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Median bundle neurons coordinate behaviours during *Drosophila* male courtship

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Throughout the animal kingdom the innate nature of basic behaviour routines suggests that the underlying neuronal substrates necessary for their execution are genetically determined and developmentally programmed[1–2]. Complex innate behaviours require proper timing and ordering of individual component behaviours. In *Drosophila melanogaster,* analyses of combinations of mutations of the *fruitless* (*fru*) gene have shown that male-specific isoforms (Fru^M) of the Fru transcription factor are necessary for proper execution of all steps of the innate courtship ritual[3–9]. Here, we eliminate Fru^M expression in one group of about 60 neurons in the *Drosophila* central nervous

system and observe severely contracted courtship behaviour, including rapid courtship initiation, absence of orienting and tapping, and the simultaneous occurrence of wing vibration, licking and attempted copulation. Our results identify a small group of median bundle neurons, that in wild-type *Drosophila* appropriately trigger the sequential execution of the component behaviours that constitute the *Drosophila* courtship ritual.

Genetic, developmental and behavioural studies of the sex determination gene *fru* (Fig. 1) show that Fru^M proteins are only produced in a small, limited and dispersed subset of central nervous system (CNS) cells[6,10,11]. These studies also suggest that Fru^M is responsible for building the potential for male courtship behaviour into the CNS during development, and that the cells that express Fru^M comprise the circuitry that controls and coordinates male courtship behaviours[1,6,9]. Here we test the proposition that subsets of Fru^M cells have specific roles in courtship by examining the behavioural phenotypes produced when Fru^M expression is eliminated in a specific subset of CNS neurons.

We identified a GAL4 enhancer trap line, *P52a-GAL4* (ref. 12), whose expression overlaps a bilateral cluster of ~60 Fru^M-expressing neurons in the suboesophageal ganglion (Fig. 2a, b; white). These neurons comprise part of the tritocerebral component of the median bundle. *P52a-GAL4* expression is not detected in any other Fru^M-expressing neurons, although it is expressed in other regions of the CNS (Fig. 2a, b; green). *P52a-GAL4*-directed expression of an RNA-mediated interference transgene (UAS-*fru^{M}IR*) targeting the male-specific amino terminus of Fru^M isoforms eliminates Fru^M expression in the ~60 *P52a*-labelled Fru^M neurons (Fig. 2c), and is not expected to affect the hundreds of other cells in which *P52a-GAL4* is expressed.
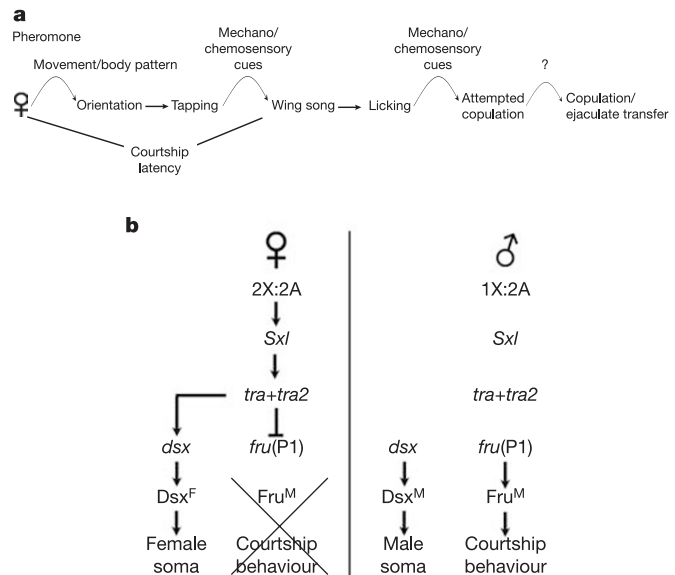

Figure 1 The *Drosophila* courtship ritual and its regulation by the *fru* branch of the *Drosophila* sex-determination hierarchy. **a,** Visual and olfactory cues used to detect and identify an appropriate female elicit orienting behaviour as the male faces and follows the female at a constant distance[3–5]. The male then orients to the female's lateral aspect and 'taps' her abdomen, perhaps facilitating the proper recognition of females and progression through the courtship ritual. The male then extends his outer wing and vibrates it to generate a species-specific courtship song, reorients towards the female's posterior, and extends his proboscis to 'lick' her genital region. Following successful licking, the male will then curl his abdomen and attempt copulation. Copulation typically lasts for about 20 min and results in the transfer of sperm and seminal fluids. **b,** The regulation of male courtship through Fru^M by the *Drosophila* sex-determination hierarchy. In males, the lack of Sxl and thus Tra activity leads to the default splicing of *fru* P1-derived transcripts, yielding Fru^M.

Males in which Fru^M expression had been eliminated in median bundle neurons by the *P52a-GAL4*-directed expression of UAS-*fru^{M}IR* (*P52a/fru^{M}IR*) were used in standard courtship assays (see Methods) to assess the Fru^M-dependent roles of these neurons in courtship. In *P52a/fru^{M}IR* males, courtship latency—the period from the initial presentation of a virgin female to the initiation of courtship behaviour, defined here as wing extension; Fig. 1a—decreased (8 ± 1 s ($\pm$s.e.m.) for *P52a/fru^{M}IR* males, compared with 94 ± 8 s for control males; Fig. 3a and Table 1). However, *P52a/fru^{M}IR* males can still distinguish females from males, because they do not sustain courtship towards each other or towards control males (data not shown), unlike previously described mutants that exhibited a rapid initiation of courtship towards both virgin females and mature males[13]. We did several controls to ensure that the rapid initiation of courtship seen in *P52a/fru^{M}IR* males is the consequence of blocking Fru^M expression in these 60 median bundle neurons. All of the individual transgenes used in these studies were backcrossed into a common genetic background before use. For each of these transgenes the courtship behaviours of males carrying that transgene alone did not differ from our controls (Fig. 3a). Additionally, the *P52a-GAL4*-directed expression of a UAS-*traF* transgene (Fig. 1b) also eliminates Fru^M expression in these 60 neurons (data not shown) and reduces courtship latency (10 ± 2 s versus 94 ± 8 s) (Fig. 3a and Table 1). On the basis of these and other controls (see Methods), we conclude that it is the elimination of Fru^M protein expression in the $\sim$60 median bundle neurons, through the *P52a*-driven expression of UAS-*fru^{M}IR*, that is responsible for the decreased courtship latency.

To address whether rapid courtship by *P52a/fru^{M}IR* males was a reflection of general heightened activity, we performed short-, intermediate and long-term locomotor assays on both control and *P52a/fru^{M}IR* males[14] (Table 1 and Fig. 3b). There were no significant differences in their activity (see Methods), suggesting that the behavioural differences observed in *P52a/fru^{M}IR* males are specific to courtship.

The longer courtship latency seen in wild-type relative to *P52a/fru^{M}IR* males suggests that initiation of courtship by wild-type males requires sensory information beyond that sufficient to elicit courtship by *P52a/fru^{M}IR* males. Whether this represents a need for qualitatively different information in the wild type (that is, specific sensory information not required to elicit courtship by *P52a/fru^{M}IR* males), or quantitative differences in the information obtained (that is, stimuli integrated over the longer latency period in the wild type), remains undetermined. These data suggest that in the wild type, Fru^M-dependent median bundle function inhibits the rapid

onset of courtship until sufficient stimuli overcome this inhibition (Fig. 4a, Supplementary Fig. 1), and that initiation requires both proper recognition of females—the cues sufficient to elicit courtship in *P52a/fru^{M}IR* males—as well as potentially a second stimulus, which acts to relieve the inhibition of courtship initiation (Fig. 4b).

P52a/fru^{M}IR males also display a marked compression of the remainder of the courtship ritual, quantified as the period from the initiation of courtship to attempted copulation. In the wild type, courtship typically involves multiple tapping interactions before progression to wing song and attempted licking, and often numerous licking attempts before attempted copulation. In control males, such courtship bouts extend on average for about 2 min (111 ± 20 s). In marked contrast, *P52a/fru^{M}IR* males attempt copulation within 4 s after courtship initiation (Table 1).

Analysis of videotaped behaviour of *P52a/fru^{M}IR* males revealed that the initial stages of courtship (orienting and tapping) were absent. These males rapidly progressed to the later stages of licking and attempted copulation, with wing vibration, proboscis extension and attempted copulation often occurring simultaneously (Fig. 3d, Table 1 and see below). Furthermore, proboscis extension was never directed towards the female's genitalia, but rather towards her dorsal posterior while copulation was being attempted, and never resulted in a successful contact of the proboscis with genital structures. Thus in *P52a/fru^{M}IR* males, the requirements for direct contact with females (tapping and licking) are bypassed, and the recognition of a female seems to immediately elicit attempted copulation.

Previous studies concluded that tapping is necessary for proper species and sexual discrimination[3,4,15]. However, our findings with respect to *P52a/fru^{M}IR* males indicate that appropriate recognition of females can occur in its absence, implying that initial visual and/or olfactory cues are sufficient for the proper recognition of females. Moreover, these cues are able to elicit all components of the courtship ritual when the requirement for positively-acting cues received by means of tapping and licking are bypassed. The information obtained by tapping, although still potentially providing sex- and species-specific cues, may function as a necessary step in wild-type situations by facilitating progression from orienting and tracking to wing song and subsequent courtship behaviours.

The complex courtship phenotype of *P52a/fru^{M}IR* males—the rapidity of courtship initiation, absence of orienting and tapping, and simultaneous occurrence of later steps—suggests that Fru^M-expressing median bundle neurons function to modulate progression through courtship. There are at least two possible models for how this may occur. First, Manning has suggested that the
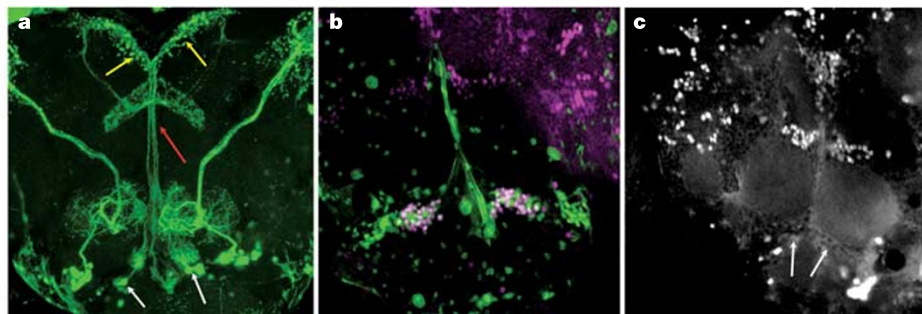

Figure 2 *P52a-GAL4* expression in Fru^M neurons of the median bundle. *P52a-GAL4* directs inhibition of Fru^M expression by a UAS-*fru^{M}IR* transgene. **a**, *P52a*-GAL4 expression in the *Drosophila* brain is shown by expression of UAS-driven cytoplasmic β-Gal. Arrows indicate cell bodies of median bundle neurons in the suboesophageal ganglion (white), their projections dorsally in the median bundle (red) and ramification in the dorsal protocerebrum (yellow). **b**, *P52a-GAL4* directs GFPnls (green) expression in Fru^M-expressing (magenta; overlap in white) suboesophageal ganglion cells. View of the anterior surface. **c**, *P52a-GAL4*-directed expression of a UAS-*fru^{M}IR* transgene successfully inhibits Fru^M expression in suboesophageal ganglion neurons. Arrows indicate the region of the suboesophageal ganglion where Fru^M expression is absent (compare with **b**)

integration of excitatory and inhibitory cues over time contribute to increasing excitation in males[5], and that progressively increasing levels of excitation then drives the progression through the courtship sequence, for example, by sequential components requiring progressively higher levels of excitation for their elicitation. Thus in wild-type males, Fru^M-dependent median bundle neuron function would be required for the system of progressively higher thresholds (Supplementary Fig. 1a). In $P52a/fru^MIR$ males, the thresholds for the various steps in courtship would be substantially lower and equivalent, such that minimal stimulation could simultaneously elicit wing song, licking and attempted copulation (Supplementary Fig.1b). An alternative model is that in the wild type, Fru^M-dependent median bundle function is necessary for tonic inhibitions that block progression from one step to the next in courtship. In the wild type, each of these tonic inhibitions can be relieved by the male receiving appropriate sensory cues (Fig. 4a). In $P52a/fru^MIR$ males, such tonic inhibitions would be absent, and thus the later steps in courtship would be simultaneously triggered by recognition of a female (Fig. 4b).

Another behavioural anomaly in $P52a/fru^MIR$ males occurs when multiple males are presented with a single female. In control situations, if two or more males are present with a single female, initiation of courtship by one male delays the initiation of courtship by the second male (Table 1). When multiple $P52a/fru^MIR$ males are with a single female, all males within the chamber immediately court and attempt to copulate with the female. The courtship by multiple $P52a/fru^MIR$ males is distinct from the lines of males that court a single female at the head of the chain, as seen with certain *fru* alleles[14], because all $P52a/fru^MIR$ males focus on the posterior of the single female, consistent with defects in behaviour downstream of proper mate recognition (Supplementary Video 1). These data suggest that in wild-type situations, cues from one male or a male–female pair act through the median bundle to inhibit the initiation of courtship behaviour by a second male (Fig. 4c).

Given the advantage that vigorous, rapid-onset courtship might confer, and the related question of why such behaviour has not been selected for as the species norm, we examined copulation by and fertility of $P52a/fru^MIR$ males. Whereas $P52a/fru^MIR$ males attempt copulation more quickly, successful copulation occurred more rapidly in control males, with 8 out of 20 successful attempts within the first 5 min, compared with 0 out of 20 in $P52a/fru^MIR$ males. Moreover, although copulations by $P52a/fru^MIR$ males were of
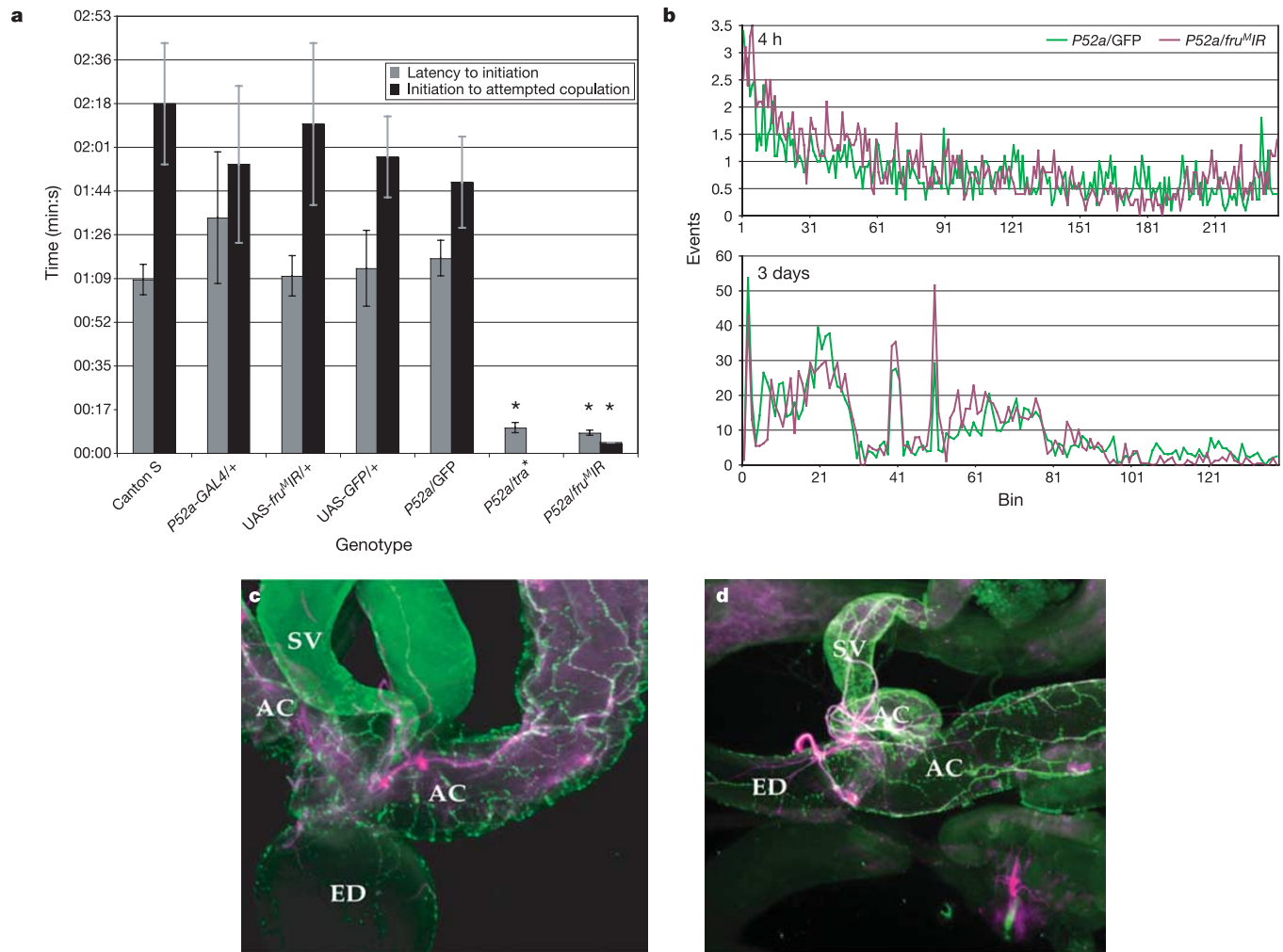

Figure 3 Inhibition of Fru^M expression in median bundle neurons leads to aberrant courtship behaviour. **a**, Average courtship latencies for control and $P52a$-manipulated males in which Fru^M expression has been reduced using directed expression of a UAS-*tra.F* (female) or UAS-*fru^{M}IR* transgene (n = 15 for Canton S, $P52a/+$, UAS-*fru^{M}IR*/+, UAS-*GFP* and $P52a/tra.F$, and 20 for $P52a/GFP$ and $P52a/fru^MIR$). $P52a$-manipulated males show decreases, relative to control males, in courtship latency when presented with virgin females. An asterisk indicates $P < 0.01$ for values compared with $P52a/GFP$ controls. Error bars indicate s.e.m. values. **b**, $P52a/fru^MIR$ (purple) and control (green) males show no differences in intermediate (top panel, $P = 0.79$) or long-term (bottom panel, $P = 0.65$) locomotor activity. **c**, **d**, Although sterile, $P52a/fru^MIR$ males (**d**), compared with control $P52a/LacZ$ (**c**), show no defects in either the innervation of internal genitalia (mAb 22C10 staining, magenta), or the serotonergic differentiation of abdominal ganglion neurons that innervate portions of the internal genitalia (anti-5HT staining, green). ED, ejaculatory duct; SV, seminal vesicle; AC, accessory gland.

normal duration (17.4 min versus Canton S 17.5), these matings were all sterile, despite the presence of motile sperm in their testes (Table 1). Because sperm and seminal fluid transfer are dependent on the functioning of eight Fru^M-expressing serotonergic neurons that provide the sole innervation to much of the male internal genitalia[9], we compared the innervation of internal genitalia in fertile control and infertile $P52a/fru^MIR$ males (Fig. 3d, e). We found no differences in either the patterns of innervation by, or serotonergic differentiation of these neurons. These findings suggest that the infertility of $P52a/fru^MIR$ males stems from a defect in a median bundle function necessary for the proper transfer of sperm and seminal fluids (Fig. 4b), which is known to occur sequentially in this species[16]. Consistent with this notion, after copulation with $P52a/fru^MIR$ males, the presence of sperm and a mating plug were rarely detected in female internal genitalia and never together, whereas they were consistently seen in control matings (Supplementary Fig. 2).

Whereas the courtship behaviours of $P52a/fru^MIR$ males represent striking abnormalities in the *D. melanogaster* courtship ritual, it is worth noting that these aberrant mating strategies are similar to the normal strategies in other insect species. Numerous fly species (such as *Ephemeroptera* and *Fannia canicularis*)[17,18] with aerial mating behaviour begin to track females almost immediately after encountering them (or indeed any object with comparable size and movement), and such tracking is immediately followed by attempts at copulation. Whereas multi-male courtships are abnormal for *D. melanogaster*, they are the norm in various other insect species, such as the solitary honeybee (*Anthophora plumipes*)[19]. The fact that the manipulation of a small set of Fru^M neurons in *D. melanogaster* generates behaviours resembling those of other species raises the

possibility that the CNS circuitry underlying courtship behaviours is common to many species and that ethological differences arise from relatively subtle variations in such a common ancestral circuit. For example, in *D. melanogaster* such changes may allow for

Table 1 **Courtship and behavioural differences between $P52a/fru^MIR$ and control males**

	P52a/GFP	*P52a/fru^MIR*
Courtship assays*		
Latency to initiation	94 ± 8 s	8 ± 1 s
Initiation to attempted copulation	111 ± 20 s	4 ± 0 s
Orienting	17/20	Not observed (0/20)
Tapping	15/20	Not observed (0/20)
Wing extension	20/20	**20/20**
Proboscis extension	19/20	**20/20**
Attempted copulation	17/20	**20/20**
Fertility	15/15	0/15
Short-term locomotor assays†		
Average line crossings (10 males; 4 min)	52 ± 10 s	52 ± 5 s
Multiple-male courtship assays‡		
Multiple-male courtship (<5 min) (2 males)	(2)/15	15/15
Male pair courtship latency to initiation (first male)	80 ± 8 s	10 ± 2 s
Male pair courtship latency to initiation (second male)	$6:57 \pm 3:27$ min:s	12 ± 5 s

*Multiple courtship aberrations occur when Fru^M expression is inhibited in median bundle neurons. These include: (1) decreased courtship latency; (2) the absence of orienting and tapping; (3) a compressed courtship ritual; and (4) sterility. Values in bold indicate behaviours late in courtship that often occur simultaneously in $P52a/fru^MIR$ males.
†Short-term locomotor assays (4 min) show no differences in general activity between $P52a/fru^MIR$ and control males ($P = 0.98$).
‡Multiple-male courtship assays reveal that multiple $P52a/fru^MIR$ males invariably simultaneously court and attempt copulation with a single female. The non-courting male in pairs of control males only rarely displays courtship behaviour towards a female (2/15 times) within the first 5 min. Finally, there are differences in the courtship latencies of first and second males in pairs of control males when compared with pairs of $P52a/fru^MIR$ males.
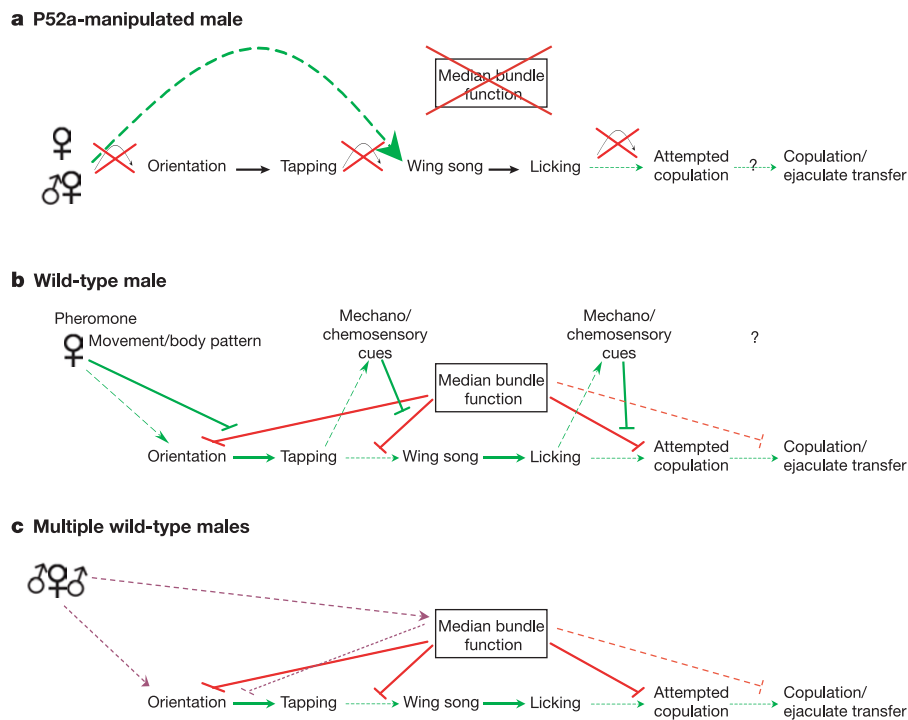

Figure 4 A functional schematic of putative median bundle roles during the *Drosophila* courtship ritual. **a**, Aberrant courtship behaviour in $P52a/fru^MIR$ males. In the absence of Fru^M-mediated median bundle function, elicitation of rapid-onset courtship by single females (green) or females in the presence of other males (purple, see **c**) bypasses initial steps (orientation and tapping) and rapidly progresses to attempted copulation. **b**, In the wild type, proper median bundle function is required for the staging of sequential courtship components and for proper transfer of seminal contents. Tonic inhibitions (red) of later behaviours are dependent upon Fru^M-expressing median bundle neurons. Disinhibtions of behaviours (green) are elicited in response to specific sensory cues.

Recognition of females initiates orienting, tracking and tapping behaviours by means of positively-acting sensory information. Additional cues may relieve the median-bundle-mediated inhibition of rapid courtship initiation and permit progression to tapping. Similar disinhibtion following successful tapping allows wing song and licking, and successful licking disinhibits attempted copulation. Median bundle function is additionally needed for successful insemination (dashed red line). **c**, In wild-type situations involving multiple males, median bundle neurons function in the inhibition of courtship initiation by a second male when a female is already being courted (dashed purple line).

social dynamics appropriate to terrestrial environments where their courtship normally occurs[20].

How do median bundle neurons function to allow the appropriate coordination of the component behaviours of the courtship ritual? Clonal analysis of the morphology of these neurons (see Methods) reveals local dendritic elaborations in the suboesophageal ganglion, consistent with functional data suggesting that median bundle neurons may integrate information from multiple sensory modalities whose axons project to this region[21,22] (Supplementary Fig. 3). Such connections would place median bundle neurons in an ideal position to modulate the initiation of component behaviours in response to appropriate, sequential, sensory cues. The projection of median bundle neurons directly onto the dorsal protocerebrum, a region generally implicated in higher-order processing, is also consistent with a role in coordinating/processing information from multiple modalities[21].

The question then remains as to how male-specific Fru^{M} expression alters neuronal function. Examination of median bundle neurons in males and females labelled by P52a-GAL4 fails to reveal any gross morphological differences between the sexes (data not shown). The existence of homologous neurons in females suggests that in this instance, Fru^{M} functions to modify neurons present in both sexes for male-specific functions. Whether Fru^{M} specifies changes in neuron morphology and/or physiology will no doubt illuminate the molecular mechanisms underlying the development of such circuitry, and may provide insights into how local development and changes in distinct subsets of neurons are then integrated and coordinated over evolutionary time to form complex learning and behavioural programs. □

Methods

Drosophila strains and cultures

The P52a-GAL4, UAS-LacZ line was provided by J. Nambu[12], and the P52a-GAL4 driver was isolated and maintained independently. The Stinger 5 UAS-GFPnls was provided by S. Barolo. The UAS-traF and UAS-GFP lines were obtained from the Bloomington *Drosophila* Stock Center. The UAS-fru^{M}IR line was generated as described below. Stocks were maintained on standard sugar media at 25 °C. Crosses were performed and progeny maintained at 29 °C. Males used in behavioural assays were collected 0–6 h after eclosion and raised in isolation on a 12 h light/dark cycle for 3–5 days before testing.

UAS-*fru*^{M}*IR* transgenic line

A pUAST-based construct designed to generate a hairpin duplex targeting amino acids 3–101 of the male-specific Fru^{M} N terminus was made as described[23] with 5′ *Eco*RI and 3′ *Kpn*I sites. Transgenic lines were generated by P-element-mediated germ line transformation. The line used in these experiments contains two insertions of the UAS-fru^{M}IR construct.

CNS dissection, immunohistochemistry and imaging

Males at 0–12 h after eclosion were dissected, fixed and stained using rat polyclonal anti-Fru^{M} antisera (1:250) and Cy3-conjugated goat anti-rat IgG (1:1000; Jackson Immunoresearch) as described[11]. Stacks of optical sections, usually at 1 μm spacing, were obtained with a Bio-Rad MRC 1024 confocal microscope, using the Laser Sharp program, then processed with NIH Image and Adobe Photoshop.

Courtship assays and analysis

Courtship assays were performed in a similar way to established protocol[14]. Males were entrained in isolation for 3–5 days after eclosion and then single or multiple males were presented with a 1–2-day-old Canton S virgin. Flies were videotaped for 20 min or until copulation occurred, then courtship behaviour was analysed.

Locomotor assays and analysis

Assays were performed on ten males for each genotype and data were then averaged. Short-term locomotor assays were performed by placing a single male in a 35 mm Petri dish and counting the number of midline crossings in a 4-min-period. A standard *t*-test was performed to determine the significance of differences. Intermediate and long-term assays were performed as previously described[14] using the Drosophila Activity Monitoring System (Trikinetics). Intermediate assays were performed for 4 h with 1-min-bins, and long-term assays were performed for 3 days, with food at one end of the monitor tube sealed with parafilm, with 30 min bins. Data were analysed using a non-parametric permutation test on all permutations to determine significance of differences.

Controls and other lines tested

In addition to the controls shown (Fig. 3a), other GAL4 enhancer trap lines (c271, c552), also expressed in the median bundle, and other Fru^{M}-expressing neurons produced

similar decreases in courtship latency in GAL4/fru^{M}IR males, although this phenotype was less reproducible in c552 (data not shown). However, owing to overlap with other areas of Fru^{M} expression in the CNS, they were not used for further analysis. In experiments to examine the roles of other groups of Fru^{M} neurons in male courtship behaviour, using over 800 independent GAL4 enhancer-trap insertion lines driving expression of UAS-fru^{M}IR, <1% of lines produced decreases in courtship latency similar to those seen in GAL4/fru^{M}IR males.

Male genital dissection, analysis and immunohistochemistry

Male genitalia were dissected from 3–5-day-old adults and either examined for motile sperm or fixed and stained with monoclonal antibody 22C10 (1:20; DSHB) and rabbit polyclonal anti-5HT antisera (1:800; Immunostar) and secondary antibodies Cy3-conjugated goat anti-mouse (1:1000; Jackson Immunoresearch) and Oregon Green 488 goat anti-rabbit IgG (1:1500; Molecular Probes).

Matings and female genital dissections

Females were mated to P52a/fru^{M}IR males bearing Don Juan–GFP fusion-labelled sperm[24]. Female internal genitalia were dissected within 45 min after the end of copulation and examined using brightfield and fluorescent microscopy.

Clonal analysis

Clones were generated as previously described[25] by delivering a 5 min 37 °C heat shock to 6–30-h-old embryos containing the hsFLP, P52a-GAL4 and UAS > CD2, y + >CD8-GFP and then raised to adulthood at 18 °C. CNS dissection and immunohistochemistry was performed as described with rat anti-mouse CD8 (1:100; Caltag Laboratories) and Alexafluor 488-conjugated goat anti-rat (1:200; Molecular Probes).

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Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1

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In Old World primates, TRIM5-α confers a potent block to human immunodeficiency virus type 1 (HIV-1) infection that acts after virus entry into cells[1–5]. Cyclophilin A (CypA) binding to viral capsid protects HIV-1 from a similar activity in human cells[4,6–8]. Among New World primates, only owl monkeys exhibit post-entry restriction of HIV-1 (ref. 1). Paradoxically, the barrier to HIV-1 in owl monkey cells is released by capsid mutants or drugs that disrupt capsid interaction with CypA[4]. Here we show that knockdown of owl monkey CypA by RNA interference (RNAi) correlates with suppression of anti-HIV-1 activity. However, reintroduction of CypA protein to RNAi-treated cells did not restore antiviral activity. A search for additional RNAi targets unearthed *TRIMCyp*, an RNAi-responsive messenger RNA encoding a TRIM5–CypA fusion protein. *TRIMCyp* accounts for post-entry restriction of HIV-1 in owl monkeys and blocks HIV-1 infection when transferred to otherwise infectable human or rat cells. It seems that *TRIMCyp* arose after the divergence of New and Old World primates when a LINE-1 retrotransposon catalysed the insertion of a *CypA* complementary DNA into the *TRIM5* locus. This is the first vertebrate example of a chimaeric gene generated by this mechanism of exon shuffling.

Post-entry restriction of HIV-1 infection is common among Old World monkeys, but owl monkeys are unique among New World primates in exhibiting this phenotype[1]. *Aotus trivirgatus* owl monkey kidney cells (OMK) restrict HIV-1 infection, but are permissive for simian immunodeficiency virus (SIV) infection[1]. HIV-1 restriction in OMK cells is completely abrogated when the interaction between HIV-1 capsid and the cellular protein cyclophilin A (CypA) is disrupted[4], either by mutations altering capsid or by treatment of target cells with the cyclophilin-binding drug cyclosporin A (CsA). This phenotype is the opposite of that seen in most human cells where the capsid–CypA interaction is required for efficient HIV-1 replication[4,6–9].

The paradoxical response to CsA in OMK cells was investigated further using two other drugs: MeIle[4]-CsA, a non-immunosuppressive analogue[9], and sanglifehrin, a structurally unrelated compound that also binds cyclophilin[10]. As with CsA, treatment of target cells with these compounds permits HIV-1 to infect OMK cells at an

efficiency similar to that of SIV (Fig. 1a). Peripheral blood mononuclear cells (PBMC) from a different owl monkey species, *Aotus nancymaae*, show the same restriction phenotype with respect to SIV, HIV-1 and CsA (Fig. 1b). Identical results were obtained with PBMC from a second animal (data not shown).

CsA, MeIle[4]-CsA and sanglifehrin bind cyclophilin family members, but not exclusively CypA. To examine the specific role of CypA, we generated stable OMK cell lines with CypA knockdown by RNAi. Of six CypA-specific small hairpin RNAs (shRNAs), three decreased CypA expression (Fig. 1c). Those shRNA constructs that decreased CypA expression abrogated HIV-1 restriction to a corresponding degree (Fig. 1d).

To determine whether disruption of HIV-1 restriction was due to CypA knockdown, the OMK knockdown cell line with the largest decrease in CypA expression and HIV-1 restriction (OMK$_{MH-CypA-147}$) was transfected with non-targetable *CypA* cDNAs (*ntCypA*) bearing silent mutations to make them resistant to the RNAi. A plasmid encoding cell surface H-2K^K was cotransfected so that transfected cells could be enriched using antibodies conjugated to magnetic particles. Although cells selected in this manner were fully restored for CypA expression, they remained deficient for HIV-1 restriction (Fig. 1e). We attempted to restore
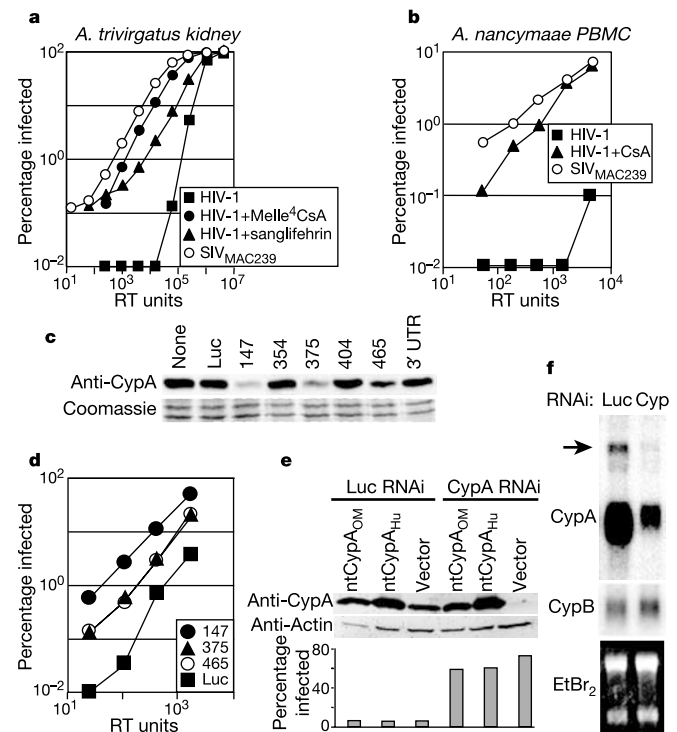

Figure 1 A CypA homologue is required for owl monkey restriction of HIV-1. **a**, **b**, *Aotus trivirgatus* OMK cells (**a**) or *Aotus nancymaae* peripheral blood mononuclear cells (**b**) were infected with GFP-transducing virions in the presence of CypA-binding drugs. GFP-positive cells were counted by flow cytometry. RT units, relative amount of virus added, as measured by reverse transcriptase activity. **c**, **d**, OMK cells were transduced with retroviruses delivering shRNAs targeting CypA mRNA at the indicated nucleotide positions. Lysates were immunoblotted (**c**) and cells were challenged with HIV-1/GFP (**d**). After antibody detection, the membrane was Coomassie-stained and a representative section is shown as a loading control. Luc is the control shRNA targeting luciferase. **e**, OMK cells transduced with shRNA-147 were selected after transfection with non-targetable-CypA expression vectors (human or owl monkey) and immunoblotted (top), or infected with HIV-1/GFP (bottom). **f**, Northern blot of total cytoplasmic RNA from shRNA-147- or shRNA-Luc-treated OMK cells, probed with *CypA* and *CypB* cDNA. The arrow indicates a ~2-kb, RNAi-responsive mRNA that hybridizes to *CypA*. EtBr$_2$, ethidium bromide.