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Genetic feminization of the thoracic nervous system disrupts courtship song in male *Drosophila melanogaster*

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Abstract

Despite the growing research investigating the sex-specific organization of courtship behavior in *Drosophila melanogaster*, much remains to be understood about the sex-specific organization of the motor circuit that drives this behavior. To investigate the sex-specification of a tightly patterned component of courtship behavior, courtship song, we used the GAL4/UAS targeted gene expression system to feminize the ventral ganglia in male *Drosophila* and analyzed the acoustic properties of courtship song. More specifically, we used the thoracic-specifying *teashirt* promoter (*tsh*^{GAL4}) to express feminizing transgenes specifically in the ventral ganglia. When *tsh*^{GAL4} drove expression of *transformer* (*tra*), males were unable to produce prolonged wing extensions. Transgenic expression of an RNAi construct directed against male-specific fruitless (*fru*^{M}) transcripts resulted in normal wing extension, but highly defective courtship song, with 58% of males failing to generate detectable courtship song. Of those that did sing, widths of individual pulses were significantly broader than controls, suggesting thoracic *fru*^{M} function serves to mediate proprioceptive-dependent wing vibration damping during pulse song. However, the most critical signal in the song, the interpulse interval, remained intact. The inability to phenocopy this effect by reducing *fru*^{M} expression in motor neurons and proprioceptive neurons suggests thoracic interneurons require *fru*^{M} for proper pulse song execution and patterning of pulse structure, but not for pulse timing. This provides evidence that genes establishing sex-specific activation of complex behaviors may also be used in establishing pattern-generating motor networks underlying these sex-specific behaviors.

Keywords

Drosophila behavior; Acoustic; *fruitless*; Sexual dimorphism; Motor control

Introduction

The behaviorally reproducible and stereotyped behavior of male *Drosophila melanogaster* courtship and its genetic amenability provide hope for a detailed and multilevel understanding of a complex and adaptive behavior. (Dickson, 2008; Villella and Hall, 2008). Completely understanding courtship behavior requires understanding its sensory transduction and integration, coordination of sub-behaviors, and its motor pattern generation. While a history of ongoing research focuses on the initiation and organization of courtship (Manoli and Baker, 2004; Datta et al., 2008; Dickson, 2008; Kimura et al., 2008), progress in understanding the motor networks themselves lags behind. Our study aims to elucidate organizational properties of one courtship motor network, the song circuit.

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D. melanogaster courtship is composed of a stereotyped sequence of motor acts including female-directed following, abdomen tapping, singing, and attempting copulation (Hall, 1994). During courtship song, the male extends and then vibrates one wing. It is believed that descending brain neurons activate a thoracic circuit producing the song motor pattern (von Schilcher and Hall, 1979; Huber et al., 1989; Konopka et al., 1996; Clyne and Miesenböck, 2008). Courtship song is controlled by direct and indirect muscles (Bennet-Clark and Ewing, 1968), both of which receive phase-locked inputs from motor neurons during song (Ewing, 1977, 1979b). This phase-locking is due to proprioceptive wing feedback upon direct flight muscle motor neurons (Ewing, 1979a; Tauber and Eberl, 2001) and reciprocal inhibition among indirect flight muscle motor neurons (Levine, 1973; Ewing, 1977; Harcombe and Wyman, 1977).

Gynandromorph studies revealed a male requirement of the dorsal brain to initiate song through wing extension (Hall, 1977), and the thoracic ganglia to generate the song motor pattern (von Schilcher and Hall, 1979). Thus, considered with the abnormal song produced by females artificially induced to sing (Clyne and Miesenböck, 2008), some thoracic song components are apparently sexually dimorphic.

Female-specific *transformer* (*tra*) controls sexually dimorphic splicing of *doublesex* (*dsx*) and *fruitless* (*fru*), and the splice isoform, fru^M, is only found in males. fru^M function has been shown to be both necessary and sufficient to initiate many of the male courtship behaviors (Baker et al., 2001; Demir and Dickson, 2005; Manoli et al., 2005), but sex-specific *dsx* function is required to fully establish the network properly (Villella and Hall, 1996; Rideout et al., 2007; Kimura et al., 2008). Mutant fru^M males exhibit disrupted courtship song production (Villella et al., 1997), but fru^M expression in females is not sufficient for song initiation (Rideout et al., 2007). Further, artificially induced song in decapitated females resulted in pulse structure like that of wild type males upon ectopic fru^M expression (Clyne and Miesenböck, 2008).

It has been proposed that fru^M function is important at all levels of the nervous system related to courtship, as fru^M is expressed in widespread neuronal populations from sensory neurons to motor neurons (Baker et al., 2001). Yet, the use of classic fru^M mutants to study song has precluded direct analysis of motor networks. We investigated the putative thoracic courtship song patterning circuit selectively by using the homeotic *teashirt* (*tsh*) gene, a transcription factor specifying thoracic and abdominal segments (Röder et al., 1992), whose expression is selectively thoracic. These genetic manipulations allowed us to investigate whether the thoracic song circuit of male *Drosophila* is sex-specifically organized, and if the sex determination genes *tra* and fru^M play a role in this organization.

Materials and Methods

Flies

Flies were maintained at 25° C in 12:12 LD conditions. All transgenes used in tsh^{GAL4} ($tsh^{GAL4\text{-}md621}$; Calleja et al., 1996) experiments were outcrossed into a common isogenic Canton-S (CS) w^{1118} background for six generations. We feminized the nervous system in the *tsh* pattern by expressing *tra* with a UAS-tra.F^{20J7} (UAS-*tra*) construct (Ferveur et al., 1995) or by reducing fru^M expression with a fru^M-RNAi construct, UAS-fru^M*IR* (Manoli and Baker, 2004). All flies carrying UAS-fru^M*IR* contain a UAS insert on the second and third chromosome (Manoli and Baker, 2004), although the genotype is subsequently abbreviated to only reflect the second chromosomal insertion. The *n-syb*^{GAL80} line drives expression of GAL80, a GAL4 inhibitor, under the control of the *neuronal-synaptobrevin* (*n-syb*) pan-neuronal promoter (DiAntonio et al., 1993) and was used heterozygously, although it is represented homozygously for clarity. The UAS-fru^M*IR* stocks were

generously provided by Bruce Baker (Manoli and Baker, 2004), and the *n-syb*^{GAL80} stock was a generous gift from Julie Simpson (Janelia Farm Research Campus). All stocks other than *n-syb*^{GAL80} and UAS-*fru*^{M}*IR* were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA). The motor neuron driver, D42 (Gustafson and Boulianne, 1996), the chordotonal driver, *ato*$^{GAL4.3.6.10}$ (Hassan et al., 2000), the proximal wing base driver, 30A (Brand and Perrimon, 1993), and the myosin heavy chain mutant, *Mhc*^{5}, previously known as *bashed* (Homyk and Emerson, 1988), have been previously described. In addition to thoracic and abdominal specification, *tsh* also functions in delineation of the thoracic-labial border (Dezulueta et al., 1994), establishment of domains along the proximo-distal axis of the developing wing and leg via *wingless* and *nubbin* (Zirin and Mann, 2007), as well as specification of the eye (Singh et al., 2002). All transgenic lines were in a w^{1118} background, while the wildtype control used here (+ / +) was Canton-S (all in the same isogenic background) to control for eye color.

Behavioral assays and song analysis

Courtship observations were made on a focal 5–6 day old male and a 3–5 day old CS virgin subject female, both isolated as virgins within 6 hours of eclosion. Individual flies were aspirated into a 10 × 6 mm cylindrical chamber with an acrylic ceiling and a fine mesh copper flooring mounted over a heat block held at 25° C. Acoustic recordings were made using a calibrated velocity sensitive microphone (Tauber and Eberl, 2001; Cator et al., 2009) beneath the chamber. Courtship and songs were recorded using digitally sampled audio (48 kHz) and video (standard NTSC). Recordings were made for five minutes or until successful copulation, whichever occurred first. The frequency response of the recording apparatus was flat within 2 dB up to 2 kHz. Recordings were high-pass filtered at 50 Hz, anti-alias filtered, and digitally resampled at 4 kHz, and passed through an integrating filter to compensate for the frequency-amplitude relationship of particle velocity measurements.

Courtship index (CI) and wing extension index (WEI), the proportion of time spent courting and extending a wing, respectively, were calculated from video. A wing extension (Figure 1) began with the promotion of the wing and ended with the retraction of the wing to resting position or initiation of another wing promotion. Pulses were initially detected through thresholding using Matlab software written for courtship song analysis and confirmed through inspection of audio and video (unilateral wing extension) records, and pulse times were logged as time of midpoint of total energy within the pulse. Pulse trains were defined as a sequence of at least 3 pulses separated by no more than a specified window (Wheeler et al., 1988) of 60 ms. A longer window of 100 ms affected the measured IPI of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males by less than 1.0% (data not shown). Pulse width was defined as the smallest window necessary to encompass 90% of the pulse energy. Sine song was detected as sinusoidal hums coinciding with unilateral wing extension (only hums longer than 100 ms were scored). Also, some *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males display wing generated output coinciding with wing extension that was not classifiable as either pulse or sine song (data not shown). Although these wing generated outputs coincided with unilateral wing extension and courtship behavior, they did not share the tonal properties of sine song or the rapid amplitude modulation of pulse song. These were taken to be failed courtship song attempts, and were classified as neither pulse nor sine song. Calculation of sound particle velocity levels (SPVL) used a standard reference of 50 nm s^{-1}. Each datum measuring the song in general (Fig. 2) represents the mean within a fly, while each datum measuring pulse characteristics (Fig. 3) represents the median within each fly due to the larger sample size.

Immunocytochemistry

The rat α-Fru^{M} antibody, kindly provided by Bruce Baker (Janelia Farm Research Campus), targets the male-specific 101-amino acid sequence at the N-terminus of the peptide (Lee et

al., 2000). Within 6 hours of eclosion, adult w; *tsh*^{GAL4}, UAS-mCD8-GFP/ CyO male central nervous systems were dissected out and fixed in 3.5% paraformaldehyde, incubated in 1:300 α-Fru^{M} overnight, and incubated in 1:1000 TRITC-conjugated goat anti-rat (Jackson ImmunoResearch, West Grove, PA, USA) for two hours. Preparations were viewed with a TCS SP2 Leica confocal microscope system.

Flight assays

Flight ability was measured in an assay adapted from Drummond et al. (1991). Three- to five-day-old males were released on a platform in the center of an open-topped cylinder 45 cm wide and 54 cm high with a light source at the top. Flies were recorded as having landed on the bottom, landed on the side, or flown above the top of the cylinder. If no flight was initiated within 30 seconds, the fly was reapplied to the platform. Flies that never left platform after at least five trials were eliminated from the study.

Results

Ectopic *tra* expression reduces wing extension

We utilized the trunk-specific expression of *teashirt* (*tsh*) to specifically manipulate gene expression in this area, allowing us to investigate the putative thoracic song patterning circuit. We used the *tsh*^{GAL4} allele to express GAL4 in a *tsh*-expressing cell-specific pattern (Brand and Perrimon, 1993; Duffy, 2002) to cell-specifically activate a UAS promoter to invert male-specific gene expression. We first investigated wing extension, a prerequisite step to producing courtship song. Video analysis of courtship trials showed that the feminizing construct UAS-*tra* driven by *tsh*^{GAL4} had no detectable effect on courtship intensity measured by proportion of time spent courting (CI), compared to wild-type control males (+) or control males carrying *tsh*^{GAL4} alone (*tsh*^{GAL4}/+) (Fig. 1A; Kruskal-Wallis, $p = 0.76$). During courtship, however, *tsh*^{GAL4}/UAS-*tra* males showed a significant decrement in (1) proportion of time spent extending a wing toward the female (wing extension index, WEI) as compared to *tsh*^{GAL4}/+ controls (Fig. 1B; Kruskal-Wallis: $p < 0.005$, Tukey-Kramer: $p < 0.05$) and (2) median wing extension duration (Fig. 1C; Kruskal-Wallis, $p < 0.005$, Tukey-Kramer, $p < 0.05$). Furthermore, these *tsh*^{GAL4}/UAS-*tra* males display an unusual wing extension profile consisting of many rapid wing extensions, too fast to be measured with standard video. *tsh*^{GAL4}/UAS-*tra* males showed more rapid wing extensions (Kruskal-Wallis: $p < 0.01$, Tukey-Kramer: $p < 0.05$) and fewer sustained wing extensions (Kruskal-Wallis: $p < 0.05$, Tukey-Kramer: $p < 0.05$) (defined as being shorter or longer than 0.5 s, respectively) than *tsh*^{GAL4}/+ controls (Fig. 1D). However, there was no significant effect of genotype across all wing extension events (Kruskal-Wallis: $p = 0.0517$).

Since the lack of wing extension exhibited by *tsh*^{GAL4}/UAS-*tra* males precluded courtship song production, we asked if elimination of *fru*^{M}, a downstream target of *tra* in the sex-determination hierarchy, would more selectively produce defective song that could be analyzed for its defects. We utilized an RNAi construct directed at *fru*^{M} transcripts (UAS-*fru*^{M}*IR*) to reduce *fru*^{M} expression (Manoli and Baker, 2004) in a *tsh*-specific pattern. As observed in *tsh*^{GAL4}/UAS-*tra* males, males carrying both *tsh*^{GAL4} and UAS-*fru*^{M}*IR* showed no defect in CI (Fig. 1A). However, unlike *tsh*^{GAL4}/UAS-*tra* males, measurements of wing extension revealed no differences between *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males and controls (Fig. 1B–D). We therefore continued our study of courtship song utilizing the UAS-*fru*^{M}*IR* transgene.

fru^M RNAi reduces amount of courtship song

Courtship song is comprised of a pulse component ("pulse song"), consisting of a train of discrete, single pulses composed of one to several cycles, and a sinusoidal (125–200 Hz) component ("sine song") as seen in flight, but slower (Bennet-Clark and Ewing, 1968).

Utilization of the UAS-*fru^MIR* transgene to express RNAi directed at *fru^M* in *tsh*-expressing neurons produced a strong phenotype of reduced courtship song. All control flies included in this analysis exhibited pulse song, but 42% (5/12) of *tsh^{GAL4}*/UAS-*fru^MIR* males exhibited no detectable pulse song (Fig. 2A) despite vigorous courtship (Fig 1A), a statistically significant effect (Pearson's χ^2, experiment-wide: $p < 0.0005$, pair-wise with Bonferonni: $p < 0.05$). The 58% of *tsh^{GAL4}*/UAS-*fru^MIR* males exhibiting song sang at a significantly lower rate measured by pulse trains per minute (Fig 2B), as compared to *tsh^{GAL4}*/+ control males (Kruskal-Wallis: $p < 0.005$, Tukey-Kramer: $p < 0.01$). They also exhibited fewer pulses per pulse train than control flies (Fig 2C; Kruskal-Wallis: $p < 0.001$, Tukey-Kramer: $p < 0.05$). The high proportion of *tsh^{GAL4}*/UAS-*fru^MIR* males that did not produce song or sang at a decreased rate indicates that expression of *fru^MIR* in a tsh-specific pattern disrupts execution of pulse song.

To ensure that the reduction in courtship song had a neural basis rather than being due to broad *tsh* expression across thoracic tissues, we included another transgene expressing the GAL4 inhibitor GAL80 in a pan-neuronal, *n-synaptobrevin* (*n-syb*) pattern to block the effects of *fru^MIR* in the nervous system. The pulses per train deficit was fully rescued in *tsh^{GAL4}*/UAS-*fru^MIR; n-syb^{GAL80}* (abbreviated from w; *tsh^{GAL4}*/UAS-*fru^MIR; n-syb^{GAL80}*/ UAS-*fru^MIR*, see Materials and Methods) rescue males (Fig. 2), and trended towards a rescue in pulse train rate, arguing that the phenotype is indeed neuronal.

fru^M RNAi disrupts song structure

Representative data show that pulse song typically consists of a train of many pulses, each consisting of one or two cycles as seen in *tsh^{GAL4}*/+ controls (Fig. 3A) and *tsh^{GAL4}*/UAS-*fru^MIR; n-syb^{GAL80}* rescue flies (Fig. 3B). Those *tsh^{GAL4}*/UAS-*fru^MIR* males that sang show several defects, such as decreased amplitudes (Fig. 3C) and polycyclic pulses (Fig 3D), in which extra cycles are present before and after the peak particle velocity.

Wild-type control males exhibited a peak pulse particle velocity of 99.2 ± 1.0 db SPVL (re: 50 nm s^{-1}, mean ± SE). Pulse amplitudes from *tsh^{GAL4}*/UAS*fru^MIR* males showed a non-significant trend towards reduction of amplitude compared to tsh^{GAL4}/+ controls and a significant decrease compared to wildtype controls (Fig. 4A; Kruskal-Wallis: $p < 0.01$, Tukey-Kramer: $p < 0.01$). Although *tsh^{GAL4}*/UAS-*fru^MIR; n-syb^{GAL80}* rescue males showed a trend of increased amplitude compared to *tsh^{GAL4}*/UAS-*fru^MIR* males, these males also sang at a decreased amplitude compared to wild-type controls ($p < 0.05$), but not compared to tsh^{GAL4}/+ controls. Peak intrapulse frequencies and peak sine song frequencies were not found to differ among genotypes (Fig. 4B; Kruskal-Wallis: $p = 0.43$ and $p = 0.80$, respectively). The similarity of mean pulse and sine peak frequencies in *tsh^{GAL4}*/UAS-*fru^MIR* males should not be interpreted as a single underlying frequency for both song modes, as pulse and sine song frequency did not correlate in each fly examined, and intermale variation was quite high. Pulse widths were broader (Kruskal-Wallis: $p < 0.001$, Tukey-Kramer: $p < 0.05$) in *tsh^{GAL4}*/UAS-*fru^MIR* males as compared to *tsh^{GAL4}* and wildtype controls (Fig. 4C). Pulse widths of *tsh^{GAL4}*/UAS-*fru^MIR* and *tsh^{GAL4}*/UAS-*fru^MIR; n-syb^{GAL80}* males were not significantly different (Tukey-Kramer). However, because *tsh^{GAL4}*/UAS*fru^MIR; n-syb^{GAL80}* males serve as rescue males, an a priori prediction of narrower pulse width in rescue versus *tsh^{GAL4}*/UAS-*fru^MIR* males could be made. Using

a one-tailed test, the pulse width phenotype was rescued in *tsh*^{GAL4}/UAS*fru*^{M}*IR; n-syb*^{GAL80} vis-à-vis *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males (Tukey-Kramer: $p < 0.05$).

Pulses within a pulse train are separated by a species-specific interpulse interval (IPI) (Bennet-Clark, 1971) which itself oscillates in a species-specific manner (Kyriacou and Hall, 1980). In many species IPI is critical to maximizing a male's chance of copulation (Bennet-Clark and Ewing, 1969; Kyriacou and Hall, 1982, 1986). Interestingly, males of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* did not show a disrupted IPI (Fig. 4D, Kruskal-Wallis: $p < 0.05$, no effects from Tukey-Kramer), the most important feature of the courtship song.

tsh^{GAL4} is expressed in Fru^{M} domains

To identify the specific regions of the nervous system affected by the *tsh*^{GAL4}/UAS-*fru*^{M}*IR* genotype, we analyzed the *tsh*^{GAL4} expression pattern in the CNS of recently emerged males using a membrane-bound GFP reporter gene (mCD8-GFP) and determined its corresponding overlap with Fru^{M} immunoreactivity. Expression of *tsh*^{GAL4}-driven mCD8-GFP in the brain was very limited (occasionally absent) and inconsistent with no observed overlap with Fru^{M} immunoreactivity (Fig. S1). In contrast, *tsh*^{GAL4} driven mCD8-GFP was widely expressed throughout the somata and neuropils of the ventral ganglia, including all neuromeres (Fig. 5A). No obvious sexual dimorphisms were detected in GFP labeled neurons (data not shown), but the extensive labeling by *tsh*^{GAL4} makes definitive analysis difficult. Colocalization of GFP and Fru^{M} immunoreactivity was observed in all five previously described (Lee et al., 2000) groups of Fru^{M} ventral ganglia neurons (Fig. 5B–F). The most extensive colocalization of Fru^{M} and *tsh*^{GAL4}-driven GFP expression was observed at the anterior margin of the ventral mesothoracic neuromere (Fig. 5C). Although *tsh*^{GAL4} expression was observed in somata of widely varying size, Fru^{M} immunoreactivity was only detected in those *tsh*^{GAL4}-expressing neurons with small somata (~ 5 μm in diameter), consistent with previous reports of limited motor neuron Fru^{M} expression (Ryner et al., 1996;Manoli et al., 2005). The somata of direct flight muscle motor neurons (DFMns) are known to be located in the anterior mesothoracic region of the ventral ganglion (Trimarchi and Schneiderman, 1994), raising the possibility that DFMns require *fru*^{M} for proper courtship song functioning. However, we did not observe colocalization of GFP and Fru^{M} immunoreactivity in neurons with larger somata (≥ 10 μm) characteristic of DFM motor neurons (Trimarchi and Schneiderman, 1994).

Elimination of *fru*^{M} in motor neurons and wing sensory neurons does not affect song

Motor neurons and proprioceptive sensory neurons are known to be critical members of pattern generating networks (Levine, 1973; Harcombe and Wyman, 1977; Ewing, 1979a), and *fru*^{M} is expressed in subsets of motorneurons and wing sensory neurons (Manoli et al., 2005; Rideout et al., 2007). Feminizing the entire pool of thoracic neurons resulted in a strong courtship song deficit (Fig. 2–4), so we used additional GAL4 tools to eliminate *fru*^{M} in a subset of these neurons. We first looked at motor neurons by driving *fru*^{M}*IR* with the GAL4 driver D42 (Gustafson and Boulianne, 1996) that expresses in all motor neurons. Since power and timing of sound pulses is provided by the direct and indirect flight muscles (Ewing, 1977, 1979b), we hypothesized that *fru*^{M} activity in the motor neurons that innervate these muscles may be critical for proper song production. We eliminated expression of *fru*^{M} in motor neurons by driving *fru*^{M}*IR* with D42^{GAL4}, which is expressed in direct and indirect flight muscle motor neurons (Gustafson and Boulianne, 1996; Usui-Aoki et al., 2000). No differences between D42^{GAL4}/UAS-*fru*^{M}*IR* and UAS-*fru*^{M}*IR* controls were observed. This is consistent with another motor neuron GAL4 driver, P103.3 (Consoulas et al., 2002), in that P103.3^{GAL4}/UAS-*fru*^{M}*IR* males also exhibited no detectable song defects ($n = 4$, data not shown). As motor neurons receive wing proprioceptive input entraining the song pattern and some sensory organs at base of the wing are known to express *fru*^{M}

(Manoli et al., 2005), we hypothesized that *fru*^{M} function in the sensory organs themselves may be necessary for proper courtship song production. The *ato*^{GAL4} construct drives expression in proprioceptive organs (Hassan et al., 2000), such as those expressing *fru*^{M} at the wing base, but exhibited no detectable effect on courtship song when driving UAS-*fru*^{M}*IR* (Fig. 6). Similarly, driving expression of UAS-*fru*^{M}*IR* with 30A, a GAL4 driver that is expressed at the presumptive wing base of the imaginal disc, including precursors for the wing proprioceptive organs (Brand and Perrimon, 1993), does not produce a detectable courtship song phenotype.

fru^{M}*IR* does not affect flight

Because flight and courtship song use overlapping motor components but only courtship song behavior is sexually dimorphic, we tested the flight ability of males exhibiting courtship song defects. In an assay adapted from Drummond et al. (1991), males were released in the center of a large cylinder, allowed to fly freely, and their landing site was recorded. We compared *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males with wild-type controls and a known flight mutant, *Mhc*^{5}, which has been previously reported as a “poor flier”, rather than “flightless” (Homyk and Emerson, 1988). Flies that never left the platform after four 30-second trials were excluded from analysis (1 of 24 wild-type controls, 4 of 41 *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males, and 1 of 20 *MHC*^{5} males). As expected, *Mhc*^{5} mutants exhibited a significant flight defect compared to wild-type and *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males (Pearson’s χ^2, experiment-wide: $p < 0.0005$, pair-wise with Bonferonni: $p < 0.0005$). Interestingly, no difference in flight ability was detected between wild-type males and *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males (Fig. 7), suggesting the abnormalities in wing coordination induced by reducing *fru*^{M} expression were specific to song production.

Discussion

Although the role of sex-determination genes in the initiation of courtship behaviors is well studied, very little is known about what role these genes play in the neuronal circuits directly controlling these behaviors. Previous studies of *fru*^{M} mutants exhibiting aberrant courtship song (Ryner et al., 1996; Villella et al., 1997) were ambiguous as to whether *fru*^{M} is needed during the initiation of courtship song (i.e., the brain) or the execution of courtship song (i.e., the thoracic ganglia). Other experiments providing data in support of a role for *fru*^{M} in the song circuit rely on artificial activation of networks in a decapitated preparation (Clyne and Miesenböck, 2008). Our results indicate *fru*^{M} function is necessary in the thoracic song circuit itself for normal courtship song execution. To our knowledge, this perturbation of the song circuit leaving the descending song control intact is the first in an intact, freely behaving animal. Reducing *fru*^{M} expression in motor neurons and sensory neurons resulted in normal song, suggesting that the *fru*^{M} requirement is restricted to interneurons.

Our data provide evidence that the courtship song pattern generator is sex-specifically organized. It has been previously argued that the song circuit in females is intact but remains latent and is simply not activated (Clyne and Miesenböck, 2008), as photoexcitation of *fru*^{M} neurons in decapitated females was sufficient to induce a motor output recognizably resembling courtship song, but this motor output was non-functional (i.e., it did not increase mating when played back during courtship). Considering our evidence that disruption of *fru*^{M} expression in the song circuit strongly disrupts song in males, that expression of *fru*^{M} in the song circuit functionally improves artificially-induced song in females (Clyne and Miesenböck, 2008), and a sex-specific thoracic focus critical for courtship song was identified through gynandromorphic analysis (von Schilcher and Hall, 1979), we argue that an essential component of the song circuit vital for its proper function is indeed male-specific and *fru*^{M} is responsible for establishing the most important aspects of the circuit’s function. Further, these *fru*^{M}-dependant idiosyncrasies appear to selectively affect wing

coordination with respect to courtship song, as males unable to sing normally exhibited no detectable flight deficit. These unidentified fru^M-dependant circuit modifications allow coordination of wing control mechanisms specific to song production over those required for flight.

fru^M and IPI

The interpulse interval (IPI), and the rate at which it oscillates during singing, encodes the male's species-identity and provides the most salient feature of courtship song throughout *Drosophila* (Bennet-Clark et al., 1976; Talyn and Dowse, 2004; Markow and O'Grady, 2005). Despite the strong disruption of courtship song by elimination of fru^M in tsh^+ neurons, IPI, the most critical song parameter, is unaffected, reflecting its important courtship role. Villella et al. (1997) have shown that fru^M mutant males have an extended mean IPI. Our data suggests that this dependence of IPI on fru^M is not found in the thorax but in the brain, or that this dependence is conferred by other fru^M, non-tsh^+ thoracic neurons. The finding that fru^M expression has no bearing on IPI in a decapitated fly (Clyne and Miesenböck, 2008) favors the former. An expansive enhancer trap study demonstrates that song features (e.g., IPI) are likely shaped by the function of a distributed set of neuropil regions (Moran and Kyriacou, 2009). The role of the circadian rhythm gene, *period*, on the cycling of IPIs is conferred in the thoracic ganglia (Konopka et al., 1996), so the mean IPI might be regulated by the brain while the thoracic ganglia might be responsible for instilling the oscillation of IPI. The descending IPI information might be encoded by tonic drive, whereby stronger song circuit excitation results in shorter IPIs (Bentley, 1977), such that the long IPIs of flies photoinduced to sing (Clyne and Miesenböck, 2008) may result from photoactivation providing less circuit activation than endogenous excitation.

fru^M and wing extension

Males expressing *tra* in a tsh^{GAL4} pattern were unable to produce prolonged wing extensions, suggesting *tra*-dependent sex-specification of *tsh*-expressing neurons is critical for extending a wing long enough to sing. This thoracic execution requirement is in addition to the previously identified initiation requirement of male tissue in the dorsal brain (Hall, 1977). Recently, Koganezawa et al. (2009) showed that blocked activity of G32a-expressing neurons, like those found in tarsi, increases abnormal bilateral wing extension. Interestingly, feminizing tsh^+ neurons suppresses wing extension, while blocking $G32a^+$ neurons reduces the suppression of wing extension, suggesting these distinct neural populations are antagonistic or manipulations of the overlapping populations resulted in opposite effects. Alternatively, *tra* expression may result in a subtle wing cuticle or muscle phenotype in males, as *tsh* is important for determining the proximal wing domain (Zirin and Mann, 2007). In either case, the normal wing extensions observed in tsh^{GAL4}/UAS-fru^M*IR* males suggest thoracic fru^M, downstream of *tra*, is not responsible for the wing extension phenotype, emphasizing the emerging role that other downstream genes (e.g. *doublesex*) play in the organization and function of the courtship network (Rideout et al., 2007).

Potential substrates for fru^M's role in song

The fru^M song phenotype may be produced by affecting any of four song circuit components: (1) descending inputs, (2) motor neurons, (3) proprioceptive neurons, and (4) local interneurons. First, the selective expression of *tsh* in the thorax and abdomen eliminates the role of descending brain interneurons in this fru^M-dependent song phenotype. Second, elimination of fru^M in motor neurons using $D42^{GAL4}$ did not result in a detectable phenotype, despite the fact that invertebrate flight motor neurons integrate sensory afferents and related motor neuron efferents, thus are critical for flight pattern generation (Levine, 1973; Harcombe and Wyman, 1977). This is consistent with fru^M's lack of an effect on innervation patterns of direct flight muscles, although it is expressed in one of them (Rideout

et al., 2007). Third, eliminating *fru*^{M} in proprioceptive organs using *ato*^{GAL4} and at the developing wing base using 30A^{GAL4} had no detectable effect on courtship song, including pulse duration. Wing sensory neurons limit the number of cycles per pulse and ensure short pulse durations, as sensory information entrains elements damping the wing vibration (Ewing, 1979a; Tauber and Eberl, 2001), and *fru*^{M} is expressed in sensory sensilla at the wing base (Manoli et al., 2005). Taken together, our results argue against a motor neuron, sensory neuron, or descending interneuron courtship song requirement for *fru*^{M}, suggesting local interneurons have a sex-specific *fru*^{M} requirement to properly assemble the song patterning circuit.

Although *fru*^{M} interneurons are widely distributed throughout the thoracic ganglia, a large cluster of *tsh* and *fru*^{M} co-expressing neurons is located ventrally along the anterior margin of the mesothoracic segment. This region has been speculated to be important in courtship song production (von Schilcher and Hall, 1979; Rideout et al., 2007), particularly since *dsx*^{M} and *fru*^{M} are highly co-expressed in this region, both of these factors are important in proper song production (Rideout et al., 2007), and this region is responsible for control of wing movement (Trimarchi and Schneiderman, 1994). These *fru*^{M}-dependent song circuit neurons may overlap with the previously identified pool of sexually dimorphic neurons (Rideout et al., 2007) and neurons putatively expressing the *tsh* paralog, *tiptop*, which affect pulse width and IPI (Datta et al., 2009; Moran and Kyriacou, 2009).

Mechanisms of *fru*^{M} function in the song circuit

The *fru*^{M} requirement for courtship song demonstrated here may control wiring of the song circuit by controlling the song circuit's connection to the descending command system or by sculpting the connections among song circuit components themselves. In the former case, a *fru*^{M} "identity" signal in the song circuit may be required to receive projections from the *fru*^{M}-expressing song command system in the brain, perhaps through a thoracic *fru*^{M}-specific projection that is absent when *fru*^{M} is eliminated (Datta et al., 2008; Kimura et al., 2008). Thus, song circuits not expressing *fru*^{M} may not receive descending excitation (or insufficient descending excitation), resulting in aberrant courtship song. On the other hand, *fru*^{M} may be required for the song patterning circuit itself to be wired properly. This is consistent with the observation of Clyne and Miesenböck (2008) that *fru*^{M} expression in females improves song circuit function. Although we have focused on *fru*^{M}'s developmental ability to mediate sex-specific differences in neuronal projection patterns (Kimura et al., 2005; Datta et al., 2008; Kimura et al., 2008), we cannot rule out a neurophysiological role for *fru*^{M}, despite a limited set of studies addressing this issue (Datta et al., 2008).

Interpretation of the abnormal courtship song parameters in males with disrupted thoracic *fru*^{M} expression may provide insights into how *fru*^{M} affects neuromuscular control of song. The neuromuscular mechanism producing pulse song is still unclear, but one hypothesis is that pulse timing is achieved by direct, timed inputs onto an unidentified direct flight muscle that moves the wing and distorts the thorax to trigger a contraction of a power-delivering, stretch-activated fibrillar muscle (Ewing, 1977). Lagging sensory input via proprioceptive feedback (Ewing, 1979a; Reddy et al., 1997; Tauber and Eberl, 2001) damps the ensuing antagonistic indirect flight muscle contraction directly by activating opposing direct flight muscles or indirectly by releasing tension in the thoracic box to prevent its stretch-activation and the subsequent polycylic pulse. In this model, the decreased amplitude of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* pulse song suggests that indirect flight muscles may not be sufficiently recruited in the absence of *fru*^{M}. The broadened, polycyclic pulse width suggests that damping may not occur properly.

Courtship song intensity

To our knowledge, our recordings are the first calibrated measure of acoustic output of a courting male fly reported in the literature. The mean sound particle velocity level (SPVL) of a wild-type male sound pulse as measured here is 99.2 ± 1.0 db SPVL (re: 50 nm s^{-1}, mean ± s.e.m.). As males were allowed to freely move around the chamber while singing at distances from 1 – 6 mm from -- and unrestricted angles to -- the microphone, a standardized output intensity is confounded by distance and directional effects, as well as complex near-field propagation physics. Nonetheless, this figure corresponds well to the predictive figure calculated by Bennet-Clark (1971) of 95 dB SPVL at 5 mm in front of the courting male. The near-field dipteran auditory organ, the arista, attenuates the vibration amplitude at 166 Hz by approximately only 2.5 dB (Gopfert and Robert, 2002), thus it is obviously sensitive enough to detect sounds we measured.

This report provides evidence that sex-specification via fru^M function is required specifically in the thoracic ganglia to perform courtship song, and suggests other courtship-specific motor patterns (e.g., genitalia licking, copulation attempts) may be controlled by circuits requiring fru^M. However, fru^M does not affect the most important feature of that pattern, IPI. Identification of neurons critical to patterning the song in a fru^M-dependent matter will further our understanding of how complex behaviors are produced by the nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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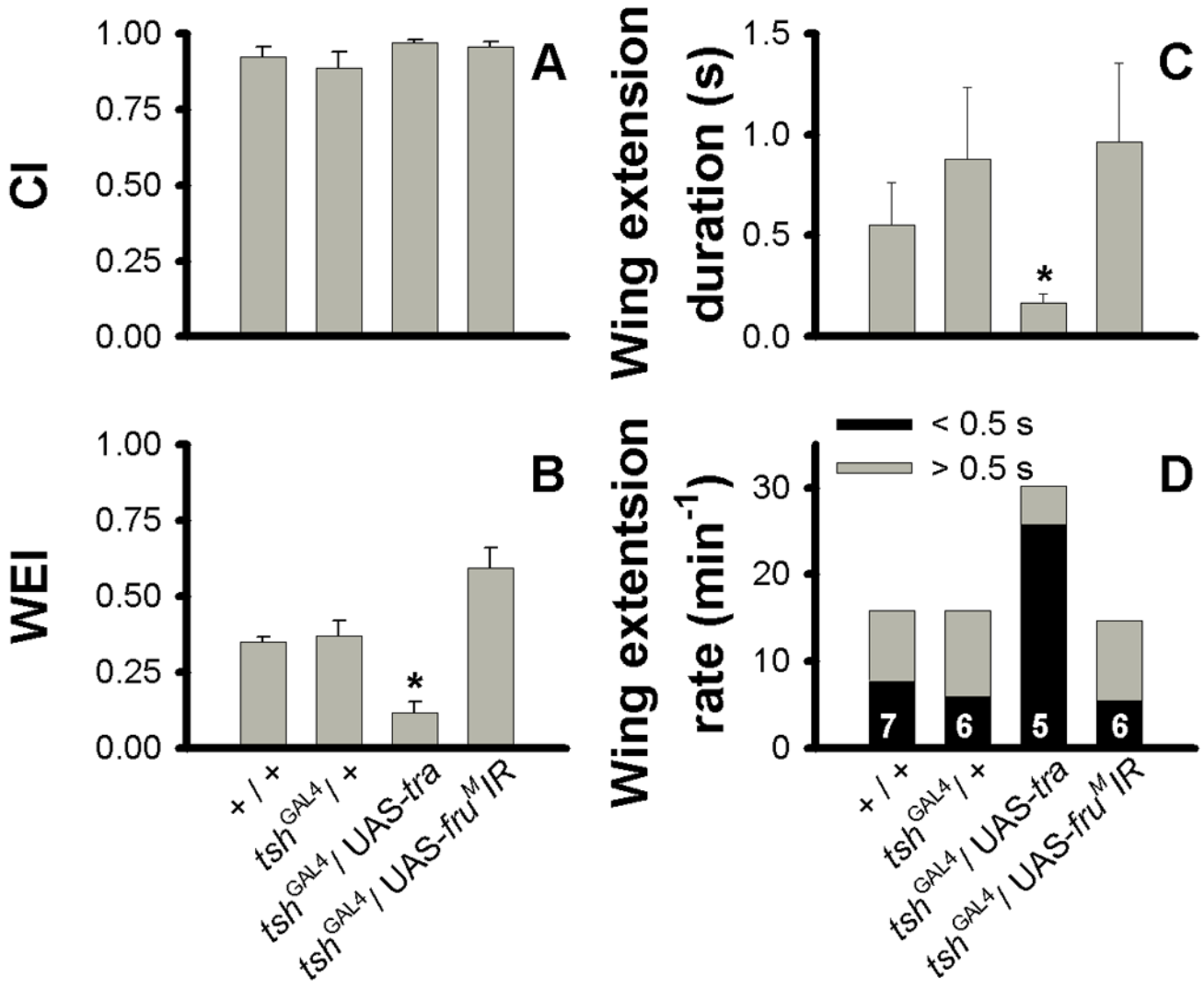

Figure 1.

Measurements of wing extension behavior. (A) No differences in courtship index (CI) were observed among genotypes (mean ± s.e.m.). (B) *tsh*^{GAL4}/UAS-*tra* flies had a significantly decreased median wing extension duration compared to *tsh*^{GAL4}/+ controls, while *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males were no different than controls. (C) Mean of a fly's median wing extension duration. *tsh*^{GAL4}/UAS-*tra* males display significantly shorter wing extensions than *tsh*^{GAL4}/+ controls. (D) Wing extension frequency, separating extensions shorter and longer than 0.5 s. tsh^{GAL4}/UAS-*tra* flies are not different in frequency of total wing extensions, but exhibit more frequent wing extensions shorter than 0.5 s and less frequent wing extensions longer than 0.5 s compared to *tsh*^{GAL4}/+ controls. There is no difference between *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males and *tsh*^{GAL4}/+ control males. Sample size indicated within bars in (D). *: $p < 0.05$.

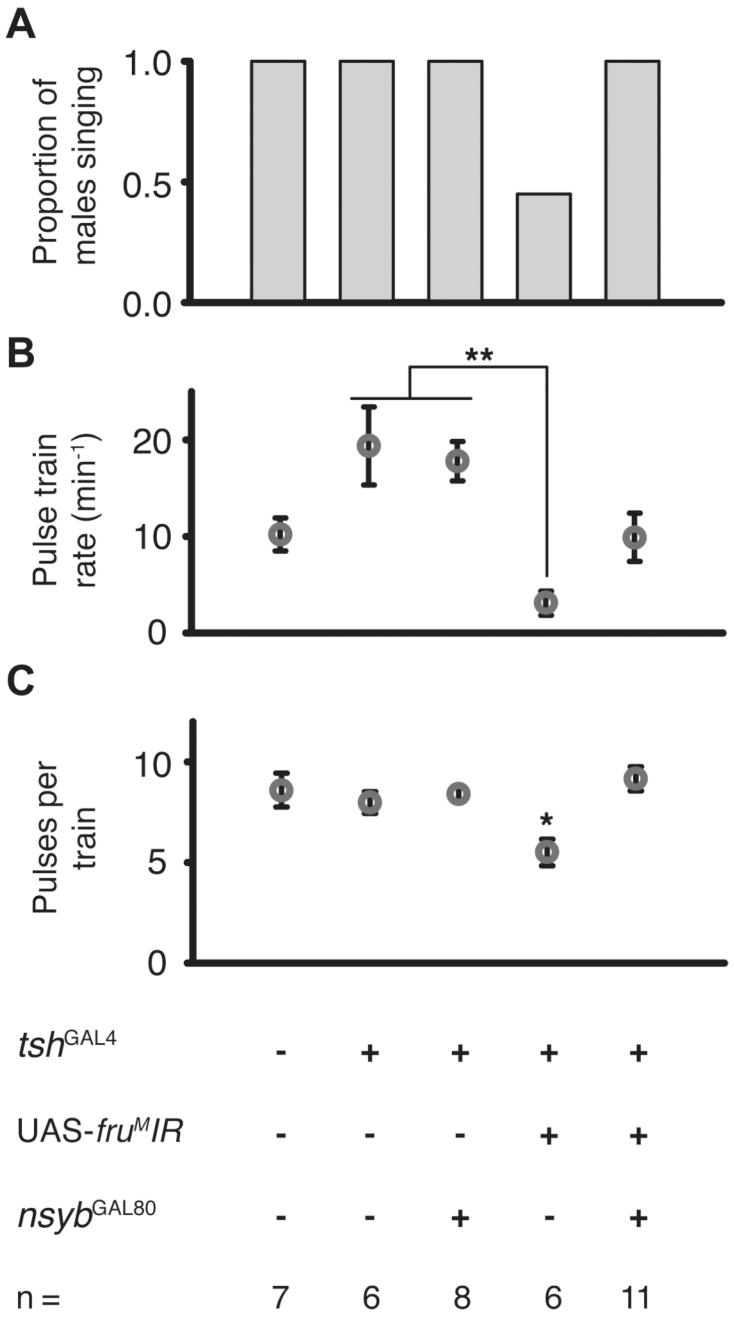

Figure 2.

Expression of *fru*^{M}*IR* in *tsh*-specific pattern reduces amount of courtship song. Proportion of flies that produced audible output classified as pulse song (A). Only *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males failed to produce courtship song. *tsh*^{GAL4}/UAS-*fru*^{M}*IR* also males exhibited fewer pulse trains per minute (B) and pulses per train (C) than *tsh*^{GAL4}/+ controls, while *tsh*^{GAL4}/UAS-*fru*^{M}*IR; n-syb*^{GAL80} males were no different than controls. *tsh*^{GAL4}/UAS-*fru*^{M}*IR; n-syb*^{GAL80} males rescued the decreased pulses per train in *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males. *: $p < 0.05$, **: $p < 0.01$.

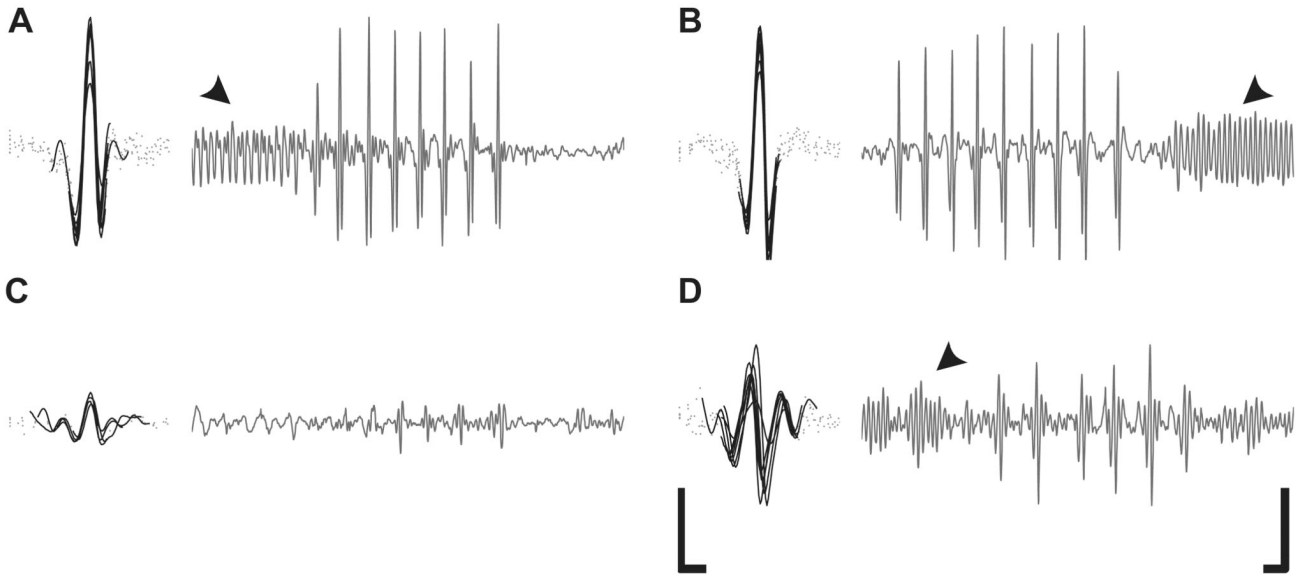

Figure 3.

Representative traces of courtship song output. The left panel displays individual pulses within a pulse train aligned by midpoint of energy. Solid lines indicate amount of trace required to include 90% of the signal's energy (pulse width). Right panel displays the whole trace. Arrowheads indicate sine song. (A) tsh^{GAL4}/+ controls, (B) tsh^{GAL4}/UAS-$fru^{M}IR$; $n\text{-}syb^{GAL80}$ rescue flies, (C) representative small amplitude and (D) polycyclic nature of tsh^{GAL4}/UAS-$fru^{M}IR$ courtship song. Scale bars: Left panel, horizontal 5 ms, vertical 5 mm s^{-1}. Right panel, horizontal 25 ms, vertical 5 mm s^{-1}.

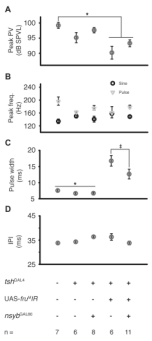

Figure 4.

Intrapulse data. (A) Peak particle velocity within a pulse is reported here as a mean of medians in dB SPVL, using 50 nm s^{-1} as a reference. There no significant differences were detected in dB SPVL, but there was a trend for a reduction in pulse amplitude for *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males. (B) Peak frequency of sine songs (circle) and individual pulses (triangle). No significant differences were observed. (C) Pulse widths from *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males were significantly broader compared to *tsh*^{GAL4}/+ controls. This broadened pulse width is rescued in *tsh*^{GAL4}/UAS-*fru*^{M}*IR; n-syb*^{GAL80} males. (D) Mean interpulse interval (IPI) was unaffected by genotype. *: $p < 0.05$, **: $p < 0.01$, ‡: one-tailed, $p < 0.05$.

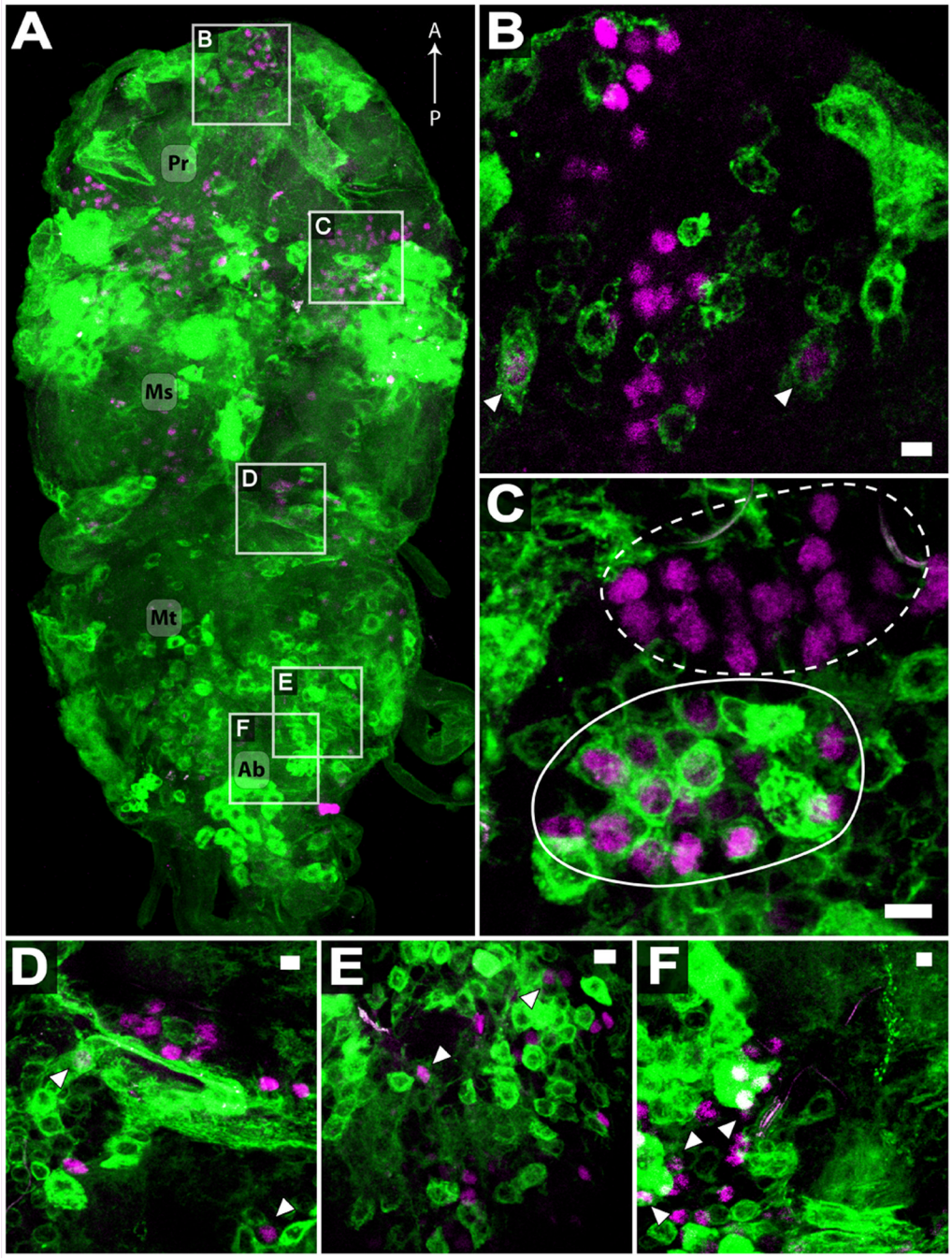

Figure 5.

Immunocytochemistry of adult w; *tsh*^{GAL4}, UAS-mCD8-GFP/CyO CNS, visualizing endogenous, membrane bound GFP (green) and Fru^{M} immunoreactivity (magenta). (A) Dorsal-ventral view of adult ventral ganglia. Extensive labeling is visible in prothoracic (Pr), mesothoracic (Ms), metathoracic (Mt), and abdominal (Ab) segments. Anterior-posterior axis is indicated. (B–F) 3 – 5 μm representative sections of the five groups of *fru*^{M} neurons in the ventral ganglia, according to (Lee et al., 2000). Fru^{M} neural cluster 16 (B), 17 (C), 18 (D), 19 (E), and 20 (F). Arrowheads indicate examples of neurons coexpressing Fru^{M} and mCD8-GFP. In (C), a Fru^{M}-expressing cluster clearly coexpresses GFP (solid line), while an adjacent Fru^{M} cluster does not (dashed line). Scale bars (B–F) represent 5 μm.

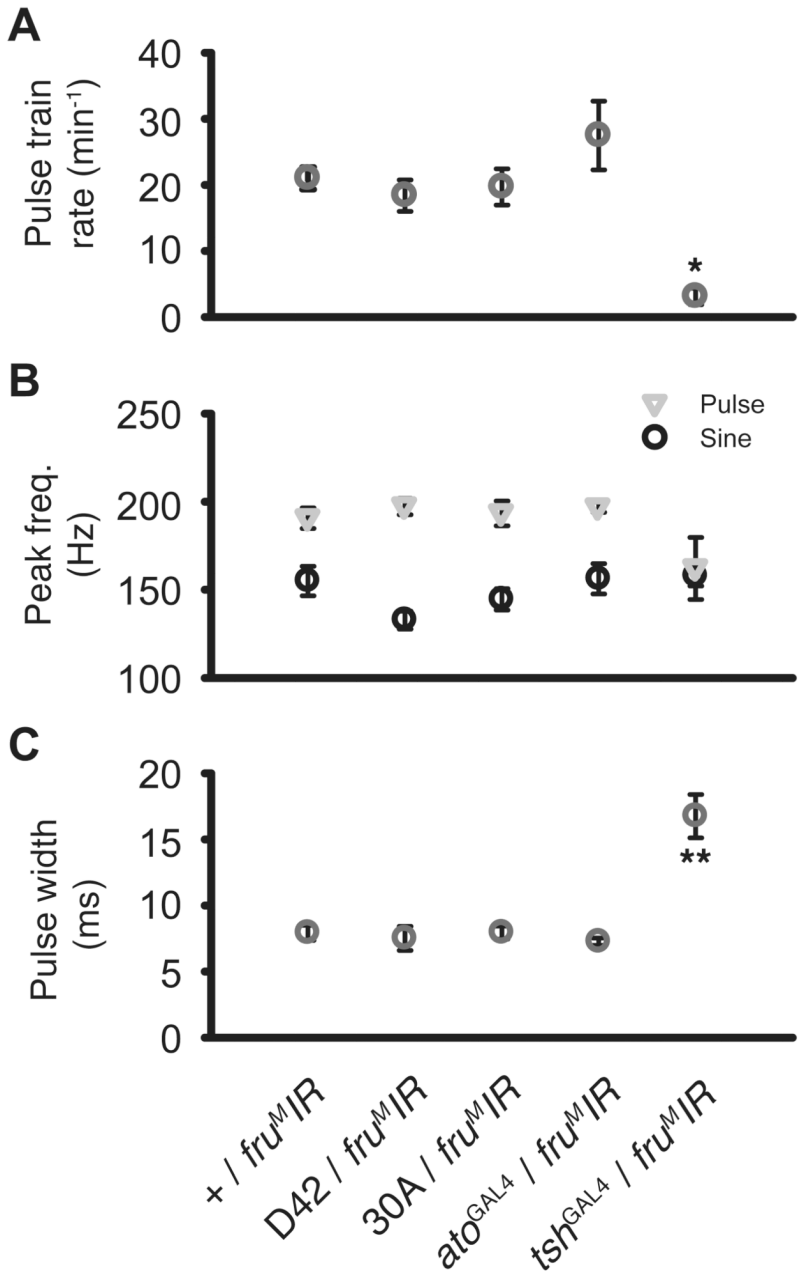

Figure 6.

Courtship song is unaffected by driving *fru*^{M}*IR* in motor neurons and sensory neurons. No significant effects of genotype were found on (A) pulse rate, (B) pulse and sine song peak frequency, or (C) pulse width compared to UAS-*fru*^{M}*IR* controls. The *tsh*^{GAL4}/UAS-*fru*^{M}*IR* mutant phenotype is replotted from Fig. 2 and Fig. 4 for comparison. n = 6 – 8. *: $p < 0.005$, **: $p < 0.001$.

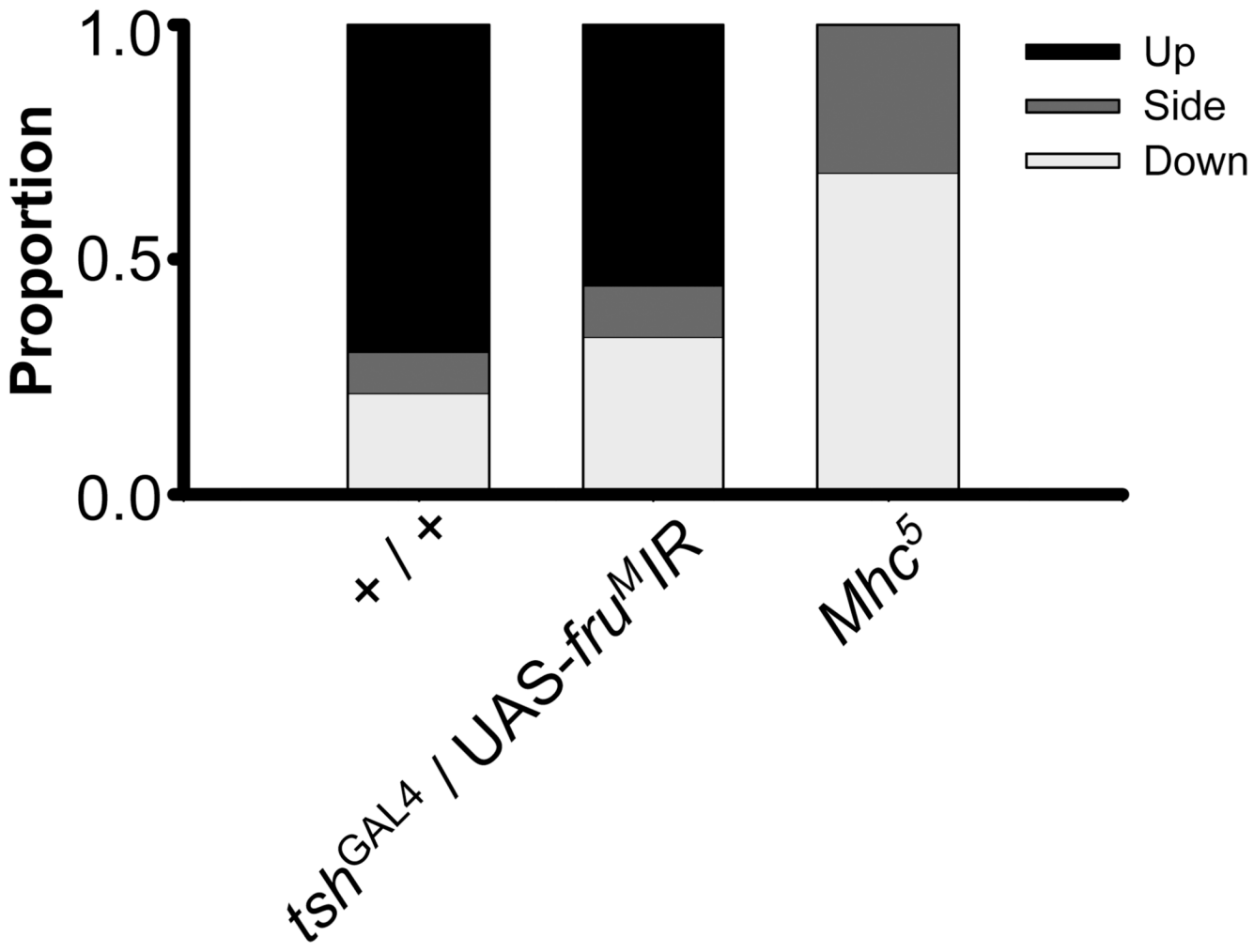

Figure 7.
Flight ability of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males. Males were placed in the center of a cylinder and allowed to freely fly. Landing sites were recorded as the bottom (white), side (gray), or out of the cylinder (black). There was a significant effect of genotype on flight performance ($p < 0.001$). Flight performance of control + / + males (n = 23) was not significantly different from *tsh*^{GAL4} / UAS-*fru*^{M}*IR* males (n = 27), but both performed better than the known flight mutant, *Mhc*^{5} (n = 19) ($p < 0.0005$).