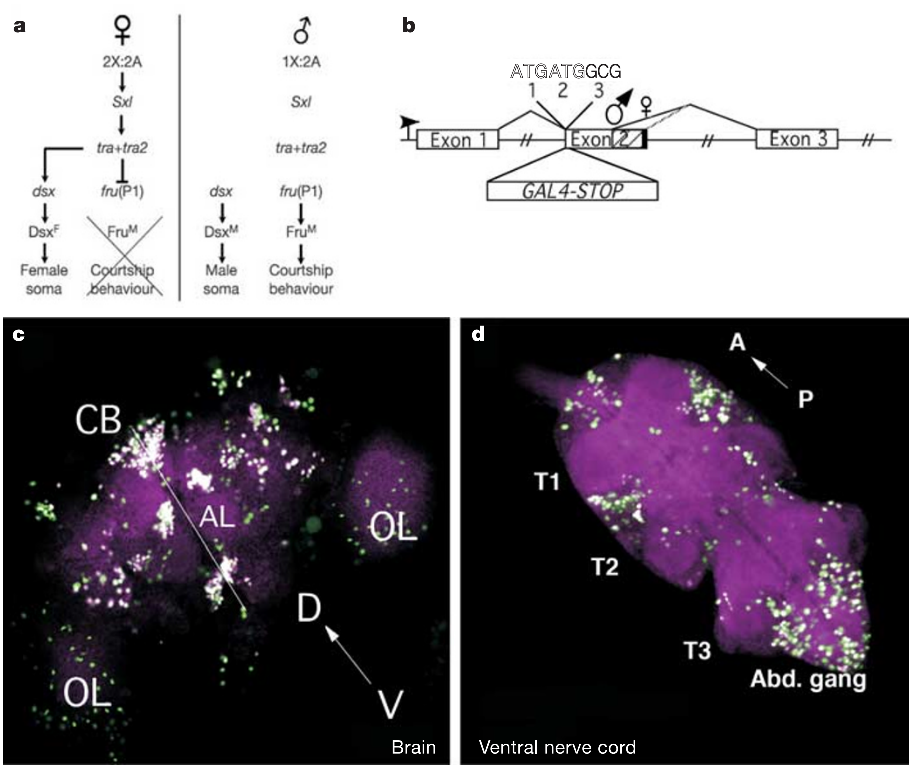

Figure 1 | Male-specific *fruitless* regulates courtship. a, In male flies, the absence of *Sex lethal* (*Sxl*) and *transformer* (*tra*) activity results in the default splicing of P1-*fru* transcripts to produce male-specific isoforms (Fru^{M}) that are required for courtship behaviour. **b**, The generation of *fruP1-GAL4*. A diagram of the *fru* locus indicates the insertion point of the yeast *GAL4* transcription factor into the P1-*fru* open reading frame by homologous recombination. The arrowhead shows the P1 transcriptional start site. Male and female splice sites are indicated, and the Tra/Tra-2 binding region is shown in black. Codons 1 and 2 (outline) were deleted upon recombination. **c**, **d**, *fruP1-GAL4*-directed expression accurately reproduces endogenous Fru^{M} expression patterns. *fruP1-GAL4*-driven nuclear GFP (green) and endogenous Fru^{M} (magenta) expression in the anterior brain (**c**) and ventral nerve cord (**d**) of a male two-day-old pupa coincide (white) throughout the CNS. Abbreviations used: A, anterior; Abd. gang., abdominal ganglion; AL, antennal lobes (line shows the midline); CB, central brain; D, dorsal; OL, optic lobes; P, posterior; T1–T3, thoracic segments 1–3; V, ventral.

driver), or neurons projecting to the glomeruli labelled by *fruP1-GAL4* (through the *SG18.1-GAL4* driver), resulted in sustained male–male courtship after 1 h of pairing, whereas males expressing a control UAS-*GFPIR* transgene typically showed a decrease in courtship levels[18,19] (Fig. 4a). Thus, Fru^M function in olfactory receptor neurons and/or secondary olfactory neurons is required for male–male habituation.

As second-order olfactory projection neurons project to the mushroom bodies, we looked for expression of *fruP1-GAL4* in mushroom bodies. Anti-Fru^M staining is not seen in pupal mushroom bodies, but weak Fru^M staining has been seen in adults in the region of Kenyon cell nuclei[3,15]. Examining *fruP1-GAL4*-driven UAS-*mCD8GFP* expression in adult flies revealed substantial expression in mushroom body γ-neurons (arrows in Fig. 2d), and in a small number of α/β-neurons (arrowheads) that appeared ~24 h after eclosion, when sexual maturity is attained (Fig. 2d and Supplementary Fig. S4).

Male mushroom body γ-lobes, although not necessary for courtship itself, are necessary for courtship conditioning to mated females (that is, males learn not to court recently mated females, which display high levels of rejection[20]; J. M. Dura, personal communication). To determine whether Fru^M function in mushroom body neurons was necessary for such conditioning, we analysed conditioning in males in which Fru^M expression was inhibited in sets of mushroom body neurons by UAS-*fru^{M}IR* expression. Inhibition of Fru^M expression throughout the mushroom bodies (using an *OK107-GAL4* driver) and in γ-neurons (using *H24-GAL4* and *201y-GAL4* drivers) reduced the conditioning response. Restricting the expression of interfering RNAs to only α/β-neurons (using the *17D-GAL4* driver) had less of an effect (Fig. 4b). Thus, Fru^M functions in mushroom bodies to regulate courtship conditioning to mated females. The large number of Fru^M-expressing neurons in the mushroom bodies suggests that a significant fraction of the mushroom bodies might function in a manner that is at least in part sex-specific.

There is only minimal *fruP1-GAL4* expression in 'higher-order' centres such as the central complex and much of the proto- and deuterocerebrum, structures previously implicated in the generation and coordination of general motor programmes and behaviours in insects[21] (Fig. 2d and Supplementary Fig. S4). This suggests that Fru^M neurons are unlikely to be involved in general processing and coordination of behaviour (see below). *fruP1-GAL4* expression is also not detected in most motor neurons in the ventral nerve cord. This again suggests that Fru^M-expressing neurons might modulate, rather than directly mediate, behavioural output (data not shown). One example of such courtship-specific control of conserved neural modules is the generation of song, as the same motor neurons that drive flight also generate courtship song[9]. However, Fru^M-expressing neurons might directly control certain outputs of courtship behaviour; for example, Fru^M-expressing motor neurons innervate the male-specific muscle of Lawrence, and about eight serotonin-containing, Fru^M-expressing neurons provide the sole innervation to some male internal genital organs[5,15,22,23]. Thus Fru^M-expressing neurons might directly mediate output through male-specific structures, and indirectly modulate output dependent on structures common to both sexes.

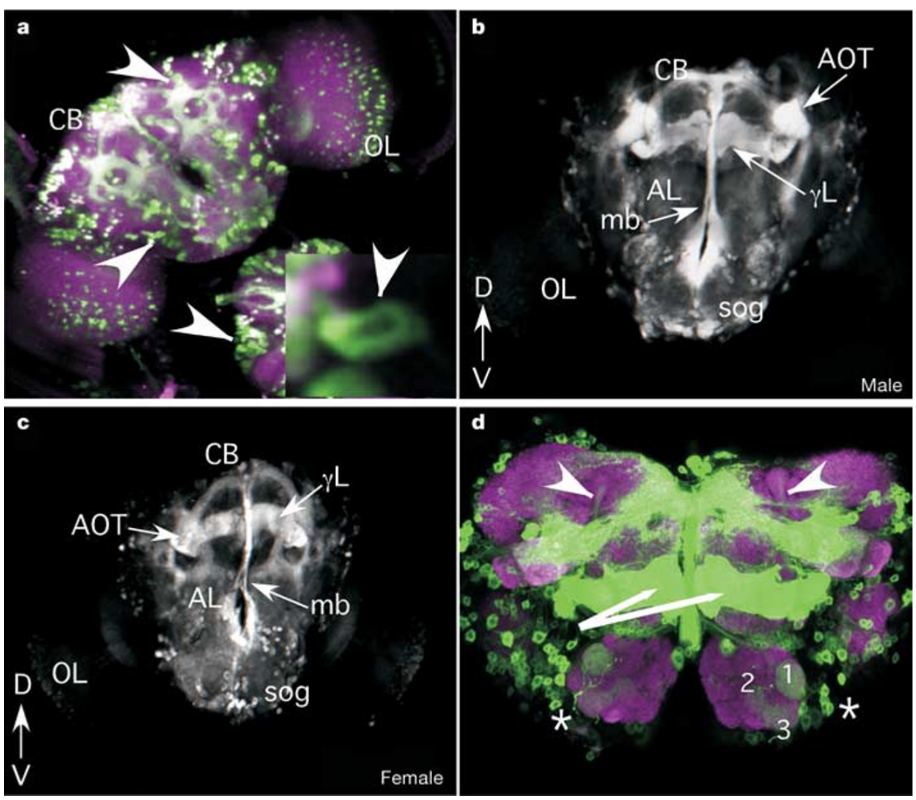

Figure 2 | *fruP1-GAL4* expression in the central nervous system. **a**, *fruP1-GAL4*-driven expression of membrane and nuclear GFP (green) and Fru^M (magenta) in pharate adults reveals a limited number of neurons showing membrane GFP expression but neither nuclear GFP nor Fru^M staining. This suggests that nuclear GFP and Fru^M may be degraded in these cells (arrowheads and inset). **b**, **c**, There are no major differences in *fruP1-GAL4*-driven expression of membrane-bound GFP between males (**b**) and females (**c**), suggesting that Fru^M does not specify basic neuronal structures or tracts. Abbreviations used: AOT, anterior optic tubercle; γL, mushroom body γ-lobes; mb, median bundle; sog, suboesophageal ganglion (additional abbreviations provided in Fig. 1 legend). **d**, *fruP1-GAL4* is expressed in the olfactory system, including in projections from olfactory receptor neurons to antennal lobe glomeruli DA1 (1), VA1l (2), VA1m (3) and VL2 (not shown), projection neurons innervating these glomeruli (asterisks), and mushroom body γ- (arrows) and α/β-neurons (arrowheads). Membrane GFP is shown in green, and neuropil (nc82) staining in magenta.

To determine whether the function of Fru^{M}-expressing neurons during courtship is necessary, we used *fruP1-GAL4*-directed expression of a temperature-sensitive dynamin allele (shi^{TS}) to transiently inactivate these neurons. Transient inactivation of Fru^{M}-expressing neurons in males at restrictive temperature (31 °C) abolishes courtship behaviour (Fig. 4c; $n = 20$), but grooming, walking and flight behaviours are normal (Supplementary Video S1), suggesting that Fru^{M}-expressing neurons are largely dedicated to courtship.

We asked whether expression of Fru^{M} in these neurons is both necessary and sufficient to confer the potential for male courtship by using *fruP1-GAL4*-driven expression of UAS-*tra2IR* to inhibit transformer-2 (Tra-2) expression and thus masculinize just the Fru^{M}-expressing neurons in a female[5,24] (see Fig. 1a). Strikingly, *fruP1-GAL4*/UAS-*tra2IR* masculinized females all (10/10) displayed the initial stages of courtship behaviour—orientation and tapping—when paired with a wild-type virgin female (Fig. 4d), but wing and proboscis extension and attempted copulation were not seen. When paired with a wild-type male, these masculinized females were always courted, but showed male-like rejection behaviours, including wing flicking and kicking, and never showed the female rejection response of ovipositor extrusion seen in control females (Fig. 4d).

Similarly, *fruP1-GAL4*-directed expression of individual Fru^{M} isoforms (as UAS-*fru* or UAS-fru^{M} constructs) in females also conferred certain aspects of courtship behaviour (Fig. 4d). However, the lower level and extent of courtship behaviours in these females suggest that each isoform functions in a non-redundant manner.

We wondered whether such masculinized females might have the potential for more aspects of male courtship than they displayed. As hearing male song is sufficient to induce courtship behaviour in wild-type males[25], we placed multiple *fruP1-GAL4*/UAS-*tra2IR* masculinized females with a single wild-type male. Indeed, in 10 out of 13 groups containing three *fruP1-GAL4*/UAS-*tra2IR* females and one wild-type male, male singing was sufficient to elicit wing extension and vibration as well as occasional proboscis extension in a masculinized female that was not being courted (Fig. 4d and Supplementary Fig. S5). No attempts at copulation were observed, perhaps owing to the anatomical restrictions of a female abdomen. Thus *fruP1-GAL4* masculinized females have the potential for more male courtship behaviour than they display when with a single female. This could be because the masculinization/transformation by UAS-*tra2IR* was incomplete or because male identity in tissues other than *fruP1*-expressing neurons is necessary for proper stimulation. The observation that Fru^{M} function in a distinct subset of neurons is both necessary and largely sufficient to confer the potential for courtship strongly supports the idea that the circuitry underlying innate behaviours might be controlled by dedicated genetic programmes[2].

Our findings offer new insights into the neuronal circuitry underlying complex behavioural programmes. The existence of Fru^{M} expression in subsets of all peripheral sensory systems implicated in courtship, as well as second- and third-order neurons in the two sensory systems examined, suggests that specific parts of sensory systems mediate the detection and initial processing of sensory cues relevant to courtship. The lack of overt sexual dimorphism in Fru^{M}-expressing neurons suggests that Fru^{M} proteins function to alter fine neuronal connectivity and/or physiology in order to process and transmit information relevant to courtship arousal. That Fru^{M}-expressing neurons have little (if any) role in other behaviours suggests that these neurons modulate conserved elements

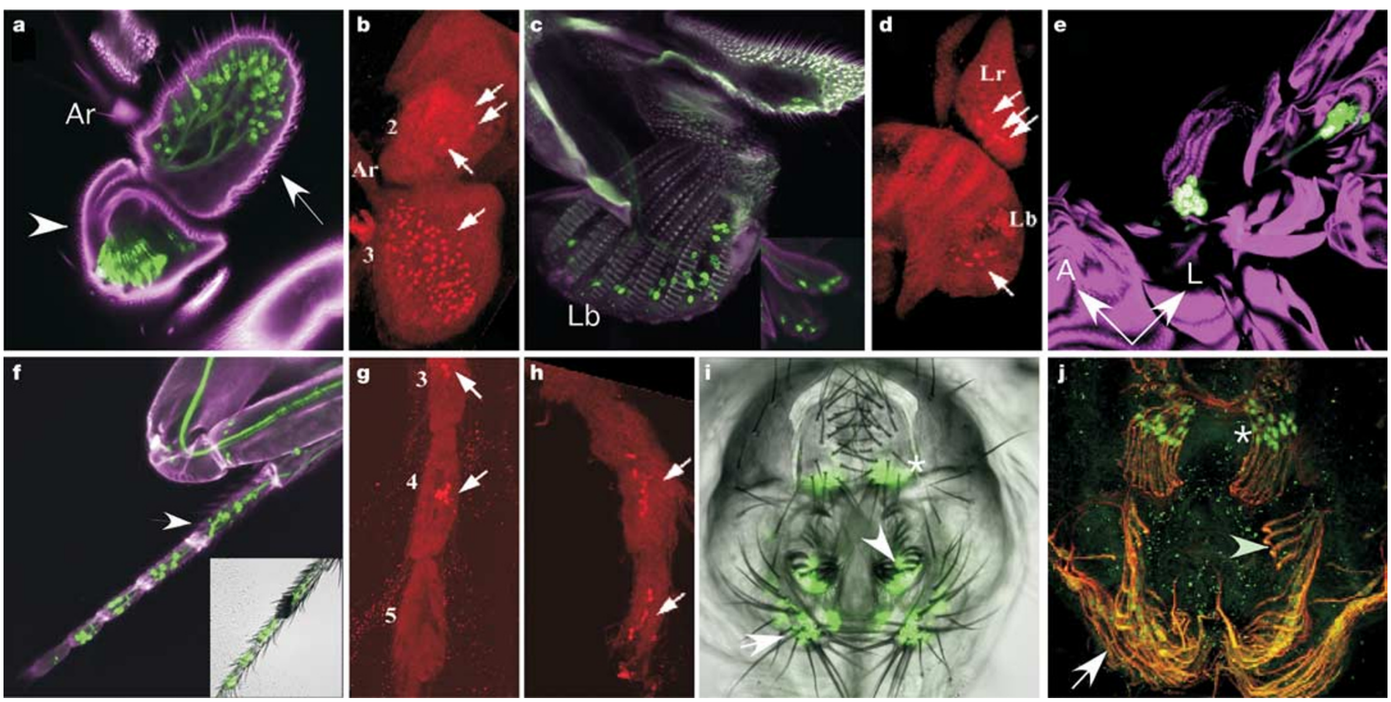

Figure 3 | *fruP1-GAL4* reveals Fru^{M} expression in regions of the peripheral nervous system implicated in courtship behaviours. Shown are *fruP1-GAL4*-expressing neurons (membrane GFP, green) and autofluorescence (magenta/grey; **a**, **c**, **e**, **f**, **i**) in peripheral nervous system structures. Endogenous Fru^{M} is found in these locations (arrows in **b**, **d**, **g**, **h**, **j**). **a**, **b**, In the antenna, *fruP1-GAL4* labels 100–150 olfactory sensory neurons in the third antennal segment (arrow in **a**) and auditory neurons of Johnston's organ in the second segment (arrowhead in **a**; Ar, arista). **c**, **d**, In the proboscis, 20–30 gustatory neurons express *fruP1-GAL4*, and 4 olfactory neurons in the maxillary palps are labelled (inset). Lb, labellum; Lr, labrum. **e**, In the wing joint, *fruP1-GAL4* labels two clusters of proprioceptive neurons (A, anterior; L, lateral). **f**–**h**, In the prothoracic leg, *fruP1-GAL4* labels gustatory neurons and mechanosensory neurons associated with the sex combs (arrow in **f**, inset shows brightfield image of leg and sex comb; proximal tarsus segments numbered in **g**; distal tarsus shown in **h**). **i**, **j**, In the male external genitalia, *fruP1-GAL4* labels distinct clusters of mechanosensory neurons associated with bristles on the lateral plates (arrow), the claspers (arrowhead), and the ventral-most part of the analia (asterisk in **i**, **j**), neuronal projections (22C10) are shown in red (**j**).

of the nervous system for courtship-specific behavioural output. Thus, the specification of distinct circuitry for complex innate behavioural programmes might involve the establishment of elements that (1) discriminate specific stimuli from background, (2) integrate such information from multiple sensory modalities, and (3) relay ethologically relevant input to and output from conserved components of the nervous system to generate specific behavioural states, as well as elements that coordinate distinct behavioural modules[4]. A precedent for such a circuit involved in mating behaviour, in which sensory cues detected through male-specific neurons mediate the coordination of centrally generated behaviours, is seen in nematodes[26].

We can now begin to characterize the molecular and cellular processes regulated by Fru^M proteins, and examine how these processes act during development to build the potential for male sexual behaviour. Understanding the apparently subtle but nevertheless critical function of Fru^M as a transcription factor might help to elucidate the evolutionary strategies through which behavioural programmes are built from or into general components of the nervous system[27]. We can now also address how specific neurons function to detect or transmit behaviourally relevant sensory cues, integrate this information to perceive the external environment, and process such information to generate and modulate meaningful behavioural output.

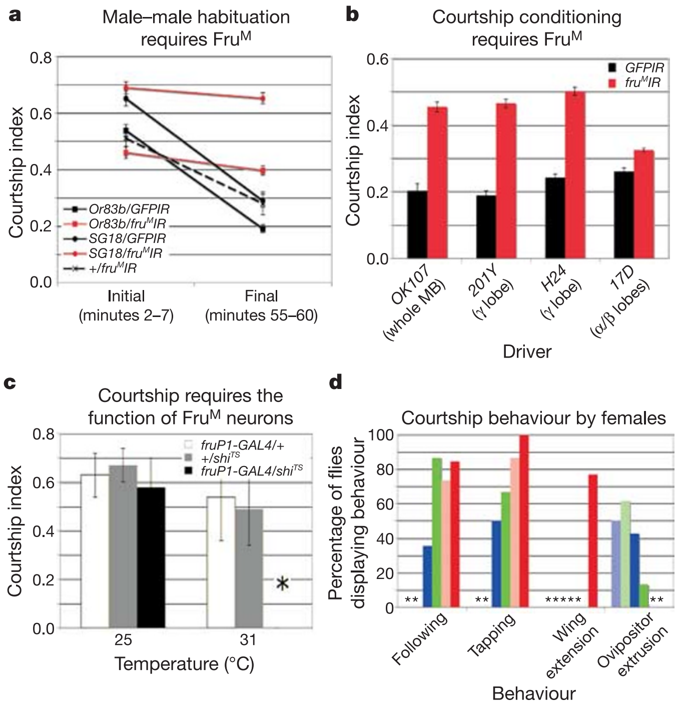

Figure 4 | Function of Fru^M neurons in courtship. **a**, Inhibition of *fru^M* expression in primary and/or secondary olfactory neurons reduces male–male habituation. Shown are courtship index (CI) values for pairs of males with *fru^M* inhibition by UAS-*fru^{M}IR* expression (*SG18*, $n = 20$; *OR83b*, $n = 12$), control males expressing UAS-*GFPIR* ($n = 10$ for *SG18* and *OR83b* drivers) or with UAS-*fru^{M}IR* alone ($n = 10$). Males showed persistent male–male courtship after the habituation period (*SG18*, $F_{1,18} = 114.7$; *OR83b*, $F_{1,18} = 87.6$; $P < 0.001$) in *fru^{M}IR* but not control animals. **b**, Inhibition of *fru^M* expression in mushroom bodies reduces courtship conditioning in response to mated females. Shown are CI values for males with virgin wild-type females after exposure to a mated wild-type female ($n = 10$ for all groups). RNAi effects, $F_{1,72} = 459.7$; driver effects, $F_{3,72} = 12$; interaction, $F_{3,72} = 30.5$, $P < 0.001$. Homogeneity groups between lines for each treatment: *GFPIR*, all lines; *fru^{M}IR*, OK107/201Y, H24, 17d. **c**, Inhibition of synaptic transmission in *fruP1-GAL4*-expressing neurons in males abolishes courtship. Shown are CI values for *fruP1-GAL*/+ ($n = 10$), UAS-*shi^{TS}* ($n = 10$) and *fruP1-GAL4*/UAS-*shi^{TS}* ($n = 20$) males at permissive (25 °C) and restrictive (31 °C) temperatures. Following a burst of wing extension (14/20 males, 62 ± 7 s), *fruP1-GAL*/UAS-*shi^{TS}* males thereafter displayed no courtship. **d**, Expression of Fru^M in and masculinization of *fruP1-GAL4*-expressing neurons in females confers components of courtship behaviour. Females expressing Fru^M zinc-finger isoforms A or C show following and tapping behaviour towards a virgin CS female, and decreased levels of ovipositor extrusion when placed with a CS male. Only females masculinized in *frup1-GAL4*-expressing neurons show wing and sometimes proboscis extension when grouped and placed with a CS male. UAS-transgenes used: sex-common isoforms are light green (*fru^C*; $n = 10$) and light blue (*fru^A*; $n = 13$); male-specific isoforms are green (*fru^{MC}*; $n = 14$), blue (*fru^{MA}*; $n = 15$) and pink (*tra2IR*; $n = 15$). Red bars represent groups containing 1 CS male and 3 *tra2IR* females ($n = 13$ groups). Asterisks (**c**, **d**) indicate no behaviour observed. All error bars indicate s.e.m.

METHODS

***Drosophila* stocks and culture.** The *fruP1-GAL4* line was generated as described below. The UAS-mCD8GFP, UAS-*traF* and UAS-*tra2IR* lines were obtained from the Bloomington Drosophila Stock Center. The Stinger 5 nuclear GFP (UAS-*GFPnls*) line was a gift from S. Barolo. The UAS-*fru* lines were a gift from S. Goodwin[28]. The UAS-*shi^{TS}* line was provided by T. Kitamoto[29]. The UAS-*GFPIR* line (RNA inhibitory to GFP) was a gift from the Krasnow laboratory. The UAS-*fru^{M}IR* line has been previously described[4]. All stocks and crosses were maintained at 25 °C except for those using UAS-*shi^{TS}*, UAS-*tra2IR* and UAS-*fru^{M}IR* flies, for which crosses were performed at 18 °C, 29 °C and 29 °C, respectively.

Generation of *fruP1-GAL4* through homologous recombination. The techniques for homologous recombination were adapted from previous studies[6]. Fragments containing ~3 kb of sequence 5′ and 3′ to the *fru^M* start codon were independently cloned. The first three codons of the GAL4 coding sequence were added to the 3′ end of the 5′ fragment, with codons 2 and 3 of GAL4 altered to create a *Hin*dIII site, and a *Sac*II site was added to the 5′ end of the fragment. The 3′ fragment began with codon 3 of the *fru^M* coding sequence (the first 2 codons were deleted), and was flanked on the 5′ end by a *Bam*HI site and on the 3′ end by a *Stu*I site. The *GAL4* coding sequence was amplified using primers with mutations to change codons 2 and 3, and included a *Bam*HI site after the stop codon. Fragments were ligated into the pWhiteOut2 P-element transformation vector (a gift from J. Sekelsky) and transformants were generated using standard techniques.

After transformation, multiple lines containing the donor element (pWhiteOut2 construct) were crossed to a UAS-*mCD8GFP* line to verify absence of ectopic GAL4 expression. Donor lines were then crossed to obtain progeny that contained the donor elements as well as heat-shock inducible FLPase and I-Sce. Larvae were heat shocked for 1 h on days 3 and 4. Individual progeny containing all three elements were then crossed to a UAS-*mCD8GFP* line and progeny were examined for GFP expression, indicating mobilization of the donor element, splicing and expression of GAL4. Approximately 1,500 indivdual crosses were screened and eight independent insertion events were isolated and confirmed using genomic PCR. These lines were then crossed to a nuclear GFP reporter, and co-expression in Fru^M-expressing neurons in the CNS was verified by immunohistochemistry using standard techniques[4].

Tissue dissection, staining and imaging. CNS and peripheral tissue were dissected and fixed using standard techniques[4]. Additional *fruP1-GAL4*-expressing neurons were seen in specific peripheral locations with two copies of the reporter transgene. Analysis presented is from animals with one reporter.

Rat anti-Fru^M antibody was used at 1:300, rat anti-HA (Roche) was used at 1:100, mouse monoclonal nc82 was used at 1:20, and Cy3-conjugated goat anti-rat and goat anti-mouse antibodies were used at 1:1,000 (Jackson Immunoresearch). For colorimetrically-visualized tissue, flies were cryosectioned and visualized as described[30], but were labelled with anti-Fru^M antibody (1:300) and an alkaline-phosphatase-conjugated goat anti-rat secondary antibody (1:200). For the whole mounts, fixed tissue was incubated for 5 min in PBS with 5% Triton X-100, rinsed and processed using anti-Fru^M antibody (1:300) and goat anti-rat AlexaFluor555-conjugated secondary antibody (Molecular Probes/Invitrogen). The samples were mounted in Vectashield mounting media (Vector Labs) and imaged using a BioRad MRC 1024 microscope, or mounted in ProLong reagent (Molecular Probes; for antibody and *in situ* hybridization preparations of peripheral tissue), and imaged on a Zeiss LSM510 Meta scanning confocal microscope.

In situ hybridization on 20-μm tissue sections was performed using the previously described S1 riboprobe [30].

Behavioural assays. Courtship assays were performed at ZT (circadian time) 6–10 h with males entrained in isolation for 3–5 days in 12 h light/dark cycles, and 3–5-day-old virgin females; assays were performed at 25 °C except as noted below[4]. Courtship index (CI) was calculated as the percentage of time spent courting (including following, tapping, wing and proboscis extension and attempted/successful copulation) divided by the total observation time. For habituation assays, sibling males were paired for 1 h and the courtship index was calculated for minutes 2–7 and 55–60. For courtship conditioning assays, males were paired in a mating chamber with a mated CS (Canton-S) female for 45–60 min, and then placed into a new chamber with a virgin CS female. For experiments using UAS-shi^{TS} flies, crosses were performed and the flies were raised in isolation for 6–10 days after eclosion at 18 °C, entrained at 25 °C for two days (as above) and then assayed at 25 °C and 31 °C. Isolated animals were warmed for 10–15 min at 31 °C before courtship assays.

Statistical analysis. For comparisons of male habituation, final values of CI for males expressing $fru^{M}IR$ or *GFPIR* were compared using a one-way analysis of variance (ANOVA). As the driver lines did not have a common genetic background, lines were analysed independently to determine whether changes in final CIs were significant. For comparison of mushroom-body-mediated effects on courtship conditioning, a two-way ANOVA showed a significant effect for both *GAL4* lines and $fru^{M}IR$ expression (see Fig. 4 legend). Tukey and Bonferroni post-tests were use to determine homogeneity between drivers for each treatment.

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