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Neuronal Control of *Drosophila* Courtship Song

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SUMMARY

The courtship song of the *Drosophila* male serves as a genetically tractable model for the investigation of the neural mechanisms of decision-making, action selection, and motor pattern generation. Singing has been causally linked to the activity of the set of neurons that express the sex-specific *fru* transcripts, but the specific neurons involved have not been identified. Here we identify five distinct classes of *fru* neuron that trigger or compose the song. Our data suggest that P1 and pIP10 neurons in the brain mediate the decision to sing, and to act upon this decision, while the thoracic neurons dPR1, vPR6, and vMS11 are components of a central pattern generator that times and shapes the song's pulses. These neurons are potentially connected in a functional circuit, with the descending pIP10 neuron linking the brain and thoracic song centers. Sexual dimorphisms in each of these neurons may explain why only males sing.

INTRODUCTION

Many animals use acoustic signals to coordinate their social behaviors. Among these are the songs that males of various insect species, including grasshoppers, crickets, and cicadas, produce to attract or arouse females. These mating calls are astonishing in their diversity and, often, their volume. Male crickets, for example, rub their front wings together to produce a calling song that attracts females from a distance, and a courtship song that stimulates them during mating behavior (Hedwig, 2006). *Drosophila melanogaster* males produce their courtship song by extending and vibrating one wing (Bennet-Clark and Ewing, 1967). Although not as spectacular as the songs of crickets and cicadas, the *Drosophila* song offers an ideal opportunity to apply molecular genetic approaches to the investigation of the neural mechanisms of acoustic communication.

The courtship song of *Drosophila melanogaster* consists of two components: sine song and pulse song (von Schilcher, 1976). The sine song is a humming sound with a fundamental frequency of 140–170 Hz; it has been proposed to prime the female for the pulse song (von Schilcher, 1976). The pulse song consists of a train of 2–50 pulses, each containing one to three cycles (cycles per pulse, or CPP) with a carrier frequency of 150–300 Hz. Pulses are separated by a pause that lasts an average of ~35 ms (the interpulse interval, or IPI). The pulse song is a key factor in mating success, with the IPI providing a critical signature for song and species recognition (Bennet-Clark and Ewing, 1969; Kyriacou and Hall, 1982).

Normally only male flies sing. Initial attempts to map the neural centers responsible for song production thus relied on the construction of sex mosaics, or gynandromorphs, in order to delineate the parts of the nervous system that must be male for a fly to sing. These studies demonstrated that a region of the dorsal posterior brain must be male to initiate singing (Hall, 1977; von Schilcher and Hall, 1979), while regions of the mesothoracic ganglia need to be male to ensure the correct song structure (von Schilcher and Hall, 1979). Accordingly, fly song is thought to rely on a neural architecture in which a local and largely autonomous central pattern generator (CPG) produces rhythmic motor patterns subject to the control of descending "command" neurons in the brain. Such an architecture has been documented in crickets, for example, with the identification of command neurons that activate a thoracic CPG for stridulation (Hedwig, 1994, 2000; Howse, 1975).

More recent studies have begun to exploit molecular genetic approaches to map the fly's song circuitry more precisely. These studies have also been guided by the fact that only males sing, and thus focused on the two genes that control almost all aspects of sexual differentiation in *Drosophila*: *fruitless* (*fru*) and *doublesex* (*dsx*). Of these, *fru* plays the predominant role in the sexual differentiation of the nervous system and behavior, including song production. Male-specific *fru* isoforms (fru^M) are essential for males to sing (Ryner et al., 1996; Villella et al., 1997), and, if produced aberrantly in females, are sufficient to enable them to sing (Demir and Dickson, 2005). The songs of fru^M females are not, however, perfect renditions of the male song, but become so if male-specific *dsx* isoforms are also present (Rideout et al., 2007). Male *dsx* isoforms on their own are neither necessary nor sufficient for pulse song (Taylor et al., 1994; Villella and Hall, 1996).

fru^M is expressed in ~2000 neurons distributed in small clusters throughout the male central nervous system (CNS) (Lee et al., 2000). Genetic access to these neurons has been gained through targeted insertion of sequences encoding the GAL4 or lexA transcriptional activators, or the FLP recombinase, into the *fru* locus (Manoli et al., 2005; Mellert et al., 2010; Stockinger et al., 2005; Yu et al., 2010). These genetic reagents can now be used to target the expression of genetically encoded activity modulators specifically to the *fru*-expressing neurons in males or their counterparts in females. Silencing these neurons in males impairs courtship performance, including song production (Manoli et al., 2005; Stockinger et al., 2005). Conversely, light-triggered activation of the *fru* neurons in beheaded flies of

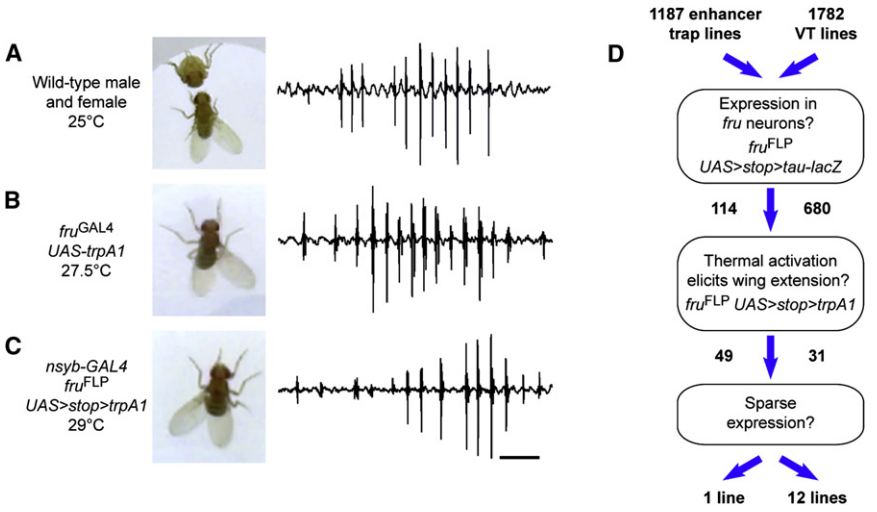

Figure 1. Thermal Activation of *fru* Neurons with TrpA1 Evokes Courtship Song

(A–C) Wing extension (left) and pulse song production (right) of (A) a wild-type male courting a female, (B) an isolated fru^{GAL4} *UAS-trpA1* male at 27.5°C (see also Movies S1 and S2), and (C) an isolated *nsyb-GAL4* fru^{FLP} *UAS>stop>trpA1* male at 29°C (see also Movies S3 and S4). Scale bar: 100 ms.

(D) Overview of thermal activation screen to identify sparsely expressed GAL4 lines capable of driving wing extension in combination with fru^{FLP} and *UAS>stop>trpA1*. Initial identification of lines expressed in *fru* neurons is according to Yu et al. (2010) and our unpublished results (VT lines).

either sex elicits singing and other aspects of the courtship ritual (Clyne and Miesenböck, 2008). These experiments have established a causal link between the activity of the *fru* neurons and song production. The specific neurons involved have not been identified.

We have recently used the fru^{FLP} allele in an intersectional genetic approach to subdivide the set of *fru* neurons into some 100 distinct neuronal classes (Yu et al., 2010). This work not only provided a cellular resolution map of the *fru* network, but also the genetic tools needed to selectively express activity modulators in small subsets of these neurons. Here, we use these tools, together with a thermal activation strategy (Hamada et al., 2008; Pulver et al., 2009), to identify and functionally characterize specific neurons involved in pulse song production. We identify two types of neuron in the brain, P1 (pMP4) and pIP10 neurons, that are capable of eliciting an authentic song. The pIP10 neuron is a descending neuron with axonal termini in the mesothoracic ganglia, and P1 is likely to be one of its inputs. Three other types of neuron in the thoracic ganglia, dPR1, vPR6, and vMS11, appear to control distinct features of wing extension and pulse song. We propose that dPR1, vPR6, and vMS11 neurons are components of a thoracic CPG for pulse song, controlled by signals from P1 and the pIP10 command neuron. The P1, pIP10, and dPR1 neurons are all male specific, potentially explaining why only males can sing.

RESULTS

Thermal Activation of *fru* Neurons with TrpA1 Elicits Pulse Song

Photoactivation of the *fru* neurons using fru^{GAL4} and the $P2X_2$ system (Lima and Miesenböck, 2005) elicits courtship song in isolated flies (Clyne and Miesenböck, 2008). Similarly, we found that thermal activation with TrpA1 also induced singing, often together with other courtship behaviors such as abdominal bending (Figures 1A and 1B and Movie S1, available online). One important difference, however, is that robust singing with the $P2X_2$ system was only observed with beheaded flies (Clyne and Miesenböck, 2008), whereas thermal activation also triggered singing in intact flies. Aside from this exception, the two activation methods gave similar results, in that both produced pulse songs with somewhat longer IPIs than normal (55.7 ± 1.5 ms for thermal activation at 27.5°C, n = 14), and elicited singing in females as well as males. For both methods, greater input energy was required to induce females to sing (for thermal activation, above ~28.5°C for females and ~26°C for males), and female pulse songs had even longer IPIs (72.8 ± 1.6 ms at 29°C, n = 14) and were more often polycyclic (11% ± 4% of pulses in female songs had more than two cycles, n = 5 flies, compared with 2% ± 1% of male songs, n = 5 flies; p = 0.012, Mann-Whitney test). Whereas photoactivated and thermally activated males generally extended only one wing, as in natural songs, females often extended both wings simultaneously, and to a lesser degree (Movie S2).

We obtained qualitatively similar results when we used fru^{FLP} and the panneuronal driver *nsyb-GAL4* to thermally activate the *fru* neurons, in this case using a combinatorial *UAS>stop>trpA1* transgene (Figure 1C and Movies S3 and S4; "*>stop>*" indicates a transcriptional stop cassette flanked by FLP recombinase target [FRT] sites, and thus excised only in the cells that express fru^{FLP}). This intersectional approach required slightly higher activation temperatures (above ~28.5°C for males and ~31.5°C for females) than the direct fru^{GAL4} *UAS-trpA1* strategy, possibly due to differences in TrpA1 expression levels from the two transgenes. Despite this minor difference, thermal activation of *fru* neurons using fru^{FLP} also produced songs with pulses that were more widely spaced than those of natural songs, and which were often polycyclic in females (IPIs of 51.2 ± 1.9 ms in males at 29°C, n = 10; 64.7 ± 2.2 ms in females at 32°C, n = 8; 64% ± 6% of pulses polycyclic in females, n = 5, 1.0% ± 0.0% in males, n = 5).

The robust song response of thermally activated fru^{FLP} flies, together with the intersectional genetic approach fru^{FLP} enables (Yu et al., 2010), provided an efficient and reliable assay for a thermogenetic screen to identify specific neurons involved in song production (Figure 1D). To this end, we screened a set of 794 GAL4 lines known to drive expression in one or more subclasses of *fru* neuron, consisting of 114 enhancer trap lines (Yu et al., 2010) and 680 molecularly defined enhancer-GAL4

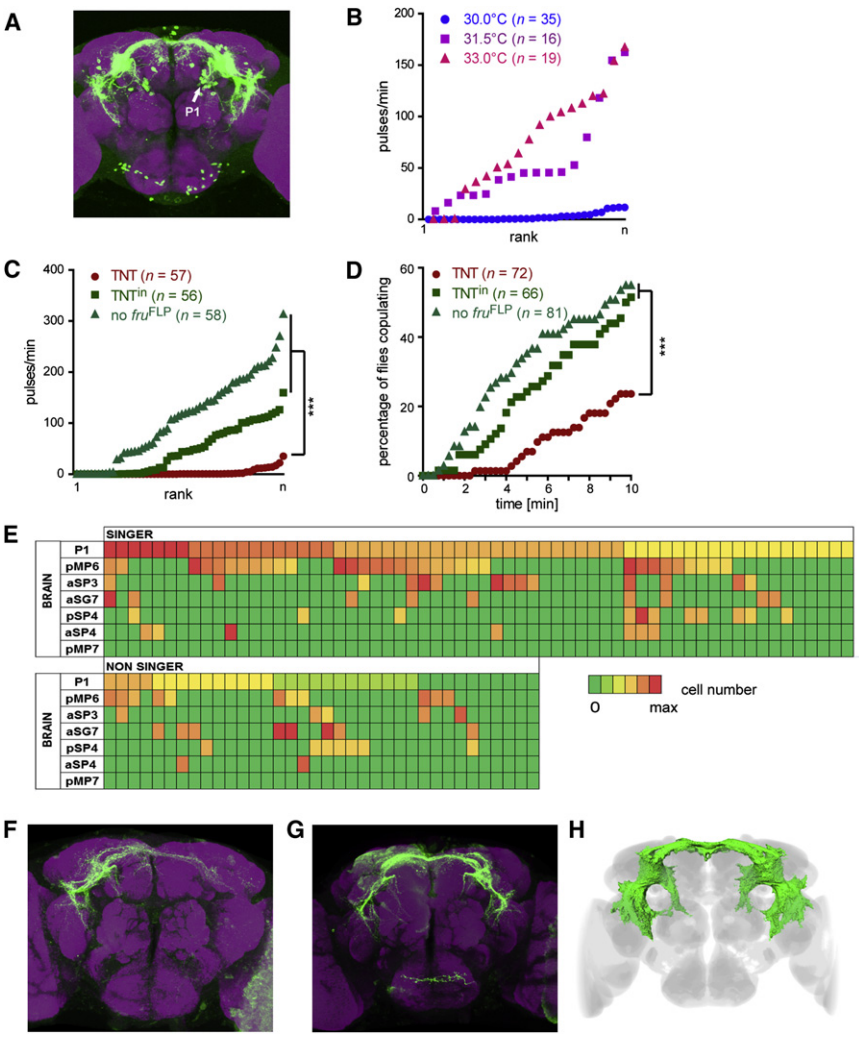

Figure 2. P1: A Brain Neuron that Triggers Pulse Song

(A) Brain of an *NP2361 fru*^{FLP} *UAS>stop>mCD8-GFP* male stained with anti-GFP (green) and the synaptic marker mAb nc82 (magenta).

(B) Song production of isolated *NP2361 fru*^{FLP} *UAS>stop>trpA1* males at different temperatures. Each data point represents a single fly, ranked by the amount of pulse song (see also Movie S5).

(C) Song production of *NP2361 fru*^{FLP} *UAS>stop>TNT* (TNT), *NP2361 fru*^{FLP} *UAS>stop>TNT*^{in} (TNT^{in}), and *NP2361 UAS>stop>TNT* (no *fru*^{FLP}) males paired with wild-type virgin females. Each data point represents a single fly, ranked by the amount of pulse song. ***$p < 0.0001$, Mann-Whitney test.

(D) Copulation success of males of the same genotypes as in (C). ***$p < 0.0009$, Fisher's exact test.

(E) TrpA1^{myc} expression in each of the *NP2361*$^{+}$ *fru*$^{FLP+}$ neuronal classes in 62 singing and 36 non-singing *NP2361 hs-mFLP5 fru*^{FLP} *UAS>stop>*$\geq$ *stop*$\geq$ *trpA1*^{myc} males subjected to a brief heat shock during development. Each vertical column represents one fly; each row, one cell type. Color-coding indicates the approximate number of cells labeled, with maxima (red) of 40+ for P1, 10 for pMP6 and aSP3, 2 for aSG7, 6 for pSP4, and 5 for aSP2. Green indicates no labeling. The six different shades for P1 indicate bins of 0, 1–10, 11–20, 21–30, 31–40, and over 40 cells, respectively, from green to red. pMP7 was never labeled. Neuronal classes are as described previously (Yu et al., 2010) and as in Figure S1A.

(F) Male brain with unilateral TrpA1^{myc} expression in P1 neurons, as visualized with anti-myc (green) and mAb nc82 counterstain (magenta).

(G) Male brain with bilateral TrpA1^{myc} expression in P1 neurons, stained as in (F).

(H) Segmented arborization of the P1 class.

transgenes (VT lines; C.M., S.B., T.L., V. Belyaeva, M. Kinberg, and B.J.D., unpublished data; Pfeiffer et al., 2008). Each line was crossed into the *fru*^{FLP} *UAS>stop>trpA1* background, and four to eight isolated male progeny were gradually warmed from 25°C to ~32°C during a 10 min video recording. Lines in which the majority of flies showed unilateral wing extension and/or vibration were scored as positive. Of 80 such lines recovered in the screen, we restricted our further analysis to 13 lines with relatively sparse expression in the CNS.

P1: A Brain Neuron that Triggers Pulse Song

The one positive GAL4 enhancer trap line from our screen was *NP2361*, which labels seven classes of *fru* neuron in the brain and none in the ventral nerve cord (VNC) (Yu et al., 2010; Figures 2A and S1A available online). In video and audio recordings, we found that *NP2361 fru*^{FLP} *UAS>stop>trpA1* males sang reliably at temperatures between 30°C and 33°C (Figure 2B and Movie S5), producing monocyclic pulses organized into distinct trains that were indistinguishable from those of natural courtship songs (Table 1). Moreover, in contrast to the extended IPIs of songs produced upon activation of all *fru* neurons using *nsyb-GAL4*, the IPIs of songs elicited at 30°C using the *NP2361* driver were comparable to those of natural song at the same temperature (Table 1). Artificial activation of one or more of the *NP2361*$^{+}$ *fru*$^{FLP+}$ neurons in the brain is thus sufficient to trigger a pulse song very close to the natural rendition.

To test whether the activity of these neurons is also required for normal song production, we combined *NP2361* and *fru*^{FLP} with a *UAS>stop>TNT* transgene. *TNT* encodes tetanus toxin light chain (TeTxLC), which cleaves synaptobrevin and thereby inhibits synaptic transmission (Sweeney et al., 1995). Males were paired with wild-type virgin females for either a 3.5 min recording session to monitor song production or a 10 min video assay to assess copulation success. Compared to control males that either expressed an inactive TeTxLC protease (*TNT*^{in}) or lacked *fru*^{FLP}, test males sang less often and with fewer pulse trains (Figure 2C and Table S1 available online). They were also less successful in their courtship attempts (Figure 2D and Table S1).

These data suggest that the activity of one or more of the seven classes of *NP2361*$^{+}$ *fru*$^{FLP+}$ neuron in the brain is both

Table 1. Pulse Songs Elicited by Thermal Activation of Specific *fru* Neurons

Neuronal Class	GAL4 Line	Temperature	Singers (%)	n	Pulses/Min	n	IPI (ms)	n	CPP	n
P1	NP2361	30.0°C	63	35	4.2 ± 0.8	22	28.4 ± 0.7	11 (311)	1.01 ± 0.01	8 (207)
		31.5°C	100	16	57.9 ± 11.4	16	29.4 ± 0.4	16 (1850)	1.00 ± 0.00	6 (712)
		33.0°C	89	19	84.2 ± 10.7	17	33.7 ± 0.6	16 (2567)	1.00 ± 0.00	6 (872)
pIP10	VT40556	29.0°C	100	20	25.1 ± 4.9	20	33.3 ± 0.6	14 (722)	1.01 ± 0.01	5 (335)
		31.5°C	100	16	245.8 ± 29.9	16	34.1 ± 0.7	14 (6965)	1.09 ± 0.02	5 (945)
		33.0°C	100	15	285.7 ± 26.8	15	35.4 ± 0.7	15 (6875)	1.13 ± 0.06	5 (999)
dPR1	VT41688	31.5°C	43	35	21.3 ± 4.9	15	46.3 ± 0.9	10 (522)	1.01 ± 0.00	6 (453)
		33.0°C	63	35	34.8 ± 7.0	22	45.7 ± 1.0	16 (1308)	1.01 ± 0.00	6 (635)
vPR6	VT19579	27.5°C	95	20	30.4 ± 5.1	19	63.1 ± 0.7	16 (1381)	1.31 ± 0.05	6 (540)
		29.0°C	100	16	105.7 ± 16.5	16	59.0 ± 0.7	16 (4420)	1.19 ± 0.02	5 (625)
		31.5°C	100	15	168.1 ± 23.9	15	43.5 ± 0.8	15 (7360)	1.18 ± 0.02	5 (754)
	VT5534	29.0°C	100	16	123.0 ± 21.8	16	60.1 ± 0.5	16 (4962)	1.36 ± 0.07	6 (755)
		31.5°C	100	16	144.5 ± 19.7	16	40.5 ± 0.7	16 (6164)	1.22 ± 0.02	6 (795)
	VT57239	29.0°C	100	15	98.6 ± 13.1	15	60.6 ± 0.7	15 (3923)	1.29 ± 0.03	5 (596)
		31.5°C	100	15	246.4 ± 19.2	15	43.0 ± 0.8	15 (10677)	1.32 ± 0.05	5 (721)
		33.0°C	100	17	78.0 ± 11.7	17	36.2 ± 0.7	16 (3354)	1.23 ± 0.03	5 (576)
	VT46099	29.0°C	95	22	15.7 ± 5.1	21	68.3 ± 1.2	14 (632)	1.18 ± 0.05	5 (261)
		31.5°C	94	17	44.4 ± 12.2	16	60.6 ± 0.9	16 (3936)	1.18 ± 0.03	5 (629)
		33.0°C	100	18	122.9 ± 18.5	18	47.7 ± 1.1	16 (6185)	1.31 ± 0.05	5 (614)
	VT17258	30.0°C	72	25	10.8 ± 2.7	18	67.9 ± 1.3	11 (523)	1.13 ± 0.03	6 (312)
		31.5°C	100	16	104.6 ± 23.7	16	61.5 ± 0.8	16 (4744)	1.23 ± 0.04	6 (709)
		33.0°C	100	16	118.0 ± 17.5	16	59.1 ± 1.1	16 (4606)	1.13 ± 0.04	6 (719)
n/a	wild-type courtship	27.5°C	–	–	–	–	30.9 ± 0.5	11 (645)	1.02 ± 0.01	6 (784)
		30.0°C	–	–	–	–	27.9 ± 0.4	11 (692)	1.01 ± 0.01	6 (740)
		33.0°C	–	–	–	–	25.5 ± 0.4	8 (606)	1.01 ± 0.00	6 (892)

Values for pulses/min are mean ± SEM of n flies that sung. Values for IPI and CPP are grand mean ± SEM, i.e., the mean of the mean per fly, for n flies. Numbers in parentheses indicate the total number of IPIs and pulses, respectively. Wild-type courtship indicates songs recorded from a Canton S male courting a virgin Canton S female.

necessary and sufficient to trigger pulse song. We used a stochastic approach to identify the specific neuronal type(s) involved. As each neuronal class is represented by multiple cells, we feared that cellular redundancy might preclude the identification of these neurons by stochastic silencing of single neurons. In contrast, activation of single or few neurons in a given class may be sufficient to trigger song production. To enable such a stochastic activation approach, we thus modified the *UAS>stop>trpA1* transgene to tag the TrpA1 protein with a c-*myc* epitope, and in addition, inserted a second transcriptional stop cassette flanked by mutant FRT sites (mFRT71, denoted here as "≥"). These mutant FRT sites are not recognized by the wild-type FLP protein, but are efficiently excised by a mutant FLP protein, mFLP5 (Hadjieconomou et al., 2011; Voziyanov et al., 2003). In this tripartite strategy, the canonical *>stop>* cassette is excised, as before, with fru^{FLP}, while the additional *≥stop≥* cassette is removed using *hs-mFLP5*. Thus, by subjecting *NP2361 hs-mFLP5* fru^{FLP} *UAS>stop>≥stop≥*$trpA1^{myc}$ males to a brief heat shock during larval development, we could restrict $TrpA1^{myc}$ expression to a random subset of the $NP2361^{+}$ fru^{FLP+} cells. After testing individual adult males for song, we dissected and stained their brains to identify these cells.

From a total of 98 flies tested, 62 produced pulse song and 36 did not (Figure 2E). In all 62 singers, neurons of the P1 class were labeled, and in 16 of them these were the only labeled cells (Figures 2E and 2F). None of the other six classes of neuron were consistently labeled in singers. P1 neurons were also labeled in 25 of the 36 flies that did not sing, but these flies generally had fewer labeled P1 cells than the singers (Figure 2E). The P1 class comprises 15–20 individual, and possibly heterogeneous, neurons per hemisphere (Yu et al., 2010). P1 neurons are male specific, and their ectopic presence in female gynandromorphs correlates with male-like courtship behavior (Kimura et al., 2008). Clusters of at least 10 individual P1 neurons were labeled in all of the singers (62/62), but few of such clusters (14/36) were labeled in the nonsingers. Moreover, the number of labeled P1 cells in singers positively correlated with the amount of produced pulse song (Figure S1B). The pMP6 neurons were also more often labeled in singers (34/62) than in nonsingers (11/36, $p = 0.02$). However, as pMP6 was not labeled in all singers, and among the singers pMP6 labeling did not correlate with the amount of song produced (Figure S1C), we infer that P1 neurons alone are primarily responsible for the song production observed with *NP2361*, and that a threshold number of P1

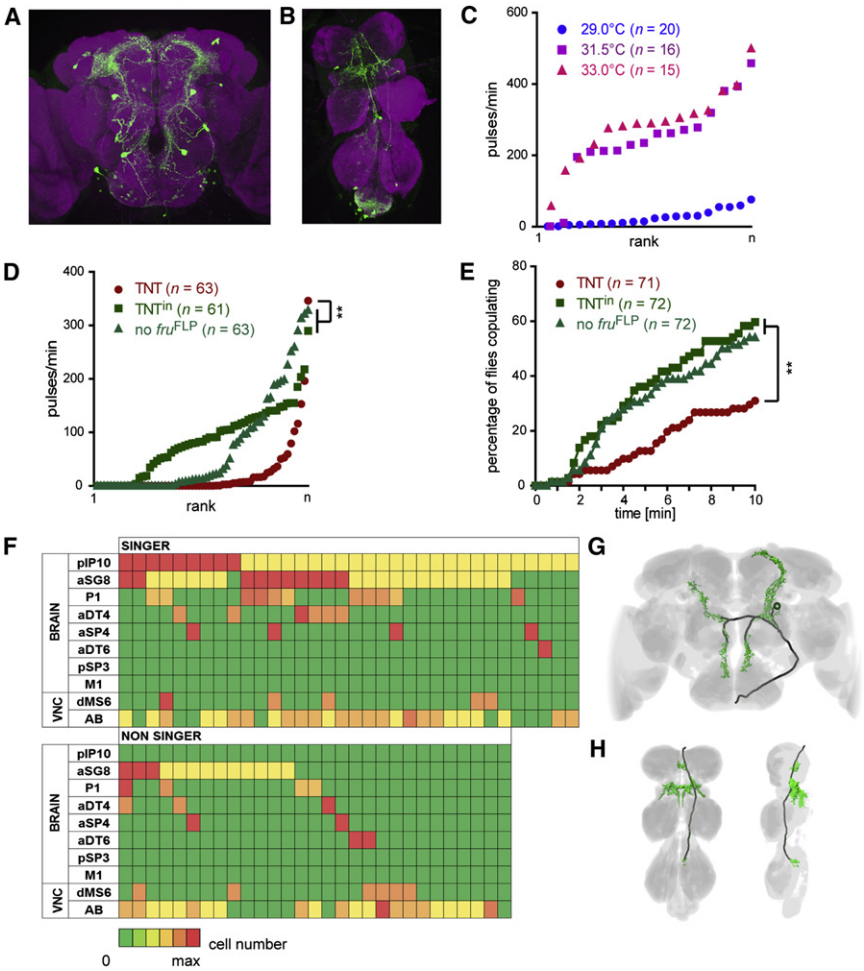

Figure 3. pIP10: A Descending Command Neuron for Pulse Song

(A and B) Brain (A) and VNC (B) of a *VT40556 fru*^{FLP} *UAS>stop>mCD8-GFP* male stained with anti-GFP (green) and the synaptic marker mAb nc82 (magenta).

(C) Song production of isolated *VT40556 fru*^{FLP} *UAS>stop>trpA1* males at different temperatures. Each data point represents a single fly, ranked by the amount of pulse song (see also Movie S6).

(D) Song production of *VT40556 fru*^{FLP} *UAS>stop>TNT* (TNT), *VT40556 fru*^{FLP} *UAS>stop>TNT*^{in} (TNT^{in}), and *VT40556 UAS>stop>TNT* (no *fru*^{FLP}) males paired with wild-type virgin females. Each data point represents a single fly, ranked by the amount of pulse song. **p < 0.002, Mann-Whitney test.

(E) Copulation success of males of the same genotypes as in (D). **p < 0.007, Fisher's exact test.

(F) TrpA1^{myc} expression in each of the *VT40556*$^{+}$ *fru*$^{FLP+}$ neuronal classes in 34 singing and 29 non-singing *VT40556 hs-mFLP5 fru*^{FLP} *UAS>stop>*≥ *stop*≥*trpA1*^{myc} males subjected to a brief heat shock during development. Each vertical column represents one fly; each row, one cell type. Color-coding indicates the approximate number of cells labeled, with maxima (red) of two for pIP10, aSG8, aDT4, and dMS6, four for P1 and AB, and one for aSP4 and aDT6. Green indicates no labeling. pSP3 and M1 were never labeled. Neuronal classes are as described previously (Yu et al., 2010) and as in Figure S2A.

(G and H) Segmented arborization of the pIP10 class, showing the brain (G) and the ventral (H, left) and lateral (H, right) views of the VNC.

neurons, or possibly a specific subtype, must be activated to trigger song.

To assess whether unilateral activation of P1 neurons preferentially leads to song generation with one or the other wing, we analyzed the wing extensions of six flies with unilateral expression of TrpA1 in P1 neurons (Figure 2F), as well as 12 with bilateral expression (Figure 2G). In almost all cases (5/6 and 11/12, respectively), flies variously extended either the ipsilateral or contralateral wing, but never both simultaneously. P1 neurons evidently do not control the laterality of wing extension.

In summary, we infer from these data that activity of P1 neurons (Figure 2H) is necessary and sufficient to trigger song production, and that song structure and wing choice are under the control of subordinate neural circuits.

pIP10: A Descending Neuron that Triggers Pulse Song

Another GAL4 line from our initial screen that elicited seemingly natural pulse songs was *VT40556* (Figures 3A and 3B). As with *NP2361*, the thermally induced pulse songs obtained using *VT40556* consisted of monocyclic pulses organized in trains with IPIs in the natural range (33.3 ± 0.6 ms at 29°C to 35.4 ± 0.7 ms at 33°C; Figure 3C, Table 1, and Movie S6). To test whether activity of *VT40556*$^{+}$ *fru*$^{FLP+}$ neurons is also required for song, we silenced these neurons with *UAS>stop>TNT* and tested these males for song production and copulation success in pairings with wild-type virgin females. These *VT40556* test males sang less and copulated less than each of the corresponding controls (Figures 3D and 3E and Table S1).

To identify the specific subset of *fru* neurons labeled by *VT40556*, we replaced *UAS>stop>trpA1* with a *UAS>stop>mCD8-GFP* transgene, inserted at the same genomic location. Staining brains and VNCs from these animals with anti-GFP revealed expression in eight classes of *fru* neuron in the brain and in two *fru* clusters in the VNC (Figures 3A, 3B, and S1B). The P1 neurons were among those cells labeled in the brain. However, *VT40556* labels only 2.7 ± 0.2 P1 neurons per hemisphere (n = 12). Judging from the results of our stochastic activation experiments using *NP2361*, this could be too few P1 cells to account for song production in *VT40556* flies. We therefore suspected that some other cell type might be responsible for eliciting songs in these flies.

As previously with *NP2361*, we used the stochastic activation approach with *VT40556* to identify the specific cell type responsible, recovering in this case 34 males that sang and 29 that did

not (Figure 3F). One cell type was labeled in all singers and in none of the nonsingers: the pIP10 neurons (Figure 3E, $p < 0.0001$, Fisher's exact test). P1, in contrast, was no more often labeled in singers than in nonsingers (Figure 3E, $p = 0.14$). aSG8 neurons were slightly more frequently labeled in singers (27/34) than in nonsingers (13/29, $p = 0.008$). However, in contrast to pIP10, aSG8 was labeled in many nonsingers and not labeled in all singers. Furthermore, more pulse song was consistently produced by flies with bilateral labeling of pIP10 than those with unilateral labeling ($p = 0.009$, Figure S2B), whereas no such effect was observed with aSG8 ($p = 0.37$, Figure S2C). We conclude therefore that the pIP10 neurons alone account for song production in thermal activation experiments with *VT40556*.

The pIP10 neuron has its soma located in the medial posterior brain (Figure 3G). In *VT40556 fru*^{FLP} *UAS>stop>mCD8-GFP* males we observed just a single pIP10 neuron either bilaterally (14 of 18 males) or unilaterally (4 of 18 males). A corresponding cell type was never seen in females (n = 8). pIP10 extends neurites bilaterally, branching ventrally to innervate the periesophageal region and dorsally to innervate the lateral protocerebral complex. Both of these regions are richly innervated by fibers of other *fru*$^{+}$ neurons, including the P1 neurons in the lateral protocerebral complex (Yu et al., 2010). Another long process descends to the VNC, where it arborizes extensively within the wing neuropil of the anterior mesothoracic ganglia (Figure 3H). The pIP10 neuron was not characterized in our previous genetic dissection of the *fru*^{FLP} neurons (Yu et al., 2010), presumably because it is not targeted by any of the enhancer trap GAL4 lines in our collection. pIP10 is however similar to a cell type observed within the male-specific clone pIP-a in a MARCM analysis of the *fru*^{GAL4} neurons (Cachero et al., 2010).

We selected 20 singers for analysis of wing usage, 13 with unilateral labeling of pIP10 and 7 with bilateral labeling. In almost all cases (10/13 and 6/7, respectively), flies variously extended either the left or the right wing only. In the case of those flies with unilateral expression of TrpA1^{myc}, there was no obvious bias for the ipsilateral or contralateral wing.

In summary, we conclude that activity of pIP10 neurons, just like P1 neurons, is necessary and sufficient to trigger song production, but also does not encode specific features of the song.

dPR1: A Prothoracic Song Neuron

As none of the remaining positive lines from our screen labeled either P1 or pIP10, the singing observed with these lines was presumably due to activation of some other class of *fru* neuron. One of these, *VT41688*, labels three distinct clusters of *fru* neuron in the VNC: dPR1, dMS7, and a heterogeneous set of cells in the abdominal ganglia (AB; Figure 4A). It does not label any *fru* neurons in the brain. Typically, ~50% of *VT41688 fru*^{FLP} *UAS>stop>trpA1* males produced pulse songs when warmed above ~31.5°C (Figure 4B, Movie S7, and Table 1). Like natural songs, these were organized into distinct trains of monocyclic pulses, but with significantly longer IPIs (46.3 ± 0.9 ms at 31.5°C, n = 10, and 45.7 ± 1.0 ms at 33.0°C, n = 16, $p = 0.0001$). Conversely, silencing these neurons with *UAS>stop>TNT* significantly reduced both the song production ($p < 0.0001$, Figure 4C and Table S1) and copulation success ($p < 0.0004$, Figure 4D) when males were paired with wild-type virgins. The frequency of wing extension was however similar in both test and control males ($p < 0.1$, Mann-Whitney test, Table S1), suggesting that males with silenced *VT41688*$^{+}$ *fru*$^{FLP+}$ neurons extend their wings but do not produce pulse song.

To determine which *VT41688*$^{+}$ *fru*$^{FLP+}$ neurons are involved in song production, we took advantage of the fact that the expression in each cell type is somewhat stochastic in *VT41688*$^{+}$ *fru*$^{FLP+}$ flies. This inherent stochasticity may explain why not all flies sang in the thermal activation experiments, and some still did in the silencing experiments. Using *UAS>stop>trpA1*^{mCherry} and *UAS>stop>trpA1*^{myc} transgenes, we sorted individual flies into singers (n = 57) and nonsingers (n = 54) and then dissected and stained their VNCs (Figure 4E). dPR1 was labeled in all 57 singers but only 37 of the 54 nonsingers ($p < 0.0001$, Fisher's exact test). Moreover, dPR1 labeling was bilateral in 52/57 singers but only in 7/54 nonsingers ($p < 0.0001$). In contrast, neither dMS7 nor AB neurons were more frequently labeled in singers versus nonsingers ($p < 0.66$). These data strongly suggest that dPR1 neurons are responsible for pulse song production in thermally activated *VT41688* flies.

The morphology of dPR1 is consistent with a role in song production (Figures 4A and 4F). In *VT41688 fru*^{FLP} *UAS>stop>mCD8-GFP* males we typically observed one or two cell bodies located medially in the anterior region of the prothoracic ganglion. Processes of these neurons extended bilaterally to innervate the wing neuropil of the anterior mesothoracic segment. We have not observed this neuron in *VT41688 fru*^{FLP} *UAS>stop>mCD8-GFP* females (n = 10), implying that it is either absent or does not express GAL4 in *VT41688* females. The former is consistent with data from the MARCM study (Cachero et al., 2010): dPR1 is likely contained within the dPR-b clone, which in females lacks the arborization that we attribute to dPR1 in males. The location and dimorphism of dPR1 further suggest that it may correspond to a subtype of the *dsx*$^{+}$ TN2 neurons (Rideout et al., 2010). In support of this, double stainings with anti-Fru^{M} and anti-Dsx^{M} revealed that dPR1 neurons are Dsx^{+} (Figure S3A).

vPR6: A Mesothoracic Neuron that May Encode the IPI

Of the remaining 10 positive GAL4 lines from the *trpA1* screen, 9 are expressed in the vPR6 neurons of the thoracic ganglia. As vPR6 is the only class of *fru* neuron common to all nine lines, these neurons are most likely responsible for song production within each of these lines. We focused our further analysis on the five lines with the most restricted expression patterns: *VT19579*, *VT5534*, *VT57239*, *VT40699*, and *VT17258* (Figures 5A and S4). When combined with *fru*^{FLP} and *UAS>stop>mCD8-GFP*, each of these lines consistently labeled two to five vPR6 cells per hemisphere. These neurons are located laterally near the border of the prothoracic and mesothoracic ganglia, and extend processes medially and posteriorly within the wing neuropils (Figures 5A and 5B). In some cases, we also observed weakly stained processes that extended anteriorly and may also arise from these cells. Similar cells were not observed in females with four of these GAL4 lines (n = 4–7); *VT17258* additionally labels a similar but probably distinct cell type in both sexes.

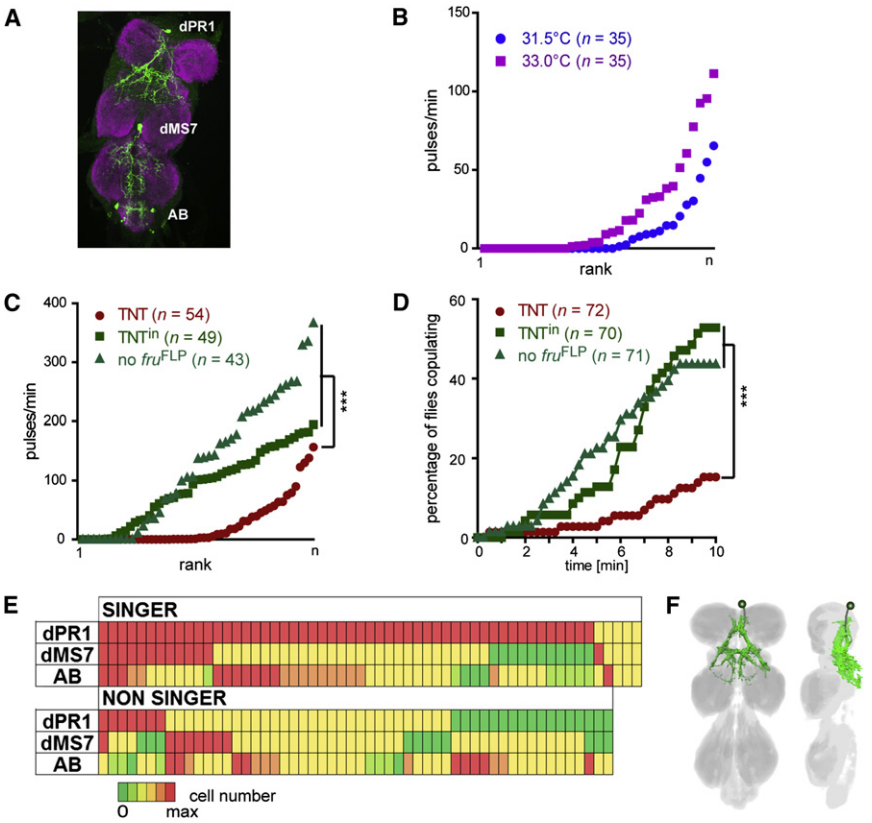

Figure 4. dPR1: A Prothoracic Song Neuron

(A) VNC of a *VT41688 fru*^{FLP} *UAS>stop>mCD8-GFP* male stained with anti-GFP (green) and the synaptic marker mAb nc82 (magenta).

(B) Song production of isolated *VT41688 fru*^{FLP} *UAS>stop>trpA1* males at 31.5°C and 33.0°C. Each data point represents a single fly, ranked by the amount of pulse song (see also Movie S7).

(C) Song production of *VT41688 fru*^{FLP} *UAS>stop>TNT* (TNT), *VT41688 fru*^{FLP} *UAS>stop>TNT*^{in} (TNT^{in}), and *VT41688 UAS>stop>TNT* (no *fru*^{FLP}) males paired with wild-type virgin females. Each data point represents a single fly, ranked by the amount of pulse song. ***$p < 0.0001$, Mann-Whitney test.

(D) Copulation success of males of the same genotypes as in (C). ***$p < 0.0004$, Fisher's exact test.

(E) Expression of tagged TrpA1 in each of the *VT41688*$^{+}$ *fru*$^{FLP+}$ neuronal classes in 57 singing and 54 nonsinging *VT40556 fru*^{FLP} *UAS>stop>trpA1*^{mCherry} or *UAS>stop>trpA1*^{myc} males. Each vertical column represents one fly; each row, one cell type. Color-coding indicates the number of cells labeled. Red, yellow, and green indicate 2, 1, and 0 cells, respectively, for dPR1 and dMS7, and 4, 2, and 0, respectively, for AB.

(F) Segmented arborization of the dPR1 class, showing ventral (left) and lateral (right) views of the VNC.

The location, morphology, and sexual dimorphism of vPR6 neurons suggest that they may represent a subclass of the *dsx*$^{+}$ TN1 neurons (Rideout et al., 2010). Double stainings for Fru^{M} and Dsx^{M} confirmed that vPR6 neurons are indeed Dsx^{+} (Figure S3B).

Songs were reliably induced with *fru*^{FLP} *UAS>stop>trpA1* and each of the five selected vPR6 GAL4 lines (Figure 5C, Movie S8, and Table 1). Songs were generally produced in the temperature range of 27.5°C–33°C, but the five lines varied in their optimal activation temperature (Table 1). For example, *VT19579* and *VT5534* flies began to sing above 27.5°C, and did so most robustly around 29°C–31°C. *VT17258* flies, on the other hand, only began to sing above 30°C and were most active around 33°C. Within their respective temperature ranges, songs from all lines were consistently organized into distinct trains of predominantly monocyclic pulses (Table 1).

We used the two most restricted GAL4 lines, together with *fru*^{FLP} and *UAS>stop>TNT*, to test whether synaptic activity of vPR6 neurons might also be essential for normal song production and courtship success. With both *VT19579* and *VT5534*, fewer flies sang when paired with virgin females, and those that did so sang less than the corresponding controls (Figures 5D and 5E and Table S1). The test males were also less successful in mating (Figures 5F and 5G and Table S1). The simplest interpretation of these data is that activity of vPR6 neurons is both necessary and sufficient for robust song production.

In the thermal activation experiments, IPIs decreased markedly with temperature for all five vPR6 GAL4 lines tested (Figures 5H and 5I and Table 1). Overall, mean IPIs decreased at a rate of 5.4 ± 0.7 ms/°C. The mean IPI of natural song also decreases slightly with temperature (Shorey, 1962), but only at a rate of 1.0 ms/°C within this temperature range (Figure 5I and Table 1). Moreover, IPIs did not decrease with temperature with any of the GAL4 lines that trigger songs by activating neurons other than vPR6 (Table 1). This is particularly notable in the case of dPR1 (*VT41688*), which elicits songs with IPIs in a similar range. The shortening of IPIs with increasing temperature is also not a trivial consequence of increased song at higher temperature, as mean IPI did not in general correlate with the number of pulses an individual fly produced (Table S2). We therefore conclude that the temperature dependence of IPI observed with all of the GAL4 lines expressed in vPR6 specifically reflects a tight inverse coupling between vPR6 activity and the IPI.

vMS11: A Regulator of Wing Extension and CPP

The final GAL4 line that we selected for detailed analysis, *VT43702*, differed from the others in that thermal activation elicited wing extension but not wing vibration (only two pulses recorded from 45 flies; Movie S9). Moreover, these wing extensions were often bilateral or, if unilateral, persistently involved one or the other wing (Figure 6A). The persistent use of one wing was even observed across repeated trials of the same fly.

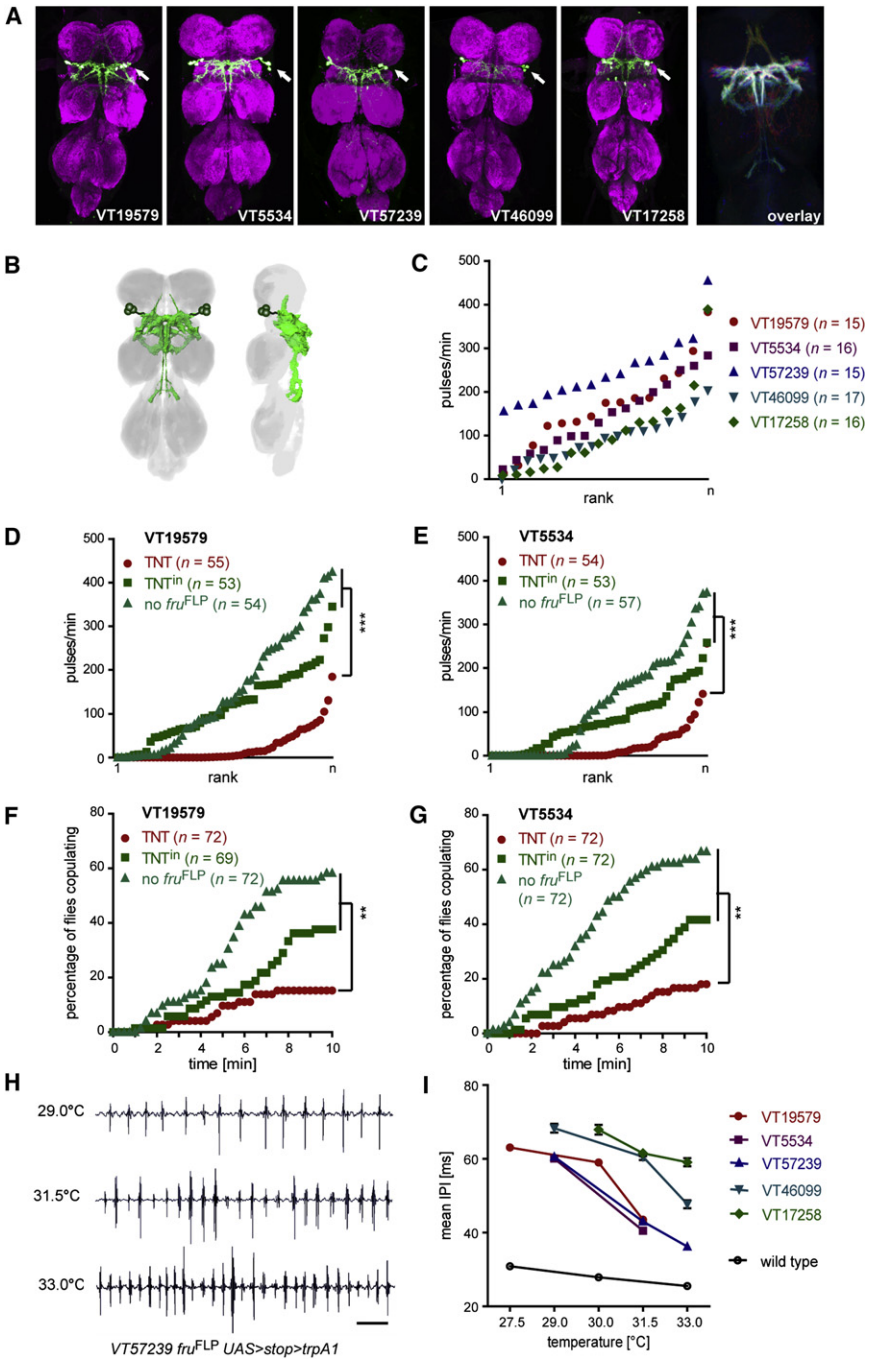

Figure 5. vPR6: A Thoracic Neuron that Influences the IPI

(A) VNCs of males carrying one of five vPR6 GAL4 lines, combined with *fru*^{FLP} *UAS>stop>mCD8-GFP*, stained with anti-GFP (green) and the synaptic marker mAb nc82 (magenta). The right panel shows an enlarged view of the overlaid registered and averaged GFP channels for *VT17258* and *VT5534* (red), *VT19579* (green), and *VT57239* and *VT46099* (blue).

(B) Segmented arborization of the vPR6 class, showing ventral (left) and lateral (right) views of the VNC.

(C) Song production of isolated males carrying one of five vPR6 GAL4 lines, combined with *fru*^{FLP} *UAS>stop>trpA1*, at 31.5°C. Each data point represents a single fly, ranked by the amount of pulse song (see also Movie S8).

(D and E) Song production of *VT19579* (D) and *VT5534* (E) males, combined with either *fru*^{FLP} *UAS>stop>TNT* (TNT), *fru*^{FLP} *UAS>stop>TNT*^{in} (TNT^{in}), or *UAS>stop>TNT* (no *fru*^{FLP}), in pairings with wild-type virgin females. Each data point represents a single fly, ranked by the amount of pulse song. ***p < 0.0001, Mann-Whitney test.

(F and G) Copulation success of males of the same genotypes as in (D) and (E), respectively. **p < 0.004, Fisher's exact test.

(H) Sample song traces of *VT57239 fru*^{FLP} *UAS>stop>trpA1* males at different temperatures. Scale bar: 100 ms.

(I) Mean IPI versus temperature for isolated males carrying one of the five vPR6 GAL4 lines in combination with *fru*^{FLP} *UAS>stop>trpA1*, as well as single wild-type males paired with virgin females (black circles).

Staining CNSs of *VT43702 fru*^{FLP} *UAS>stop>mCD8-GFP* flies revealed expression in four classes of *fru* neuron in the brain and three in the VNC (Figures 6B and S5). Because wing extensions with *UAS>stop>trpA1* were also observed in beheaded flies, and none of the brain neurons have descending projections into the VNC, we attribute the songs of *VT43702* flies to thermal activation of one or more of the *fru* neurons in the VNC. These are dMS2 neurons (Yu et al., 2010) and two previously undescribed classes, vMS11 and vMS12. All three cell types are located in the mesothoracic ganglia, with processes extending within the posterior wing neuropil.

We used the stochastic activation strategy with *UAS>stop>≥stop≥ trpA1*^{myc} to determine which of these three cell types is responsible for wing extension, selecting 34 flies that exclusively extended their left wing, 36 that extended only the right wing, and 55 that extended neither (Figure 6C; flies extending both wings were not observed in these stochastic activation experiments). The vMS11 neurons were significantly more often labeled in flies that extended their wings (53/70) than in those that did not (9/55; p < 0.0001, Fisher's exact test). Moreover, of the 44 flies with unilateral labeling of vMS11 and wing extension, it was the ipsilateral wing that was extended in all but four cases (p < 0.0001, Fisher's exact test). In contrast, there was no correlation between wing extension and the expression of TrpA1^{myc} in either dMS2 or vMS12 (p > 0.4 in both cases; Figure 6C). There are approximately three vMS11 neurons in each hemisphere, with

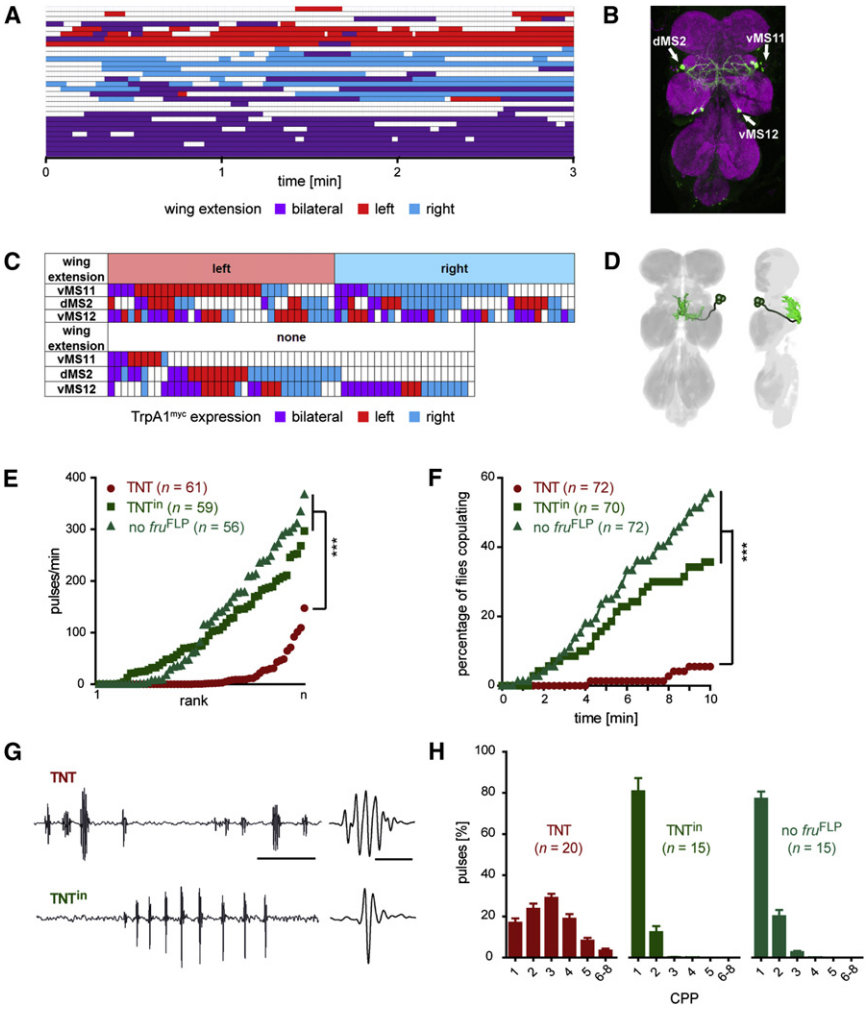

Figure 6. vMS11: A Mesothoracic Neuron that Controls Wing Extension and CPP

(A) Wing extension patterns of 30 *VT43702 fru*^{FLP} *UAS>stop>trpA1* male flies at 30.5°C. Each line represents a single fly.

(B) VNC of a *VT43702 fru*^{FLP} *UAS>stop>mCD8-GFP* male stained with anti-GFP (green) and the synaptic marker mAb nc82 (magenta).

(C) TrpA1^{myc} expression in each of the three *VT43702*$^{+}$ *fru*$^{FLP+}$ neuronal classes in the VNCs of *VT43702 hs-mFLP5 fru*^{FLP} *UAS>stop>≥stop≥ trpA1*^{myc} males subjected to a brief heat shock during development, and sorted into those that persistently extended the left wing, the right wing, or neither. Each vertical column represents one fly; each row, one cell type. Color-coding indicates whether TrpA1^{myc} was in the left hemiganglion (red), the right (blue), or both (purple).

(D) Segmented arborization of the vMS11 class, showing ventral (left) and lateral (right) views of the VNC.

(E) Song production of *VT43702 fru*^{FLP} *UAS>stop>TNT* (TNT), *VT43702 fru*^{FLP} *UAS>stop>TNT*^{in} (TNT^{in}), and *VT43702 UAS>stop>TNT* (no *fru*^{FLP}) males paired with wild-type virgin females. Each data point represents a single fly, ranked by the amount of pulse song. ***p < 0.0001, Mann-Whitney test.

(F) Copulation success of males of the same genotypes as in (E). ***p < 0.0001, Fisher's exact test.

(G) Sample song traces of *VT43702 fru*^{FLP} *UAS>stop>TNT* (TNT) and *VT43702 fru*^{FLP} *UAS>stop>TNT*^{in} (TNT^{in}) males at 30.5°C (left panels), with enlarged views of a single pulse (right panels). Scale bars: 100 ms (left), 10 ms (right).

(H) CPP distribution in songs of males of the same genotypes as in (E) and (F). Data are mean ± SEM for n = 20 flies (1608 pulses), 15 flies (2281 pulses), and 15 flies (2616 pulses).

arborizations in the dorsomedial wing neuropil (2.9 ± 1.3 vMS11 cells, n = 12 hemispheres; Figure 6D).

Synaptic silencing experiments with *UAS>stop>TNT* males confirmed that activity of *VT43702*$^{+}$ *fru*$^{FLP+}$ neurons is also essential for normal song production and copulation success. Compared to control males, these test males extended their wings less often ($p < 0.0001$, Table S1), fewer than half of them produced any pulse song at all ($p < 0.0001$, Fisher's exact test; Figure 6E and Table 1), and most failed to copulate ($p < 0.004$; Figure 6F). The songs of these flies had significantly longer IPIs than normal ($p \leq 0.0002$, Table S1) and, most strikingly, their pulses were frequently polycyclic (59% ± 4%, n = 20, pulses have over two cycles compared with fewer than 3% in each control, $p < 0.0001$, Mann-Whitney test; Figures 6G and 6H and Table S1). Thus, activity of one or more of the *VT43702*$^{+}$ *fru*$^{FLP+}$ neurons is essential for song production, and for the restriction of wing vibrations to just one or two CPP. The vMS11 neurons are obvious candidates, but we cannot exclude the possibility that these song deficits are due to silencing of dMS2, vMS12, or some of the *VT43702*$^{+}$ *fru*$^{FLP+}$ neurons in the brain.

A Neural Circuit for Courtship Song

To assess how the five distinct classes of *fru* neuron we have functionally characterized—P1, pIP10, dPR1, vPR6, and vMS11—might be integrated into a neural circuit, we examined their potential connectivity and polarity. Potential connectivity between each pairwise combination of neurons was assessed by labeling each class of neuron individually using the *UAS>stop>mCD8-GFP* marker, registering confocal images of these samples onto a common reference template, and digitally overlaying the two representations to compute the overlap between their arborizations (Yu et al., 2010). A high degree of overlap predicts (Braitenberg and Schuez, 1998), but does not establish, synaptic connectivity.

In the brain, the arborizations of P1 overlap extensively with both the ipsilateral and contralateral arborizations of pIP10 in the protocerebrum (Figures 7A and 7B). The arbors of pIP10 in

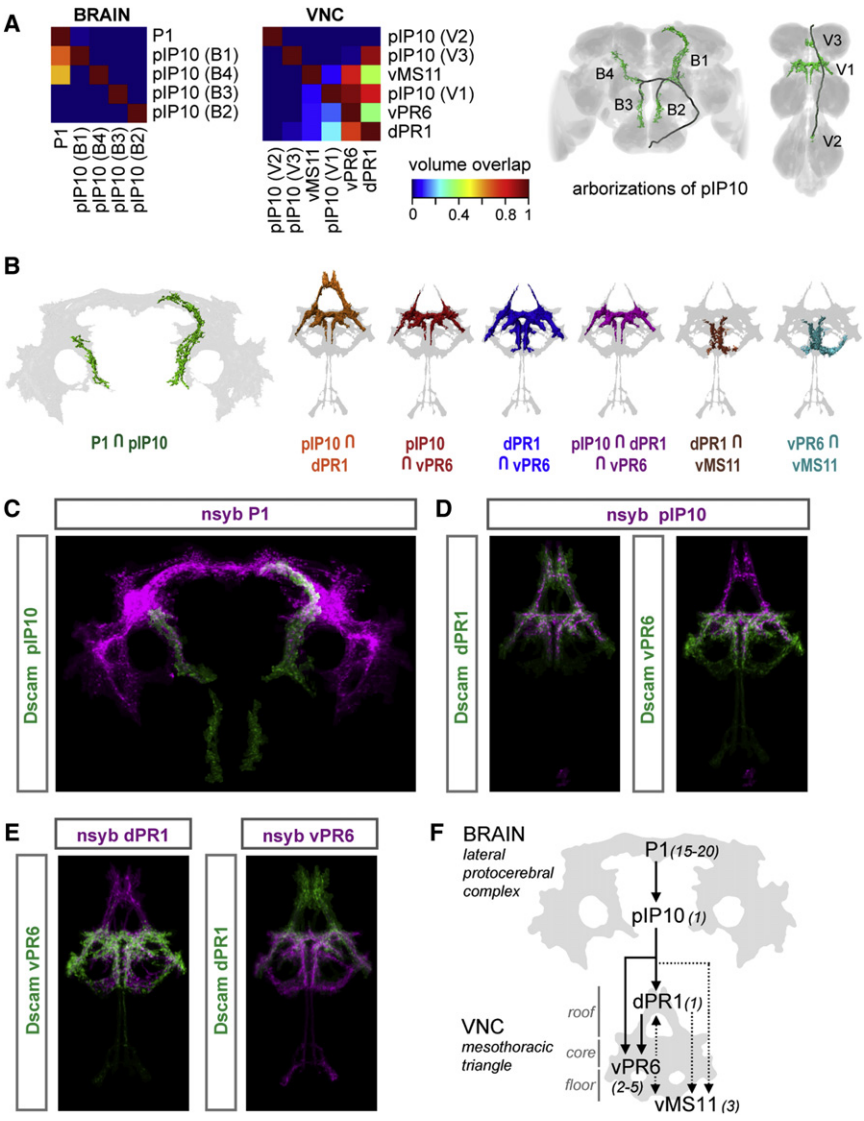

Figure 7. A Putative Neuronal Circuit for Pulse Song

(A) Calculated overlap between distinct arborization regions of P1 and pIP10 in the brain (left) and pairs of *fru* neurons in the VNC (middle), color-coded to show the fraction of the arbor indicated on the right that overlaps with the arbor indicated on the bottom. Individual arborizations of pIP10 are labeled as indicated in the segmented representation (right). Arborization volumes were segmented in both hemispheres for P1, dPR1, and vPR6, and in one hemisphere for pIP10 and vMS11.

(B) Segmented volume overlaps between the indicated sets of neurons. Gray backgrounds represent the full arborizations of P1 (for brain) or vPR6 (for VNC).

(C–E) Overlays of registered confocal images of brain (C) and VNC (D and E) samples stained to reveal either the presynaptic marker nsyb (magenta) or the dendritic marker Dscam17.1-GFP (green).

(F) A proposed neuronal circuit linking the *fru* song neurons P1, pIP10, dPR1, and vPR6. Average cell numbers per hemisphere are indicated in parentheses.

the VNC in turn overlap with those of dPR1 in the prothoracic ganglion, and in the anterior wing neuropil of the mesothoracic ganglion with both dPR1 and vPR6, and, to a lesser extent, with vMS11 (Figures 7A and 7B). dPR1, vPR6, and vMS11 arbors also overlap with each other in this region (Figures 7A and 7B).

Neuronal polarity was assessed for P1, pIP10, dPR1, and vPR6 using the presynaptic marker nsyb-GFP (Deitcher et al., 1998) and the dendritic marker Dscam17.1-GFP (Wang et al., 2004), encoded in *UAS>stop>nsyb-GFP* and *UAS>stop>Dscam17.1-GFP* transgenes, respectively (Yu et al., 2010). We confirmed previous reports (Kimura et al., 2008; Yu et al., 2010) that P1 neurons have extensive presynaptic and dendritic arborizations within the ring and arch regions of the lateral protocerebral complex. The pIP10 neuron was strongly labeled with nsyb-GFP only in the VNC, and with Dscam17.1-GFP only in the brain, as expected for a descending interneuron (Figures 7C and 7D). The dendrites of pIP10 in the brain overlap with the presynaptic sites of P1 (Figure 7C), and its presynaptic termini in the VNC overlap in turn with the dendritic fields of both dPR1 and vPR6 (Figure 7D). The respective presynaptic termini and dendritic fields of dPR1 and vPR6 overlap with each other, but there is considerably more overlap between the presynaptic termini of dPR1 and the dendritic field of vPR6 than vice versa (Figure 7E).

In summary, these data suggest that the *fru* song neurons might be interconnected in a circuit in which P1 provides input to pIP10 in the brain, which in turn conveys a descending command type signal to the thoracic neurons dPR1 and vPR6. Direct communication between dPR1 and vPR6 is likely, in particular from dPR1 to vPR6 (Figure 7F).

DISCUSSION

The courtship song of *Drosophila* serves as an ideal model system for investigating the neural mechanisms of decision-making, action selection, and motor pattern generation (Dickson, 2008). Here we have identified a set of song neurons in the *Drosophila* CNS and characterized their distinct roles in initiating or patterning the song. Artificial activation of these neurons triggers wing extension and/or vibration in isolated males deprived of the sensory inputs that would normally induce males to sing. Complementary silencing experiments suggest that these neurons also contribute to natural song production and mating success in the presence of a female.

Decision-Making and Action Selection: Song Circuits in the Brain

The male brain is presumed to contain neural circuits that integrate sensory information across multiple modalities, as well as internal information from prior experience, to create the percept of a receptive virgin female of the same species—a desirable courtship object (Dickson, 2008). These circuits would compute a decision to court the female. If acted upon, this decision would trigger courtship behavior, one prominent and critical manifestation of which is the courtship song. At any given moment, however, a male fly is likely to be confronted with multiple behavioral options, most of which are mutually exclusive. Courting a female may not be the most adaptive option, for example, in the presence of a predator or some other imminent danger. Decision-making circuits should thus be integrated with circuits that prioritize and select among alternative actions.

We propose that the P1 and pIP10 neurons are critical elements in these decision-making and action selection circuits in the fly brain. This notion rests on several lines of evidence. First, activation of either P1 or pIP10 elicits a faithful rendition of the natural song, suggesting that they trigger but do not pattern the song. Second, silencing small neuronal subsets that include either P1 or pIP10 dramatically reduces song output. Third, P1 neurons are intrinsic to the lateral protocerebral complex in the brain, where pathways from distinct sensory modalities converge (Yu et al., 2010). Fourth, pIP10 is a descending neuron that appears to collect some, but not all, of its inputs from the lateral protocerebral complex, most likely including P1.

We envision that P1 is critically involved in creating the percept of a suitable courtship object, and hence the decision to court, and that it communicates this decision to pIP10, a command-type neuron that selects and initiates the action of singing. Additional inputs to pIP10 would gate the P1 signal, so that pIP10 calls thoracic song circuits into action only if singing is judged to be the most appropriate behavioral choice at a given moment. These gating signals might also coordinate the timely execution of the courtship ritual itself, allowing the male to progress beyond singing once the female has indicated her willingness to mate. Further anatomical, physiological, and behavioral studies will test these ideas.

pIP10 is presumably not the only descending input to the thoracic song circuits. Other descending pathways might terminate the song, select between sine and pulse song (Clyne and Miesenböck, 2008), or dictate the choice of wing. Males typically sing using the wing facing toward the female, a choice governed primarily by visual (our unpublished observations) and possibly also gustatory (Koganezawa et al., 2010) cues. Unilateral activation of either P1 or pIP10 neurons does not lead to preferential extension of one or the other wing, and so if these neurons carry any laterality information at all, it must be encoded in a manner that cannot be mimicked by tonic thermal activation. Alternatively, and perhaps more likely, the choice of wing may be controlled by a separate descending pathway that collects its inputs, directly or indirectly, from the visual and gustatory centers of the brain.

Patterning the Song: Elements of a Thoracic CPG for Pulse Song

Photoactivation experiments (Clyne and Miesenböck, 2008) and gynandromorph studies (von Schilcher and Hall, 1979) have provided evidence that a CPG for song resides in the thoracic ganglia. We propose that the dPR1, vPR6, and vMS11 neurons are components of such a CPG for pulse song. In contrast to the P1 or pIP10 neurons in the brain, artificial activation of these thoracic neurons does not produce a faithful rendition of the natural song. Rather, these songs are perturbed in a characteristic fashion for each neuron, implying that each plays a distinct role in composing the pulse song.

The dPR1 and vPR6 neurons may be direct targets of the pIP10 command neuron. Songs induced by activating either of these neurons have extended IPIs. For vPR6, but not dPR1, IPI is inversely correlated with the presumed level of activation. The activity of vPR6 neurons may therefore be a critical determinant of the IPI. This prediction can now be tested by physiological investigation. If it holds up, these studies will also help to delineate the specific biophysical properties of vPR6 that determine the IPI. The corresponding genes would be candidates for the genetic changes that have diversified IPIs within the *Drosophila* genus.

The third thoracic song neuron, vMS11, appears to function in wing choice and extension. Unilateral activation of vMS11 results in the extension, but not vibration, of the ipsilateral wing. vMS11 may thus represent one of the output channels of the pulse song CPG. It may, for example, integrate song onset signals from the CPG with descending signals that convey the female's location, passing the result on to motor neurons that control the posture of the appropriate wing. A separate CPG output channel might carry precisely timed pulse signals that control wing vibration.

Synaptic silencing experiments hint that vMS11 may also control the CPP, although we cannot at present definitively assign this function to vMS11. If vMS11 is partially silenced, along with the thoracic neurons dMS2 and vMS12, fewer pulses are produced, as predicted, but most of them are also polycyclic. Feedback signals from wing sensory neurons are thought to dampen wing vibrations and limit each song pulse to one or two cycles (Ewing, 1979). Such proprioceptive signals might be blocked in these silencing experiments. If these feedback signals are conveyed by vMS11 activity, then tonic activation of this neuron might be predicted to freeze the wing in its extended position, just as we observed in the thermal activation experiments. Here too, physiological studies will further define the role of vMS11 in song production, and ultimately reveal how vMS11, vPR6, dPR1, and other song neurons function together to time and shape each pulse of the courtship song.

Sexual Differentiation of the Song Circuit

Although females do not sing naturally, photoactivation (Clyne and Miesenböck, 2008) and our thermal activation experiments imply the existence of a rudimentary song circuit in the female thoracic ganglia. This female circuit is presumably not so much a defective song circuit, but rather an overlapping circuit specialized for some other wing movements—such as those that accompany flight or aggressive displays—yet capable of

producing pulsed vibrations when inappropriately activated. That it does not normally operate in "song" mode in females suggests that this thoracic circuit might be controlled by distinct sets of descending signals in males and females. Because expression of fru^M in females endows them with the ability to sing to other females (Demir and Dickson, 2005), and also improves the song produced by photoactivated female thoraxes (Clyne and Miesenböck, 2008), we infer that fru^M masculinizes both the descending inputs from the brain and the thoracic song circuit itself. All five song neurons characterized in this study are candidates for such masculinizing influences of fru^M: P1, pIP10, and dPR1 are all male specific, and vPR6 and vMS11 appear to have sexually dimorphic arborizations within the wing neuropil.

The P1 neuron requires both fru^M and dsx^M for its male-specific differentiation (Kimura et al., 2008). Genetically mosaic females in which P1 neurons are mutant for the upstream regulator *transformer*, and hence express both fru^M and dsx^M, reportedly extended their wings, and presumably sing, to other females (Kimura et al., 2008). Not all such females courted in these experiments, and their overall courtship levels were low. Nonetheless, that some of these flies could sing at all implies that male P1 neurons can at least partially integrate into otherwise female circuits. The apparent ability of these male P1 neurons to correctly integrate inputs arriving through female sensory pathways may reflect the limited sexual dimorphism in the *fru* sensory pathways that converge upon the lateral protocerebral complex (Yu et al., 2010). That male P1 neurons could activate a female thoracic song circuit, however, is more difficult to reconcile with our notion that the male-specific pIP10 and dPR1 neurons form an essential conduit between these two centers. Although neither was specifically examined in that study (Kimura et al., 2008), both pIP10 and dPR1 were presumably lacking in most of these females. This may partly explain why these flies sang so rarely, but it does also suggest that alternative descending pathways exist, or can be recruited, to communicate between P1 neurons in the brain and the thoracic song circuits. This might include the additional descending pathways that we postulate control other aspects of song production, such as the choice of wing.

The extent to which the fru^{M+} neurons pIP10, dPR1, vPR6, and vMS11 actually require fru^M for their male-specific differentiation and function remains to be determined. The pIP10 and vMS11 neurons do not express *dsx*, and so fru^M is presumably the principle sex determinant for these neurons; dPR1 and vPR6 express and potentially require both fru^M and dsx^M. Whatever the precise genetic requirements, our functional characterization of dPR1 and vPR6 suggest that sex differences in these neurons may at least partly explain why the songs elicited by photoactivation or thermal activation of *fru* neurons in the female thorax have longer than normal IPIs. Similarly, sexual dimorphisms in vMS11 offer a potential explanation for the polycyclic pulses in these female songs.

Having delineated specific cellular components of the *Drosophila* song circuits, our work now paves the way for physiological studies to explore their operating principles in males, and how they differ in females. Genetic manipulation of individual neurons within these circuits, using strategies similar to those we have used here, should also reveal how the *fru* and *dsx* genes act through their respective target genes to control the sex-specific differentiation of these circuits, and thereby endow males and females with their distinct behavioral repertoires.

EXPERIMENTAL PROCEDURES

Fly Stocks

fru^{FLP}, *UAS>stop>mCD8-GFP*, *UAS>stop>Dscam17.1-GFP*, and *UAS>stop>nsyb-GFP* are as described in Yu et al. (2010), and *UAS>stop>TNT* and *UAS>stop>TNTQ* (TNT^{in}) are as described in Stockinger et al. (2005). *UAS-trpA1*, *UAS>stop>trpA1*, *UAS>stop>trpA1*^{myc} and *UAS>stop>trpA1*^{mCherry} were generated by standard cloning procedures, with the *trpA1* reading frame amplified by PCR from genomic DNA of *UAS-trpA1* flies provided by P. Garrity (Hamada et al., 2008). The "*>stop>*" cassette is the same as that in the *UAS>stop>TNT* constructs of Stockinger et al. (2005). These transgenes were inserted by ϕC31-mediated recombination into attP "landing sites" on the second chromosome (*UAS-trpA1* into VIE-260b and *UAS>stop>trpA1, UAS>stop>trpA1*^{myc} and *UAS>stop>trpA1*^{mCherry} into VIE-19a; K. Keleman and B.J.D., unpublished data). In *UAS>stop>≥stop≥trpA1*^{myc}, the "*>stop>*" cassette consists of a *his2A*^{V5} reporter followed by α*-tubulin84B* and *Act5C* transcriptional stop signals flanked by FRT sites, while the "*≥stop≥*" cassette contains a *lamin*^{HA} reporter followed by *Hsp70Aa* and *Hsp27* transcriptional stop signals flanked by mFRT71 sites. The *trpA1*^{myc} reading frame encodes a full-length TrpA1 protein tagged with two C-terminal c-*myc* epitopes. This transgene was inserted using ϕC31 recombinase into the VIE-19a attP site. *hs-mFLP5* was inserted into the third chromosome attP site VIE-49a (Hadjieconomou et al., 2011).

Enhancer trap GAL4 lines obtained from the *Drosophila* Genetics Resource Centre, Japan, and the collection of U. Heberlein are described in Yu et al. (2010). The VT collection of molecularly defined enhancer GAL4 lines was generated using the strategy of Pfeiffer et al. (2008) (C.M., S.S.B., A. Stark, and B.J.D., unpublished data).

Thermal Activation Experiments

trpA1-expressing flies were reared at 22°C, and males collected shortly after eclosion were aged in groups of 10–20 for 10–15 days at 22°C. For the initial GAL4 screen, four to eight males per genotype were screened for wing extension by aspirating them into chambers placed on a heating plate that was gradually heated from 25°C to 32°C–33°C during a 10 min video recording. For recording and detailed analysis of courtship songs, single males were aspirated into a metal chamber surrounded by Peltier elements containing a temperature sensor and a feedback system to maintain a constant temperature. Songs were recorded for 3.5–4.0 min.

In the stochastic activation experiments with *hs-mFLP5* fru^{FLP} *UAS>stop>≥stop≥trpA1*^{myc}, animals were heatshocked for 60–90 min at 37°C during the mid- to late-larval stage. Single males were assayed for song production and/or wing extension, then individually dissected to prepare their brains and/or VNCs for immunohistochemistry using anti-myc. For the analysis of wing extensions of *VT43702* fru^{FLP} *UAS>stop>trpA1* males, all wing extensions of at least 3 s duration and an angle of 30° were manually recorded.

Neuronal Silencing Experiments

Flies were reared at 25°C and males were collected shortly after eclosion and aged individually for 6–7 days at 25°C. For pulse song evaluation, single males were paired with a 4- to 5-day-old wild-type (Canton S) virgin and the courtship song was recorded in a soundproof chamber for 3.5–4.0 min or until copulation occurred. Analysis of courtship behavior and copulation latencies was performed as described in Demir and Dickson (2005). Wing extension frequency was determined by examining single frames of a 10 min video, taken at 15 s intervals until copulation, and counting those in which the male extended a wing at an angle of at least 30°.

Song Analysis

Pulse song was analyzed with Signal 4.0 (Engineering Design) and LifeSong (Bernstein et al., 1992) software, following manual inspection and editing to

remove background noises. LifeSong settings were generally as follows: signal/noise ratio, 5; IPI, 15–100 ms; minimum train length, 2 (for pulses/min) or 3 (for IPI). For IPI analysis, pulse trains with subthreshold pulses were excluded. CPP analysis was performed manually, scoring up to the first 100–200 pulses. Low-amplitude pulses were excluded. CPP was determined as the minimum of positive and negative peaks, counting all peaks with at least half the amplitude of the largest peak. Flies producing fewer than 10 pulses during a 3.5 min recording were excluded from the analysis of song parameters.

Immunohistochemistry and Image Analysis

Flies were reared at 25°C and aged for 4–6 days prior to dissection and staining as described in Yu et al. (2010). Antibodies used were rabbit anti-GFP (1: 6000, Torrey Pines), chicken anti-GFP (1:3000, abcam), mouse mAb nc82 (1:20, Hybridoma Bank), rabbit anti-DsRed (to detect mCherry; 1:500 or 1:1000, Clontech), rabbit anti-myc (1:6000 or 1:12,000, abcam), rabbit anti-Fru^M (Stockinger et al., 2005), rat anti-Dsx^M (Hempel and Oliver, 2007) and secondary Alexa 488, 568, and 647 antibodies (1:500 or 1:1000, Invitrogen).

Confocal stacks of stained brains and VNCs were taken with a Zeiss LSM510 with a Multi Immersion Plan NeoFluor 25×/0.8 objective and analyzed with Amira software (Visage Imaging). Nonrigid registration, segmentation, analysis of overlap, and image preparation were performed as described previously (Yu et al., 2010).

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes five figures, two tables, and nine movies and can be found with this article online at doi:10.1016/j.neuron.2011.01.011.

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