Title: Shared Song Object Detector Neurons in *Drosophila* Male and Female Brains

Drive Divergent, Sex-Specific Behaviors

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Running Title: Shared Song Detector Neurons Drive Sex-Specific Behaviors
Abstract:
Males and females often produce distinct responses to the same sensory stimuli. How such differences arise – at the level of sensory processing or in the circuits that generate behavior – remains largely unresolved across sensory modalities. We address this issue in the acoustic communication system of Drosophila. During courtship, males generate time-varying songs, and each sex responds with specific behaviors. We characterize male and female behavioral tuning for all aspects of song, and show that feature tuning is similar between sexes, suggesting sex-shared song detectors drive divergent behaviors. We then identify higher-order neurons in the Drosophila brain, called pC2, that are tuned for multiple temporal aspects of one mode of the male’s song, drive sex-specific behaviors, and show a mirrored correspondence between sensory and motor tuning. We thus uncover acoustic object detector neurons at the sensory-motor interface that flexibly link auditory perception with sex-specific behavioral responses to communication signals.
**Introduction**

Across animals, males and females produce distinct, dimorphic behaviors in response to common sensory stimuli (e.g., pheromones, visual cues, or acoustic signals), and these differences are critical for social and reproductive behaviors (Billeter and Levine, 2013; Dulac and Wagner, 2006; Kelley, 2003; Yamamoto et al., 2013; Yang and Shah, 2014). The molecular dissection of sexual dimorphisms in the nervous system of flies and mice in particular (Cachero et al., 2010; Dulac and Wagner, 2006; Rideout et al., 2010; Stowers and Logan, 2010; Yang and Shah, 2014; Yu et al., 2010) has identified neurons involved in either processing important social cues or driving social behaviors, but it remains open as to how sex-specific behaviors to common sensory signals emerge along sensorimotor pathways. It could be that males and females process sensory information differently, leading to different behavioral outcomes, or that males and females process sensory information identically, but drive different behaviors downstream of common detectors.

This issue has been most heavily investigated for pheromone processing, and these studies point to differences along sensory pathways. For instance, in *Drosophila*, the male pheromone cVA induces either aggression in males (Wang and Anderson, 2010) or receptivity in females (Billeter et al., 2009; Kurtovic et al., 2007). The pheromone is detected by shared circuits in males and females and the similarly processed sensory information (Datta et al., 2008) is then routed to sex-specific higher-order olfactory neurons (Kohl et al., 2013; Ruta et al., 2010) that likely exert different effects on behavior, although this hypothesis has not yet been tested. In the mouse, the male pheromone ESP1 triggers lordosis in females, but has no effect on male behavior. This pheromone activates V2Rp5 sensory neurons in both sexes but, analogous to cVA processing in flies, these neurons exhibit sex-specific projection patterns in the hypothalamus that drive sex-specific behavioral responses (Haga et al., 2010; Ishii et al., 2017). For pheromone processing then, the rule appears to be that early olfactory processing is largely shared between the sexes and then common percepts are routed to separate higher-order neurons or circuits for control of differential behaviors. But does this rule apply for other modalities, or for stimuli that can be defined by multiple temporal or spatial scales (e.g. visual objects or complex sounds). For such stimuli, selectivity typically emerges in higher-order neurons (Dicarlo et al., 2012; Gentner, 2008; Tsao and Livingstone, 2008) and we do not yet know if such neurons are shared between males and females, and therefore if dimorphic responses emerge in downstream circuits.
Here, we investigate this issue in the auditory system in *Drosophila*. Similar to birds (Fortune et al., 2011; Konishi, 1985), frogs (Gerhardt and Huber, 2002), or other insects (Ronacher et al., 2014), acoustic communication in *Drosophila* involves different behaviors in males and females relative to the courtship song. During courtship, males chase females and produce a species-specific song that comprises two major modes – pulse song consists of trains of brief pulses and sine song consists of a sustained harmonic oscillation (Bennet-Clark and Ewing, 1967). In contrast with males, females are silent but arbitrate mating decisions (Bennet-Clark and Ewing, 1969). Males use visual feedback cues from the female (rapid changes in her walking speed and her distance relative to him) to determine which song mode (sine or pulse) to produce over time (Clemens et al., 2017; Coen et al., 2014; 2016) – this gives rise to the variable structure of song bouts (Fig. 1A). Receptive females slow in response to song (Aranha et al., 2017; Bussell et al., 2014; Clemens et al., 2015; Coen et al., 2014; Cook, 1973; Crossley et al., 1995; F. Von Schilcher, 1976; Tompkins et al., 1982), while playback of courtship song to males can induce them to increase their walking speed (Crossley et al., 1995; F. Von Schilcher, 1976; Vaughan et al., 2014), and to display courtship-like behaviors (Eberl et al., 1997; Li et al., 2018; Yoon et al., 2013; Zhou et al., 2015). These behavioral differences surrounding song production and perception between *Drosophila* males and females, combined with the wealth of genetic and neural circuit tools, make the *Drosophila* acoustic communication system an excellent one in which to investigate whether males and females share common sensory detection strategies for their courtship song, and how divergent behaviors arise.

Each major mode of *Drosophila* courtship song, sine or pulse, contains patterns on multiple temporal scales (Arthur et al., 2013; Bennet-Clark and Ewing, 1967) (Fig. 1A) – neurons that represent either the pulse or sine mode should in theory bind all of the temporal features of each mode, similar to object detectors in other systems (Bizley and Cohen, 2013; Dicarlo et al., 2012; Gentner and Margoliash, 2003; Griffiths and Warren, 2004), and their tuning should match behavioral tuning. Historically, behaviorally relevant song features have been defined based on the parameters of the species' own song (Bennet-Clark and Ewing, 1969). However, there is now ample evidence that the preferred song can diverge from the conspecific song (Amézquita et al., 2011; Blankers et al., 2015; Ryan et al., 2001) – for instance if females prefer exaggerated song features (Rosenthal and Ryan, 2011; Ryan and Cummings, 2013) or respond to signal parameters not normally produced by their male.
conspecifics (Hennig et al., 2016). Moreover, the job of the female nervous system is not only to tell one species apart from another, but also to select a particular mate from within the species distribution. It is therefore important to define song modes by the acoustic tuning of specific behavioral outputs. This has been done for other insects (e.g. (Clemens and Hennig, 2013; D. von Helversen and O. von Helversen, 1997)) but never for flies in a systematic way that also permits a direct comparison between sexes.

To that end, we developed a behavioral assay for assessing dynamic changes in walking speed in response to sound playback in both sexes, and we then measured locomotor tuning for all features of either pulse or sine song. This reveals that males and females have similar tuning but different behavioral responses and that they are tuned for every major feature of the song. We then identified a small set of sexually dimorphic neurons, termed pC2 (Kimura et al., 2015; Rideout et al., 2010; Zhou et al., 2014), that serve as shared pulse song detectors in both sexes: the tuning of pC2 neurons is matched to behavioral tuning for pulse song – but not for sine song – across a wide range of temporal scales. We find that optogenetic activation of pC2 is sufficient to drive sex-specific behaviors produced in response to pulse song – changes in locomotion with sex-specific dynamics as well as singing in males. pC2 is also part of a sensorimotor loop, since it drives and is driven by pulse song. Finally, we establish the importance of pC2 neurons by showing that early social experience changes both the tuning of these neurons and the tuning of the behavior. Our results indicate that the fly brain contains common song object detectors in males and females which control sex-specific behavioral responses to song via downstream circuits.

Results

Comprehensive characterization of behavioral tuning for courtship song features

We designed a single-fly playback assay in which individual males or females receive acoustic stimuli in the absence of any confounding social interactions, and we implemented an automated tracker to analyze changes in locomotion relative to acoustic playback (Fig. 1B and Movie S1). The assay (which we refer to as FLyTRAP (Fly Locomotor TRacking and Acoustic Playback)) monitors dynamic changes in walking speed, which provides a readout that can be directly compared between both males and females, as opposed to slower readouts of sex-specific behaviors such as the female time to copulation (Bennet-Clark and Ewing, 1969; Zhou et al., 2014) or male-male chaining (Yoon et al., 2013; Zhou et al., 2015). Because of the high-throughput nature of our assay combined with automated tracking, we
can easily test a large number of flies and song parameters, including those only rarely produced by conspecifics but to which animals might be sensitive (Coen et al. 2014; Aranha et al., 2017; Bussell et al., 2014; Crossley et al., 1995; Eberl et al., 1997; Rybak et al., 2002a; 2002b; F. Von Schilcher, 1976; Yoon et al., 2013; Zhou et al., 2015).

Previous studies that assayed either male-female copulation rates or male-male chaining often focused on behavioral selectivity for the interval between pulses in a pulse train (inter-pulse interval (IPI), Fig. 1A) (Bennet-Clark and Ewing, 1969; Rybak et al., 2002b; F. Von Schilcher, 1976; Zhou et al., 2015). Using FLyTRAP, we systematically compared male and female locomotor tuning to 82 acoustic stimuli that span the features and timescales present in courtship song (see Supplemental Table 1). Typically, each stimulus was presented 23 times to 120 females and 120 males, generating >2500 responses per stimulus and sex (see Methods).

We started by examining behavioral tuning for IPI using the wild type strain NM91. We generated artificial pulse trains and varied only the IPI (between 16 and 96ms) – we chose a stimulus intensity of 5 mm/s since varying intensity had minimal effect on pulse song responses in females (Fig. S1A, B). Observed changes in speed were stimulus-locked, sex-specific and tuned to IPI (Fig. 1C). While females slow down to pulse trains, males exhibit transient slowing at pulse train onset followed by a long-lasting acceleration. The transient component of the locomotor response was present for all stimuli (Fig. S1C, D, S2A-C) and may correspond to an unspecific startle response to sound onset (Lehnert et al., 2013). The transient was also present in females but masked by the stimulus-dependent slowing that followed (Fig. 1C). Due to the briefness of the transient response, the integral change in speed following stimulus onset reflects mostly the speed during the sustained phase (Fig. S1C, D). For simplicity, we therefore used the full integral as an overall measure of behavioral tuning. We found that in FLyTRAP, female IPI tuning is a band-pass filter matched to the statistics of male song (Fig. 1D): the mode of the distribution of *Drosophila melanogaster* IPIs is centered between 30 and 50 ms and females decrease their speed most for the same IPI range, and less for shorter or longer IPIs. Males produced a similar band-pass tuning curve peaked at the same IPI range - but their locomotor response was opposite in sign (males accelerated, females decelerated). This is consistent with the results of other assays that have found band-pass tuning for IPI in both sexes (Bennet-Clark and
Ewing, 1969; Rybak et al., 2002b; F. Von Schilcher, 1976; Zhou et al., 2015) and a sex-specific sign of locomotor responses (Crossley et al., 1995; F. Von Schilcher, 1976).

We next systematically varied parameters that characterize pulse song to cover (and extend beyond) the distribution of each parameter within D. melanogaster male song (just as we did for IPI) (see Supp. Fig. 2). We examined behavioral tuning in both sexes for parameters that varied on timescales of milliseconds (carrier frequency, pulse duration and IPI) to seconds (pulse train duration) (Fig. 1A). We found that male and female tuning curves are of opposite sign but similar shape for all pulse song features tested across time scales (Fig. 2A, B, S2D-F, see Fig. S2A-C for speed traces), and that the behavioral tuning for pulse parameters often overlapped the distribution found in natural song (Fig. 2C). While the behavioral tuning curves for all pulse song features on short time scales are band-pass with a well-defined peak, we found that tuning for pulse train duration was monotonous: both females and males increase their locomotor response with increasing pulse train duration up to four seconds (Fig. 2A, B). During natural courtship, pulse trains longer than four seconds are rarely produced (Coen et al., 2014). Males also produce two distinct types of pulses (Clemens et al., 2017) – we find that while females appear to be broadly tuned for both types of pulses in the FlyTRAP assay, males respond preferentially to higher frequency pulses. Finally, we found that both males and females are more selective for the pulse duration versus the pulse pause, the two components of the IPI (Fig. S2D-F) – this is in contrast to other insects that produce and process song pulses (e.g. crickets, grasshoppers, katydids), and that are preferentially tuned to pulse pause, pulse period or pulse train duty cycle (Hennig et al., 2014; Ronacher et al., 2014).

We next tested locomotor tuning for the parameters that characterize sine stimuli – carrier frequency and the duration of sine trains (Fig. 1A). Both males and females slow for sine tones of different frequencies, with very low and very high frequencies eliciting the strongest responses (Fig. 2A, B and Fig. S2A-C). Notably, the frequencies inducing the strongest slowing (100Hz) are not typically produced by males (Fig. 2C). As for sine train duration tuning, we observed sustained responses that increased with duration and saturated only weakly, possibly because of the weak response magnitude.

Pulse and sine song usually co-occur within a single bout but it is not known why males produce two different modes (although females respond to both during natural courtship...
One possibility is that one mode exerts a priming effect on the other (F. V. Schilcher, 1976). To test interactions between the two song modes, we presented sequences in which a 2-second pulse train was followed by a 2-second sine tone or in which a sine tone was followed by a pulse train and compared the responses for these sequences to the responses to an individual pulse train or sine tone (Fig. S2G). The responses are well explained by a linear combination of the responses to individual sine or pulse trains. Deviations from linearity occur due to sound onset responses, but otherwise responses do not strongly depend on the order of presentation in a bout (see also (Talyn and Dowse, 2004)). This suggests that these stimuli are processed in independent pathways.

To summarize, we compared behavioral responses in males and females for all features that define the courtship song. We found that male and female speed changes were strongly correlated for both song modes but that the sign of the correlation was negative for pulse stimuli and positive for sine stimuli (Fig. 2E). The opposite sign of the correlations along with the independence of responses to sine and pulse stimuli (Fig. S2G) indicates that sine and pulse song are processed by different circuits. The large magnitude of the correlations implies that feature tuning of the behavioral responses is similar between sexes and suggests that detector neurons for each song mode should be shared between sexes.

**Hearing pulse song drives wing extension in males, but not in females**

Another sex-specific aspect of song responses is courtship: playback of conspecific song induces courtship-like behavior in males – this can even be directed towards other males, leading to the male chaining response, in which males follow other males, chasing and extending their wings (Eberl et al., 1997; F. Von Schilcher, 1976; Yoon et al., 2013). In our single-fly assay, males lack a target for courtship and the song-induced arousal likely manifests as an increase in speed. Since FLYTRAP does not permit simultaneous recording of fly acoustic signals during playback, we quantified wing extension as a proxy for singing, and examined whether song playback alone drives singing in solitary males. We found that solitary males extend their wings in response to pulse song stimuli specifically (Fig. 2F, G, Movie S2). This behavior is tuned for the inter-pulse interval (similar to the locomotor response, Fig. 1D) – the conspecific IPI of 36ms drives the most wing extension, and shorter and longer IPIs evoke fewer wing extensions. By contrast, conspecific sine song (150Hz) does not induce wing extension (Fig. 2F) (see also (Eberl et al., 1997; Yoon et al., 2013)).
We also found that playback of pulse does not elicit wing extension in females, even though females have been shown to possess functional circuitry for singing (Clyne and Miesenböck, 2008; Rezával et al., 2016) – wing extension in response to pulse song is thus sex-specific.

These results are consistent with those for locomotor tuning: pulse song, but not sine song, generates sex-specific differences in behavior. The identical tuning of the two behavioral responses in males (locomotion (Fig. 1C) and song production (Fig. 2G)) suggests that the behavioral responses are driven by a common circuit. Finally, that playback of pulse song alone is sufficient to drive singing in males implies the existence of neurons with sensorimotor correspondences, similar to “mirror neurons” (Mooney, 2014; Prather et al., 2008; Rizzolatti and Arbib, 1998) – we explore this hypothesis below.

**Drosophila male and female brains share pulse song detector neurons**

Our systematic exploration of song stimulus space using the FLYTRAP assay revealed behavioral tuning for song parameters across temporal scales (from the carrier frequencies of sine and pulse lasting milliseconds to the duration of sine and pulse trains lasting seconds). We next searched for neurons with tuning across temporal scales that could serve as object detectors of either the pulse or sine mode of courtship song. We focused on neurons expressing the Doublesex (Dsx) transcription factor that regulates sexual dimorphism in cell number and neuronal morphology between males and females. In the central brain there are ~70 Dsx+ neurons per hemisphere in females and ~140 Dsx+ neurons per hemisphere in males (Kimura et al., 2015; Rideout et al., 2010). Previous studies found calcium responses to both song-like stimuli and pheromones in Dsx+ neuron projections in females (Zhou et al., 2014) and tuning for the inter-pulse interval in males (Zhou et al., 2015). In addition, silencing subsets of Dsx+ neurons in females affected receptivity (Zhou et al., 2014). These data suggest that Dsx+ neurons could serve as the common pulse song detectors in males and females. To test this possibility, we recorded auditory responses in Dsx+ neurons and examined tuning for song features across timescales, in both males and females, to compare with our behavioral results.

We imaged neural activity using the calcium sensor GCaMP6m (Chen et al., 2013) expressed only in Dsx+ neurons and focused on the lateral junction (LJ) (Cachero et al., 2010; Ito et al., 2014; Yu et al., 2010), a site of convergence for the majority of Dsx+ neuron projections (Fig. 3A, B, S5B, C, Movie S3, S4). We found that male and female Dsx+
projections in the LJ are driven strongly by pulse, but not by sine, stimuli (Fig. 3C) similar to previous results (Zhou et al., 2014). While males overall produce weaker responses to auditory stimuli compared with females (Fig. 3C), the normalized LJ responses are highly correlated between sexes – stimuli that evoked the strongest responses in females also evoked the strongest responses in males (Fig. 3D).

In order to connect behavioral tuning to neural tuning, we validated that the genetic strain in which we imaged calcium responses possessed the same behavioral tuning as the strain used in FLYTRAP (Fig. S3A). The sex-specific sign and shape of the IPI tuning curves of flies from these two strains matches acoustic tuning measured in other playback assays: females slow for pulse song (Clemens et al., 2017; Coen et al., 2014; Crossley et al., 1995; F. Von Schilcher, 1976) and copulate most when exposed to conspecific IPIs and less for shorter or longer IPIs (Bennet-Clark and Ewing, 1969; Li et al., 2018); males in groups increase their speed (Crossley et al., 1995; F. Von Schilcher, 1976; Vaughan et al., 2014) and court other females or males most when exposed to pulse song with the conspecific IPI (Crossley et al., 1995; Yoon et al., 2013; Zhou et al., 2015). The two strains chosen for the behavioral and calcium imaging experiments thus display the species-typical acoustic tuning likely expressed during natural courtship encounters. Interestingly, the behavioral tuning for IPI in seven additional wild type strains is still sex-specific but deviates from the species-typical tuning (Fig. S3B) when assayed in FLYTRAP, even though the same wild type strains – including NM91 – display virtually identical responses to song in a courtship assay in which a male courts a female (Clemens et al., 2017; 2015; Coen et al., 2016; 2014). This indicates that these strains require additional cues (e.g., pheromones or visual cues) to fully express their preference for conspecific song features.

When examining neuronal tuning curves, we found a good match between Dsx+ LJ responses and the magnitude of changes in speed across all timescales of pulse song in both sexes (Fig. 3E, F). For example, the Dsx+ LJ tuning curve for IPI is similar in females and males with the strongest responses at 36ms, matching the behavioral tuning curves (compare with Fig. 2A,B) – moreover, Dsx+ neurons are also preferentially tuned for the pulse duration over the pulse pause (Fig. S4D, E – compare with Fig. S2E, F). At longer timescales, LJ tuning curves also match behavioral tuning curves for pulse train duration – while the integral calcium continues to increase with train duration, the peak fluorescence saturates in both sexes, similar to the behavioral response. Apart from this difference, peak
and integral $\Delta F/F$ are similar (Fig. S4F, G). Overall, LJ responses are selective for the features found in conspecific pulse song: LJ responses were strongest for stimuli with a carrier frequency of 250Hz, an inter-pulse interval of 36ms, and a pulse duration of 16ms (Fig. S4A-C). A match in only two of these three features was not sufficient to maximally drive Dsx+ neurons. Sine stimuli have lower carrier frequencies, long durations, and no pauses (they are by definition continuous) – which explains the weak responses of Dsx+ neurons to all sine stimuli (Fig. 3C).

We next directly compared Dsx+ LJ responses and behavioral responses for all stimuli and found strong correlations for pulse stimuli in both sexes – although with a sex-specific sign. That is, male neural and behavioral tuning for pulse stimuli are positively correlated ($r=0.55$, $p=9\times10^{-6}$) – high Dsx+ neuron activity correlates with the most acceleration (Fig. 3G). Female neural and behavioral tuning for pulse stimuli are negatively correlated ($r=-0.62$, $p=2\times10^{-7}$) – high Dsx+ neuron activity correlates with the most slowing (Fig. 3H). This is consistent with these neurons controlling the magnitude, but not the direction of speed changes. We observed no statistically significant correlation for sine stimuli (male: $r=-0.15$, $p=0.56$; female: $r=0.27$, $p=0.30$), as Dsx+ LJ responses to sine stimuli were weak. Note that Dsx+ LJ activity only accounts for roughly 1/3 of the variability in behavioral responses for pulse song. This suggests that the behavior is driven and modulated by additional pathways outside of the Dsx+ neurons in the LJ. Nonetheless, Dsx+ neurons that innervate the LJ have tuning properties expected for pulse song object detectors – they prefer pulse over sine stimuli, are similarly tuned in males and females, and their feature tuning matches the behavioral tuning for all pulse, but not sine, stimuli across timescales.

**Dsx+ pC2l neurons are pulse song detectors**

The Dsx+ neurons of the central brain form a morphologically heterogeneous population with several distinct, anatomical clusters many of which project to the LJ (Kimura et al., 2015; Rideout et al., 2010; Zhou et al., 2015; 2014) (Fig. 3A). Previous studies that examined auditory responses in Dsx+ neurons (Zhou et al., 2015; 2014) did not resolve which subtype carried the response. Using a stochastic labeling approach (Nern et al., 2015), we confirmed that only five out of eight Dsx+ cell types in the female brain project to the LJ (Kimura et al., 2015): pC1, pC2/l/m, pMN1, and pMN2, but not pCd1/2 and aDN (Fig. 4A and Fig. S5D, E).

We next imaged calcium responses to pulse and sine stimuli in the somata of all five Dsx+ cell types that innervate the LJ and found that a subset of neurons in the pC1 and pC2...
clusters possess auditory responses, in addition to cell type pMN2 (a female-specific neuron (Kimura et al., 2015) comprising only one cell body per hemisphere) (Fig. 4B, C, Movie S5, S6). All responsive cells preferred pulse over sine stimuli (Fig. 4D). We did not observe auditory responses in pMN1 neurons (not shown), although we cannot rule out that this neuron class has responses that are below the level of detection by the calcium indicator GCaMP6m.

The pC1 cluster contained a small number of auditory cells (2-3 cells in the female brain; we found none in the male brain) (Fig. 4C). Since pC1 and pNM2 contain only few auditory-responsive neurons and/or are present only in females, we focused on pC2 as the putative pulse song detector common to both sexes. Although there are more pC2 neurons in males versus females (~67 vs. ~26, (Kimura et al., 2015)) the number of auditory ROIs is similar in both sexes (~15). pC2 neurons can be subdivided into a lateral and a medial type, termed pC2l and pC2m (Robinett et al., 2010), and each type projects to the lateral junction via a distinct process (see Fig. 3A, S5B, C). Most auditory ROIs were lateral in the pC2 cluster (Fig. S5A), and all somata as well as the pC2l process produced strong auditory responses that were highly correlated with those we recorded in the LJ (Fig. 4F, G). From this we conclude that LJ responses reflect the tuning of pC2l neurons. Importantly, the tuning of the pC2l process matches the behavioral tuning (Fig. 4H), indicating that pC2l neurons are pulse song object detectors.

**Optogenetic activation of pC2l neurons drives sex-specific behaviors**

If pC2l neurons serve as pulse song detectors, then their activation should also be sufficient to drive the sex-specific behaviors observed for pulse song – changes in locomotion and singing that are distinct between males and females. To test this hypothesis, we used a driver line (Zhou et al., 2014; Rezával et al., 2016) that labels 11/22 female and 22/36 male pC2l neurons, in addition to 5-6 pCd neurons, but no pC2m or pC1 neurons (Fig. S6A). At least 5 of the pC2l cells in this driver line responded to song (Fig. S6B), which corresponds to ~1/3 of the auditory pC2l neurons. We expressed CsChrimson, a red-shifted channelrhodopsin (Klapoetke et al., 2014), in these neurons and optogenetically activated them in both males and females.

We first recorded behavior in a chamber tiled with microphones (Coen et al., 2014) to test whether pC2 activation was sufficient to induce singing, as we previously showed that pulse
song playback alone drives wing extension in males (Fig. 2F, G). Upon red light activation, males produced pulse song, while sine song was produced transiently after stimulus offset (Fig. 5A, Movie S7), and the amount of pulse song produced scaled with the strength of activation (Fig. 5B). The evoked pulse and sine songs were virtually indistinguishable from natural song (Fig. S6C, D). In Drosophila, retinal (the channelrhodopsin cofactor) must be supplied via feeding, and red light stimulation drove singing significantly more in males fed with retinal versus those fed regular food (Fig. S6E). Activation of a control line that only labels pCd neurons (Zhou et al., 2014) did not drive any singing (Fig. S6E), implying that song production results from the activation of the pC2 neurons in our driver. Importantly, we never observed song production upon pC2 activation in females (Fig. S6E) – pC2 neurons thus drive song in a sex-specific manner. These results also establish pC2 neurons as serving a dual sensory and motor role, analogous to “mirror neurons” in other systems (Mooney, 2014): they respond selectively to the pulse song (Fig. 3C, F) and also bias the song pathway towards producing the same song mode (Fig. 5A, B).

We next evaluated changes in locomotor speed in both sexes for multiple levels of optogenetic activation by placing flies in the FLYTRAP assay but using red light for activation (instead of sound). To account for innate visual responses to the light stimulus, we subtracted the responses of normally fed flies from retinal fed flies (Fig. S6F). Because the behavioral responses to acoustic stimuli can depend on genetic background (Fig. S3), we assessed responses to playback of pulse stimuli with varying IPI for the genotype used in the optogenetic activation experiments (see Methods for list of genotypes). For this particular strain, both males and females decreased their walking speed in response to pulse stimuli. Nonetheless, we observed sex-specific differences in both the magnitude and dynamics of the behavioral responses to sound (Fig. 5D-G), and optogenetic activation of pC2 neurons reproduced many of these sex-specific differences (Fig. 5 H-K). For instance, male responses outlasted the stimulus for both sound and optogenetic activation, while female responses were multiphasic for both conditions. To confirm that the response dynamics were sex-specific, we performed principal component analysis (PCA) on the speed traces of males and females for sound playback and optogenetic activation experiments (Fig. 5L). The first two principal components were sufficient to explain 84% of the variance in the speed traces, and the responses varied along sex-specific axes: the PCA scores for males varied most in the direction of the first principal component and female PCA scores align with the orthogonal, second principal component. In each sex, responses for acoustic and
optogenetic stimulation were co-aligned, suggesting that pC2 neuron activation recapitulates the sex-specific locomotor dynamics observed for acoustic playback in this strain.

Finally, we used the same pC2-specific driver to constitutively suppress the synaptic output of pC2 (via expression of TNT (Sweeney et al., 1995)) in females and paired them with wild type virgin males (see Methods). We quantified female song responses as the correlation between different song features and female speed (Clemens et al., 2015; Coen et al., 2014) (Fig. 5M-O). Because male song is structured via sensory feedback cues from the female (Coen et al., 2014), silencing pC2 neurons in females could affect the content of male song – however, the statistics of male song were unchanged by the female manipulation (Fig. S6G-H). pC2 inactivation specifically affected the correlation between female speed and the pulse song IPI, which changed from ~0 to 0.3 (Fig. 5M-O). While control – and wild type (Clemens et al., 2015) – females do not change their speed relative to the range of natural IPIs produced by conspecific males, females with pC2 neurons silenced accelerate more with increasing IPI. This indicates that pC2 neurons are required for the proper response to pulse song, and that other neurons that represent pulse song are tuned for longer IPIs.

While female locomotor responses to courtship song were affected by pC2 inactivation, copulation rates were not significantly reduced (Fig. S6I), consistent with previous studies (Zhou et al., 2014). In conjunction with the match between behavioral tuning and pC2 tuning, these results add to the evidence that pC2 neurons serve as pulse song object detectors and play a critical role at the sensorimotor interface – they relay information about pulse song to sex-specific downstream circuits that control either singing or locomotion, and thereby mediate acoustic communication behaviors.

**pC2 neural activity is modulated by social experience**

Many sexual behaviors change with social experience (Keleman et al., 2012; Li et al., 2018; Martin et al., 2015; Remedios et al., 2017). This plasticity could be mediated by modulating the selectivity and the gain of object detectors or that of downstream circuits. Social experience is known to affect courtship behavior in Drosophila (Ellis and Kessler, 1975; Kohatsu and Yamamoto, 2015; F. Von Schilcher, 1976), and prolonged exposure to conspecific song sharpens the IPI selectivity of the female mating decision and the male chaining response (Li et al., 2018). We first tested whether locomotor responses in FLYTRAP are also modulated by social experience. We found that males that were exposed to social cues (including the song of other males) via group-housing exhibited stronger and...
more selective changes in speed than individually housed males (Fig. 6A). Females do not
sing to other females when group housed and accordingly, their locomotor responses were
unaffected by housing conditions (Fig. 6A). These sex-specific effects of housing were
reflected in changes in the neuronal responses of pC2 neurons: calcium responses in pC2
(measured via the LJ) (Fig. 4F,G) do not change strongly with housing conditions in females,
but become more selective for IPI in group-housed males (Fig. 6B). Notably, sine song
responses and responses to pulse trains with different durations are not affected by housing
conditions (Fig. S7).

Discussion

Using a quantitative behavioral assay, we characterized locomotor responses in both males
and females to the features that define the Drosophila melanogaster courtship song. Males
and females showed similar tuning for pulse song stimuli, but nonetheless produced distinct
responses (for example, males accelerate while females decelerate; males sing while
females do not) (Fig. 1, 2). Both males and females were responsive to all features of pulse
song, across timescales, and tuning was matched to the distribution of each parameter in
the male's song. We then identified Dsx+ pC2 neurons in the brain that respond selectively
to pulse song stimuli, and whose tuning is matched to behavioral tuning (Fig. 3, 4). The
activation of pC2 neurons elicited sex-specific behavioral responses to pulse song (Fig. 5),
and social experience sharpened both behavioral feature selectivity and pC2 tuning (Fig. 6).
We thus conclude that Dsx+ pC2 neurons are pulse song object detectors that couple song
detection with the execution of sex-specific behaviors.

In other systems, object detectors are typically higher-order neurons that bind the multiple
low-level features that compose an object across spatial and temporal scales (Brincat and
Connor, 2004; Dicarlo et al., 2012): visual object detectors match visual features across
spatial scales (Freiwald and Tsao, 2010; Quiroga, 2012; Tsao et al., 2006) – for instance, a
face is recognized based on individual elements and their spatial relation (Freiwald et al.,
2009). Likewise, acoustic object detectors match acoustic features across temporal scales
(Gentner, 2008; Griffiths and Warren, 2004) – song detection neurons in the bird brain
recognize the bird's own song by both syllable identity and sequence (Doupe and Konishi,
1991; Gentner and Margoliash, 2003; Margoliash, 1983). pC2 neurons also bind different
properties of the pulse song to selectively signal the presence of conspecific pulse song –
pC2 is tuned to several features of pulse song like pulse carrier frequency, pulse duration,
inter-pulse interval, and pulse train duration and a match in only one feature is not sufficient to strongly drive these neurons (Fig. 3, S4A-C). They can therefore be considered acoustic object detectors.

**Matches between behavioral tuning and conspecific song**

Behavioral selectivity for species-specific signals is thought to serve species separation. In FLYTRAP, locomotor tuning of *D. melanogaster* females overlaps with the conspecific song – females slow to conspecific song (Fig. 2A) and do not change their speed or may even accelerate for deviant pulse parameters (Fig. S2E). However, the tuning for any single song feature is not sufficiently narrow to serve as an effective filter for conspecific song. For instance, females also slow for IPIs produced by a sibling species *D. simulans* (50-65 ms) (Bennet-Clark and Ewing, 1969). However, *D. simulans* pulses would be rejected based on a mismatch in other song features – *D. simulans* pulses are too short and of too high frequency to be accepted by females (Clemens et al., 2017; Riabinina et al., 2011).

Selectivity for multiple song features may thus enable species discrimination with relatively broad single-feature tuning (Amézquita et al., 2011). In addition, males and females are exposed to additional non-acoustic cues during courtship that may further sharpen behavioral tuning. For instance, chemical cues prevent males from courting heterospecific females (Fan et al., 2013) and likely also contribute to female rejection (Billeter et al., 2009; Rybak et al., 2002b) – it will be interesting to explore how non-auditory cues (Keleman et al., 2012; Zhang et al., 2016) modulate locomotor responses to song and whether multi-modal integration occurs in pC2 neurons or elsewhere. The absence of non-acoustic cues may also explain the diversity of locomotor responses across strains in the FLYTRAP assay (Fig. S3). Using a naturalistic courtship assay, previous studies show that these same strains exhibit similar behaviors – males pattern their song in response to the female behavior and females change their locomotor speed to the natural courtship song similarly across all strains (Clemens et al., 2017; 2015; Coen et al., 2016; 2014).

In contrast to pulse song responses, the locomotor and singing responses for sine song were less sex-specific (Fig. 2E) and the behavioral tuning did not match well the conspecific song – very low frequencies never produced by males slowed females the most (Fig. 2A, B). This implies divergent roles for the two song modes and is consistent with previous studies (Eberl et al., 1997; F. V. Schilcher, 1976) – for instance sine song does not induce male-male courtship (Yoon et al., 2013). It has been suggested that pulse song may modulate
sine song responses (F. V. Schilcher, 1976) but we did not detect strong serial interactions between the two song modes (Fig. S2G). Alternatively, responses to sine song may depend more strongly on the presence of male chemical cues (Billeter et al., 2009; Kurtovic et al., 2007) that are absent in the FLYTRAP assay. This is consistent with sine song being produced when the male is near the female (Coen et al., 2014) – that is, when these chemical cues are particularly strong.

Pathways for detecting sine and pulse

Our behavioral and neuronal results suggest that pulse and sine song are processed in parallel pathways (Fig. 2E, 3C, F-H) but it is unclear as of yet how and where sounds are split into different streams. Sine and pulse can be separated based on spectral and temporal properties (Fig. S5). In fact, the frequency tuning in auditory receptor neurons (JON) and first-order auditory brain neurons (AMMC) may already be sufficient to separate the lower-frequency sine (150Hz) from the higher-frequency pulse (>220Hz) (Azevedo and Wilson, 2017; Ishikawa et al., 2017; Kamikouchi et al., 2009; Patella and Wilson, 2018; Yorozu et al., 2009). Temporal pattern could further discriminate pulse from sine by either suppressing responses to the sustained sine via adaptation or by tuning temporal integration such that the brief pulse stimuli fail to drive neuronal spiking. A complete mapping of auditory pathways and auditory activity throughout the Drosophila brain is required to identify where and how the neural selectivity for the different song modes arises.

Here, we have identified pC2 as one of the pathways driving responses to pulse song – pC2 tuning matches the behavioral tuning for pulse song (Fig. 3G, H), pC2 activation drives sex-specific responses to song (Fig. 5), and experience-dependent modulation of pC2 tuning matches the behavioral tuning (Fig. 6). But, our data also indicate that it is not the only pathway used to detect pulse song – for example, we observed locomotor responses to pulse song even when pC2 neurons were silenced in females (Fig. 5M). Interestingly, previous studies have implied pC1 as a pulse song detector (Zhou et al., 2015; 2014). Like pC2, pC1 exists in males and females (Rideout et al., 2010), and activation drives several courtship-related behaviors in males – including singing, male-male courtship, or aggression (Koganezawa et al., 2016; Kohatsu et al., 2011; Pan et al., 2012; Philipsborn et al., 2011; Zhou et al., 2015) – and also in females (Li et al., 2018; Rézával et al., 2016; Zhou et al., 2014). All previous studies have relied on imaging activity in the lateral junction (LJ) to show that pC1 preferentially responds to pulse song (Zhou et al., 2015; 2014). However, we show
here that calcium responses of Dsx+ neurons in the LJ reflect the auditory activity of multiple
Dsx+ cell types – and we detected auditory responses in the somata of pC2, pC1 (only in females) and pMN2 (only in females) (Fig. 4). Because the number of auditory neurons
within the pC2 cluster is much larger than for pC1 or pMN2 (Fig. 4C), and because tuning in
pC2 somas matches the tuning in the LJ (Fig. 4E-H), we conclude that the LJ activity largely
reflects pC2 responses. Nonetheless, we have not exhaustively assessed the match
between the neuronal responses of female pC1 and pMN2 neurons and behavior. Those
neurons may also be critical for the female’s response to pulse song, including behaviors not
investigated here (such as oviposition (Kimura et al., 2015)).

Inputs and outputs of pC2 neurons
How the selectivity of pC2 for pulse song arises is as of yet unclear since systematic studies
of tuning for multiple pulse song features in the early auditory pathway are missing.
However, existing evidence suggests that pC2 may acquire its feature selectivity in a serial
manner – via a cumulative sharpening of tuning for song features at successive stages of
auditory processing (Kamikouchi et al., 2009; Yamada et al., 2018; Zhou et al., 2015; 2014).
Auditory receptor neurons display diverse and specific band-pass tuning for carrier
frequency (Ishikawa et al., 2017; Kamikouchi et al., 2009; Patella and Wilson, 2018; Yorozu
et al., 2009) and first order auditory B1 neurons further sharpen frequency tuning via
resonant conductances (Azevedo and Wilson, 2017). Likewise, peripheral responses are
already weakly tuned for IPI (Clemens et al., 2018; Ishikawa et al., 2017) and this tuning is
further sharpened in downstream neurons (Vaughan et al., 2014; Zhou et al., 2015) through
the interplay of excitation and inhibition (Yamada et al., 2018). This serial sharpening is
similar to how selectivity for pulse song arises in crickets, in which a delay-line and
coincidence detector mechanism produces broad selectivity for pulse duration and pulse
pause which is subsequently sharpened in a downstream neuron (Schöneich et al., 2015).
More direct readouts of the membrane voltage of auditory neurons in the fly brain are
required to determine the biophysical mechanisms that generate song selectivity in pC2.

Similarly, the circuits downstream of pC2 neurons that control the diverse and sex-specific
behaviors reported here remain to be identified. pC2 neurons may connect directly with
descending interneurons (DNs) (Cande et al., 2017; Namiki et al., 2017) that control motor
behaviors. For example, pC2 activation in males drives pulse song production, followed by
sine song production at stimulus offset (Fig. 5A). This behavior resembles that caused by
pIP10 activation (Clemens et al., 2017) – pIP10 is a sex-specific descending neuron (Philipsborn et al., 2011), but we don’t yet know if it directly connects with pC2 neurons. The fact that song responses are bi-directional – pulse song can induce both slowing and acceleration within each sex (Fig. 2A, B, S2) – implies that the sex-specificity of motor control is more than a simple re-routing from accelerating DNs in males to slowing DNs in females. Notably, song also promotes copulation, but we did not detect a significant effect of pC2 inactivation on copulation rates (Fig. S6I). This could be because our driver only labeled 1/3 of the auditory pC2 neurons or because pC2 activity does not inform the decision to mate. That is, there may exist parallel pathways that control song responses on different timescales: one pathway that accumulates song information over timescales of minutes (Clemens et al., 2015; Ratcliff et al., 2016) and ultimately controls the mating decision while another, independent pathway controls behavioral responses to song on sub-second timescales, such as dynamic adjustments in locomotion and the production of courtship song.

**Modularity facilitates plasticity of behavioral responses to song**

Our behavioral data strongly suggest that the sex-specificity of behavior arises after feature tuning – that is, the shared object detector – pC2 – determines the magnitude of behavioral responses in both sexes (Fig. 3G, H), and sex-specific aspects, such as the sign and dynamics of locomotor responses or singing in males, are driven by sex-specific circuits downstream of pC2 (Fig. 5). This is reminiscent of how sex-specific behaviors are driven to the male pheromone cVA in flies: shared detector neurons – olfactory receptor neurons and projection neurons in the antennal lobe – detect cVA in both sexes, and this information is then routed to sex-specific higher-order neurons in the lateral horn, which are thought to drive the different behaviors (Datta et al., 2008; Kohl et al., 2013; Ruta et al., 2010). This modular architecture with object detectors being flexibly routed to different behavioral outputs is beneficial if these routes are plastic. For instance, here we show that social experience can shape male responses to song (similar to (Li et al., 2018)), along with tuning at the level of the object detector neurons (Fig. 6). During mating, males transfer a sex peptide to females (Yapici et al., 2008) that alters female behavioral responses to song from slowing to acceleration (Coen et al., 2014) – these effects may be mediated at the level of the motor circuits downstream of pC2, shifting pulse song responses in females to resemble those of males. Modularity also facilitates behavioral plasticity on evolutionary time scales since only one element – the feature detector – needs to change for behavioral tuning in
both sexes to adapt to new songs that evolve during speciation (Capranica et al., 1973; Kostarakos et al., 2009). The identification of pC2 neurons as the pulse object detectors is therefore likely to benefit future studies of the evolution of song recognition.

**pC2 neurons have a dual sensory and motor role**

Strikingly, our results imply a dual sensory and motor role of pC2 neurons: they drive the production of the sensory object they detect (Fig. 3F, G, 5A-C). This dual role may guide social interactions and communication via imitation. In *Drosophila melanogaster*, hearing the song of other males induces a male to court and sing to other females and even males (Eberl et al., 1997; Yoon et al., 2013). This behavior may have originated because the song of another male indicates the presence of a female nearby. pC2 is thus reminiscent of sensorimotor correspondence neurons found in vertebrates (Mooney, 2014; Prather et al., 2008; Rizzolatti and Fogassi, 2014) – neurons that are active during the production as well as the observation of a behavior. Such neurons are hypothesized to play a role in learning (Mooney, 2014) or – as is probably the case in *Drosophila* – imitation and communication between conspecifics (Rizzolatti and Arbib, 1998). However, pC2 differs crucially from these instances of “mirror” neurons in that it directly drives the production of the acoustic object it detects (Fig. 5A-C), while other sensorimotor correspondence neurons are activated by both sensory and motor-like inputs, but do not drive the object production itself. Because we recorded pC2 activity in passively listening males, we do not yet know whether pC2 is activated by sound in an actively singing male. If so, hearing its own song could induce self-stimulation and form a positive feedback loop to maintain courtship behavior by mediating persistent behavioral state-changes (Hoopfer et al., 2016). Alternatively, auditory inputs could be suppressed during singing via a corollary discharge (Poulet and Hedwig, 2003; Schneider et al., 2014), which would allow pC2 to maintain sensitivity to the song of other males to coordinate inter-male competition during singing. Additional studies of pC2 activity in behaving animals are required to fully understand how these pulse song detector neurons integrate into the acoustic communication behavior.

In summary, we show how the circuits that recognize song to drive diverse and sex-specific behavioral responses are organized in *Drosophila*: common detector neurons – pC2 – recognize pulse song in both males and females, and this identically processed information is then routed to drive multiple sex-specific behaviors. Similar principles may underlie the
production of sex-specific behavioral responses to acoustic communication signals in other insects, song birds or mammals.

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Author contributions: JC, DD, and MM designed the study; JC primarily built the acoustic playback assay and collected behavioral data, with contributions from DD, and DD primarily collected anatomical and calcium imaging data, with contributions from JC; ST designed and built the two-photon imaging microscope; JC and DD analyzed data; JC and MM wrote the manuscript, with contributions from DD.
References


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### Methods

#### Flies

The following fly lines were used in our study:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Figures</th>
<th>Source/comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. melanogaster</em> NM91</td>
<td>1, 2, 3G,H,I, 4H, S1, S2, S3A, S6B,C</td>
<td>gift from Peter Andolfatto</td>
</tr>
<tr>
<td>8 <em>D. melanogaster</em> strains CM07, CarM03, N30, NM91, TZ58, ZH23, ZW109, and Canton S (lab stock)</td>
<td>S3B</td>
<td>Canton S is a lab stock, the 7 other strains are a gift from Peter Andolfatto</td>
</tr>
<tr>
<td>UAS-20X-GCaMP6m, UAS-tdTomato; dsx-Gal4 (Chen et al., 2013; Rideout et al., 2010)</td>
<td>3B-I 4B-D, F-H S3A, S4, S5A-C, S6, S7</td>
<td>dsx-Gal4 is a gift from Stephan Goodwin</td>
</tr>
<tr>
<td>UAS-eGFPX2; dsx-Gal4 (Rideout et al., 2010)</td>
<td>3A</td>
<td></td>
</tr>
<tr>
<td>UAS&gt;STOP&gt;CsChrimson.mVenus/LexAop-flp; dsx-LexA, 8LexAop2-flp/R42B01-Gal4 (Klapoetke et al., 2014; Zhou et al., 2015; 2014)</td>
<td>5A-L S6A-E</td>
<td>R42B01-Gal4 and dsx-LexA are gifts from Bruce Baker. Drives expression of csChrimson in pC2 neurons and in a few pCd1/pCd2 neurons</td>
</tr>
<tr>
<td>UAS-GCaMP6m, UAS-TdTom/+; R42B01-Gal4/+ (Chen et al., 2013; Zhou et al., 2015; 2014)</td>
<td>S6A, right</td>
<td>R42B01-Gal4 is a gift from Bruce Baker</td>
</tr>
<tr>
<td>UAS&gt;STOP&gt;csChrimson/LexAop-flp; dsx-LexA, 8LexAop-Flp/R41A01-Gal4 (Klapoetke et al., 2014; Zhou et al., 2014)</td>
<td>S6D</td>
<td>Used to control for pCd1 neurons in the R42B01-Dsx intersection</td>
</tr>
<tr>
<td>UAS&gt;STOP&gt;TNT/LexAop-flp; dsx-LexA/R42B01-Gal4 (Sweeney et al., 1995; Zhou et al., 2015; 2014)</td>
<td>5M-O S6F-H</td>
<td>Inhibit synaptic output of pC2 neurons in females during courtship</td>
</tr>
<tr>
<td>R71G01.AD/UAS-myrrGFP; dsx.DBD/+ (Pan et al., 2012)</td>
<td>4A (pC1)</td>
<td>R71G01.AD is a gift from Gerald Rubin, dsx.DBD is a gift from Stephen Goodwin</td>
</tr>
<tr>
<td>R57G10-flpG5/+; dsx-Gal4/10UAS&gt;STOP&gt;HA, 10UAS&gt;STOP&gt;V5,10UAS&gt;STOP&gt;FLAG (Nern et al., 2015; Rideout et al., 2010)</td>
<td>4A (pMN2), 4E, S5E</td>
<td>Bloomington #64088 crossed with ;;dsx-Gal4</td>
</tr>
<tr>
<td>R57G10-flpI/+; dsx-Gal4/10UAS&gt;STOP&gt;HA, 10UAS&gt;STOP&gt;V5,10UAS&gt;STOP&gt;FLAG (Nern et al., 2015; Rideout et al., 2010)</td>
<td>4A (pC2l), S5D</td>
<td>Bloomington #64087 crossed with ;;dsx-Gal4</td>
</tr>
</tbody>
</table>
**FLyTRAP**

Fly behavior was recorded with PointGrey cameras (FL3-U3-13Y3M-C or FL3-U3-13E4C-C). Grey color frames with a resolution of 1280x960 pixels were acquired at 30 frames per second using custom written software in python and saved as compressed videos. Sound representation was controlled using custom software written in Matlab. The sound stimuli were converted to an analog voltage signal using a National Instruments DAQ card (PCIe-6343). The signal was then amplified by a Samson s-amp headphone amp and used to drive a speaker (HiVi F6 6-1/2” Bass/Midrange). Sound intensity was calibrated as in (Clemens et al., 2015) by converting the voltage of a calibrated microphone (placed where the fly chambers would be during an experiment) to sound intensity and adjusting the sound amplification to match the target intensity. Sound and video where synchronized by placing into the camera’s field-of-view a 650nm LED whose brightness was controlled using a copy of the sound signal. The chamber consisted of an array of 12 small arenas (7 by 46 mm, made from red plastic) that was placed in front of the loudspeaker (Movie S1). The arena floor consisted of plastic mesh to let sound into the chamber and the top was covered with a thin, translucent plastic sheet. Flies were illuminated using a white LED back light from below and a desk lamp from above.

**Playback experiments**

Virgin male and female flies were isolated within 6 hours of eclosion and aged for 3-7 days prior to the experiments. Flies were raised at low density on a 12:12 dark:light cycle, at 25°C and 60% humidity. Flies were introduced gently into the chamber using an aspirator. Recordings were performed at 25°C and timed to start within 60 minutes of the incubator lights switching on to catch the morning activity peak. Stimulus playback was block-randomized to ensure that all stimuli within a set occur at the same overall rate throughout the stimulus. The stimulus set (e.g. five pulse trains with different IPIs, see Supplemental Table 1 for a list of all stimulus sets) was repeated for the duration of the experiment (2 hours). Stimuli were interleaved by 60 seconds of silence to reduce crosstalk between responses to subsequent stimulus presentations.

**Stimulus design**

Sound was generated at a sampling frequency of 10 kHz using custom Matlab scripts. Sine song stimuli were created as pure tones of the specified frequency and intensity (typically 5mm/s). Pulse song was generated by arranging Gabor wavelets in trains interleaved by a specified pause. The Gabor wavelets were built by modulating the amplitude of a short sinusoidal using a Gaussian: $\exp(-t^2/(2\sigma^2)) \sin(2\pi f * t + \phi)$, where $f$ is the pulse carrier frequency, $\phi$ is the phase of carrier, and $\sigma$ is proportional to the pulse duration. The parameters for all stimuli used along with the behavioral responses obtained in FLyTRAP are listed in Supplemental Table 1.

**Analysis of FLyTRAP data**

Fly positions where tracked using custom-written software. Briefly, the image background was estimated as the median of 500 frames spaced to cover the full video. Foreground pixels (corresponding to the fly body) were identified by thresholding the absolute values of the
difference between each frame and the background estimate. The fly center position was then taken as the median of the position of all foreground pixels in each chamber. The sequence of fly positions across video frames was then converted into a time series using the light onset frames of the synchronization LED (indicating sound onset) as a reference. From the position time series fly speed was calculated and the speed traces where then aligned to stimulus onset for each trial. Base line speed was calculated as the average of the speed over an interval starting 30 seconds and ending 2 seconds before stimulus onset. Test speed was calculated over an interval starting at stimulus onset and ending 2 seconds after stimulus offset. Tuning curves were calculated as the difference between baseline speed and test speed for each trial, averaged over trials for each stimulus and animal. Speed traces were obtained by subtracting the baseline speed from the trace for each trial and averaging over trials for each stimulus and animal. All data (tuning curves, speed traces) are presented as mean +/- s.e.m. over flies.

**Manual scoring of wing extension in FLyTRAP**

To evaluate the number of flies that extend their wings upon playback of pulse or sine song, we manually scored wing extension in the videos using the VirtualDub software. For pulse song (see Movie S2), we scored 25 stimuli/fly, choosing trials randomly but ensuring that each IPI (16/36/56/76/96 ms) was scored 5 times/fly. To avoid bias, the scorer was blind to the IPI presented to the fly in each trial. A total of 120 male flies and 36 female flies were scored (3000 and 900 single-fly responses total for pulse song). We scored wing extension only when the wing was extended in the first 1/3 second following stimulus onset, and only when the wings were not extended during the 1 second before stimulus onset. For sine song (150Hz carrier frequency), 60 males fly were scored.

**Joint tuning for pulse duration and pulse pause**

To visualize locomotor (Fig. S2E, F) and calcium (Fig. S4D,E) responses to pulse trains with different combinations of pulse duration and pulse pause we generated smooth surface plots using Matlab’s “scatteredInterpolant” function with the interpolation mode set to “natural”. The boundaries of the plots were set as follows: Pulse duration of zero corresponds to silence and the speed values were set to 0 since all speed traces are always base line subtracted. A pulse pause of zero corresponds to a continuous oscillation and we set the corresponding speed values to those obtained for a 4 second pure tone with a frequency of 250 Hz.

**Measurement of song features from natural song data**

The inter-pulse interval (IPI) is given by the interval between the peaks of subsequent pulses in a pulse train. Pulse trains correspond to continuous sequences of pulses with IPIs smaller than 200ms. Measuring the pulse durations from natural song data is non-trivial since pulses vary in their shape and can be embedded in background noise. We quantified pulse duration by 1) calculating the envelope of each pulse using the Hilbert transform, 2) smoothing that envelope using a Gaussian window with a standard deviation of 2 ms, and 3) taking as the pulse duration the full width of the smoothed envelope at 20% of the maximum amplitude of the pulse. Pulse durations for artificial stimuli used in our pulse train were defined to be consistent with this method. Pulse carrier frequency is given by the center of mass of the amplitude spectrum of
each pulse (Clemens et al., 2017). Sine carrier frequency was calculated as the peak frequency of the power spectrum of individual sine tones.

**PCA of speed traces**
For the PCA of sex-specific responses to sound and optogenetic activation of pC2 (Fig. 5L) we collected male and female speed traces for all IPIs (Fig. 5D, F) and optogenetic activation levels (Fig. 5H, J) into a large matrix. Each speed trace was cut to include only the 12 seconds after sound onset and then normalized to have zero mean and unit variance. The first two principal components explain 84% of the total data variance.

**Optogenetic experiments**
CsChrimson was expressed in pC2 neurons using the intersection between R42B01-Gal4 and dsx-LexA. 655nm light was emitted from a ring of 6 Tri-Star LEDs (LuxeonStar, SinkPAD-II 20mm Tri-Star Base) in FLyTRAP (Fig. 5D-K). Flies were fed with food that contained all-trans retinal for a minimum of three days post eclosion. Control were raised on regular fly food after eclosion. The LED was on for four seconds with 60 seconds pause between stimuli, similar to the temporal pattern used for auditory stimulus delivery in FLyTRAP (1-5mW/cm², 100Hz, duty cycle 0.5). To measure the amount of song driven by pC2 activation in solitary flies, we used a chamber whose floor was tiled with 16 microphones to allow recording of the song (Fig. 5A-C, Movie S7; see (Clemens et al., 2017)). The LED (627nm LEDs, LuxeonStar) was on for four seconds (frequency 25 Hz, duty cycle 0.5) and off for 60 seconds. We tested three different light intensities (1.8, 9, and 13 mW/cm²) that were presented in 3 blocks of 18 trials. The order of the three blocks (light intensities) was randomized for each fly. Fly song was segmented as described previously (Arthur et al., 2013; Coen et al., 2014).

**pC2 inactivation in females during courtship**
Tetanus neurotoxin light chain (TNT) (Sweeney et al., 1995). was used to block synaptic transmission in pC2 neurons in females. 3-7 days old virgin males (wild type, NM91) and females (pC2-TNT: UAS>STOP>TNT/LexAop-flp; dsx-LexA/R42B01-Gal4, pC2-control: +/-LexAop-flp; dsx-LexA/R42B01-Gal4) were paired in a custom-built chamber, designed to record fly song (~25 mm diameter, tiled with 16 microphones). Flies were allowed to interact for 30 minutes, and the percent of flies copulated as a function of time was scored (Fig. S6H). A monochrome camera (Point Grey, FL3-U3-13Y3M) was used to record the fly behavior at 60 frames per second. Fly position was tracked offline and song was segmented as previously described (Arthur et al., 2013; Coen et al., 2014). We then calculated song statistics (e.g. amount of song or number of pulses per window) and female locomotion (average female speed) in windows of 60s with 30s overlap (Clemens et al., 2015). For the rank correlations between male song features and female speed (Fig. 5M-O), we binned the female speed values into 16 bins with the bin edges chosen such that each bin was populated by an equal amount of samples (see Fig. 5M) and calculated the rank correlation between the binned female speed and the average male song feature per bin. Changes in correlation between control and experimental flies (Fig. 5O) were analyzed using an ANCOVA model with independent slopes.
and intercepts. Significance was determined based on the p-value of the interaction term (model's genotype by song-feature) after Bonferroni correction.

**Calcium imaging**

Imaging experiments were performed on a custom built two-photon laser scanning microscope equipped with 5mm galvanometer mirrors (Cambridge Technology), an electro-optic modulator (M350-80LA-02 KD*P, Conoptics) to control the laser intensity, a piezoelectric focusing device (P-725, Physik Instrumente), a Chameleon Ultra II Ti:Sapphire laser (Coherent) and a water immersion objective (Olympus XLPlan 25X, NA=1.05). The fluorescence signal collected by the objective was reflected by a dichroic mirror (FF685 Dio2, Semrock), filtered using a multiphoton short-pass emission filter (FF01-680/sp-25, Semrock), split by a dichroic mirror (FF555 Dio3, Semrock) into two channels, green (FF02-525/40-25, Semrock) and red (FF01-593/40-25, Semrock), and detected by GaAsP photo-multiplier tubes (H10770PA-40, Hamamatsu). Laser power (measured at the sample plane) was restricted to 15 mW. The microscope was controlled in Matlab using ScanImage 5.1 (Vidrio). Single plane calcium signals (Fig. 3C-I, 4F,G and pMN2 neuron in Fig 4C-E) were scanned at 8.5 Hz (256X256 pixels). Pixel size was ~0.5μmX0.5μm when imaging the lateral junction or pC2I process and ~0.25μmX0.25μm when imaging cell bodies in a single plan (Fig 4G and pMN2 in Fig. 4B-D). For volumetric scanning of cell bodies (Figs 4B-D, S5A), volumes were acquired at 0.5Hz (256*216, 20 planes, voxel size ~ 0.34μm X 0.4μm X 1.5μm), scanning one group of cells at a time (pC1, pC2, pCd).

After surgery (opening of the head capsule to reveal the brain), flies were placed beneath the objective and perfusion saline was continuously delivered directly to the meniscus. Sound playback was controlled using custom written Matlab software (Clemens et al., 2018). The software also stopped and started the calcium imaging via a TTL pulse sent to ScanImage (“external hardware trigger” mode). The sound stimulus was generated at a sampling rate of 10kHz and sent through an amplifier (Crown, D-75A) to a set of head phones (Koss, ‘The Plug’). A single ear plug was connected to one side of a plastic tube (outer-inner diameters 1/8”-1/16”) and the other tube tip was positioned 2 mm away from the fly arista. Sound intensity was calibrated by measuring the sound intensity 2 mm away from the tube tip with a pre-calibrated microphone at a range of frequencies (100Hz-800Hz) and the output signal was corrected according to the measured intensities. The pause between stimulus representation was 25 seconds. A stimulus set (26-36 stimuli) was presented to each fly for 3 times in block-randomized order as in the playback experiments. If the response decayed in the middle of a repetition (possibly because of drift in the z-axis), the whole repetition was discarded from the analysis. Typically, two full repetitions per fly were used for analysis.

Regions of interest (ROIs) for calcium response measurements (in the LJ, pC2 process and in single Dsx+ somata) were selected manually based on a z-projection of the tdTomato channel. ∆F/F of the GCaMP signal was calculated as (F(t)-F₀)/ F₀, where F₀ is the mean fluorescence in the ROI in the 10 seconds preceding stimulus onset. Integral ∆F/F (Fig. 3D, F-I) and peak ∆F/F
(Fig. 3F, inset) values were calculated in a window starting at sound stimulus onset and ending 25 seconds after sound stimulus offset. To compensate for differences in overall responsiveness across flies, we normalized ΔF/F values of each fly by dividing the integral or peak ΔF/F by the maximal value (of integral or peak ΔF/F) across all stimuli for that fly. For volumetric scanning (Fig. 4), each time series was first motion corrected using the rigid motion correction algorithm NoRMCorre (Pnevmatikakis and Giovannucci, 2017) using the tdTomato signal as the reference image.

**Light microscopy**

Flies expressing GFP in Dsx+ neurons (UAS-eGFP2X; dsx-Gal4; Fig. 3A) and flies expressing CsChrimson.mVenus in pC2 neurons (R42B01-Gal4 intersected with dsx-LexA; Fig. S6) were immunostained and scanned in a confocal microscope. 2-4 day old flies were cold-anesthetized on ice, dissected in cold S2 insect medium (Sigma Aldrich, #S0146) and fixed for 30-40 minutes on a rotator at room temperature in 4% PFA in 0.3% PBTS (0.3% Triton in PBSX1), followed by 4x15 minutes washes in 0.3% PBTS and 30 minutes in blocking solution (5% normal goat serum in 0.3%PBTS). Brains were incubated over two nights at 4°C with primary antibody, washed with 0.3%PBT and incubated for two more nights at 4°C in secondary antibody, followed by washing (4x15 minutes in 0.3%PBTS and 4x20 minutes in PBS), and mounting with Vestashield for 2-7 days before imaging. Antibodies were diluted in blocking solution at the following concentrations: rabbit anti-GFP (Invitrogen #1828014; used against GFP and mVenus) 1:1000, mouse anti-Bruchpilot (nc82, DSHB AB2314866) 1:20, goat anti-rabbit Alexa Flour 488 (Invitrogen #1853312) 1:200, goat anti-mouse Alexa Flour 633 (Invitrogen #1906490) 1:200.

Stochastic labeling of Dsx+ neurons in the female brain (Fig 4A, E) was done using multi-color-flip-out (MCFO, (Nern et al., 2015)) with three different epitope tags (HA,V5,FLAG). We followed the JFRC FlyLight Protocol ‘IHC-MCFO’ ([https://www.janelia.org/project-team/flylight/protocols](https://www.janelia.org/project-team/flylight/protocols)) for the preparation of brains. Flp was induced using R5710C10 promotor-coding sequence fusions of the flpG5 and flpl. Flies were 4-7 days old when dissected. Flies were stored at 25°C. Confocal stacks were acquired with a white light laser confocal microscope (Leica TCS SP8 X) and a Leica objective (HC PL APO 20x/0.75 CS2). A high-resolution scan of a pC2 cell (Fig 4E) was performed with an oil immersion Leica objective (HC PL APO 63x/1.40 Oil CS2, fig 4E). Images were registered to the Janelia brain template (JFRC2) (Jenett et al., 2012) using vfbaligner ([http://vfbaligner.inf.ed.ac.uk](http://vfbaligner.inf.ed.ac.uk)), which internally uses CMTK for registration (Rohlfing and Maurer, 2003). The images of the fly brain in Figs 4A and S5D were deposited by G. Jefferis (Jefferis, 2014). Image processing was performed in FIJI (Schindelin et al., 2012).
References


Figure 1

A

B

C

D

inter-pulse interval (IPI)

pulse pause

pulse duration

1 / pulse carrier frequency

1 / sine carrier frequency

inter-pulse interval [ms]

10ms

500ms

song bout duration

sine tone duration

pulse train duration

male song probability

female behavior speed [mm/s]

male behavior speed [mm/s]

female behavior Δ speed [mm/s]

male behavior Δ speed [mm/s]

inter-pulse interval [ms]

stimulus

10ms

1s

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Figure 1 – FLyTRAP assay for comparing locomotor tuning for courtship song stimuli in males and females.

A *Drosophila melanogaster* produces song in bouts that can consist of two modes: Sine song corresponds to a weakly amplitude modulated oscillation with a species-specific carrier frequency (~150Hz) and pulse song corresponds to trains of Gabor-like wavelets each with a carrier frequency between 220 and 450Hz and a duration between 6 and 12 ms. These pulses are produced at an inter-pulse interval (IPI) of 30-45 ms.

B FLYTRAP consists of behavioral chamber that is placed in front of a speaker through which sound is presented. Fly movement is tracked using a camera. Shown is a single video frame of females in the assay with fly tracks for the preceding 20 seconds overlaid in magenta. See Movie S1.

C Locomotor responses of females (magenta) and males (grey) for pulse trains with different IPIs (see legend). The gray shaded box indicates the duration of the sound stimulus. Red traces at the bottom of the plot show short snippets of the 5 IPI stimuli presented in this experiment. Baseline speed was subtracted before trial averaging.

D Speed tuning curves for different IPIs in females (magenta) and males (grey) are obtained by averaging the speed traces in the six seconds following stimulus onset. The histograms at bottom shows the IPI distribution found in male song (data from 47 males of NM91 wild type strain totaling 82643 pulses). Lines and shaded areas or error bars in C and D correspond to the mean ± s.e.m. across 112 male and 112 female flies.

See also Figure S1 and Movie S1.
Figure 2 – Responses to song playback are sex-specific and tuned for multiple features of pulse and sine song.

A, B Locomotor tuning curves for females (A, magenta) and males (B, grey) for 6 different features of pulse and sine song. Lines and error bars correspond to the mean±s.e.m. across flies (see Table S1 for a description of all stimuli and N flies).

C Distribution of the six different song features tested in A, B in the natural courtship song of Drosophila melanogaster males (data from 47 males of NM91 wild type strain totaling 82643 pulses and 51 minutes of sine song from 5269 song bouts). Histograms are normalized to a maximum of 1.0.

D Pictograms (not to scale) illustrating each song feature examined in A-C. Pulse and sine song features are marked red and blue, respectively.

E Changes in speed for males and females for all pulse (red) and sine (blue) stimuli tested (data from A, B, S2, see Table S1). Responses to sine stimuli are strongly and positively correlated between sexes (r=0.86, p=4x10^{-7}). Pulse responses are also strongly but negatively correlated (r=-0.79, p=7x10^{-18}). Blue and red lines correspond to linear fits to the responses to sine and pulse song, respectively.

F Fraction of trials for which male and female flies extended their wings during the playback of pulse song (five different IPIs as in 1C, D) and sine song (150Hz, quantified only for males). Solitary males (grey) frequently extend their wings in response to pulse but not to sine song. Solitary females (magenta) do not extend wings for pulse song. See also Movie S2.

G Fraction of trials that evoke wing extension in males (grey) and females (magenta) as a function of IPI. In males, wing extension and locomotor behavior (Figure 1D) exhibit strikingly similar tuning with a peak at the conspecific IPI. Females almost never extend their wing for any IPI.

See also Figure S2 and Movie S2.
Figure 3

A

B

C

D

E

F

G

H

inter-pulse interval [ms]

D. male norm. integral $\Delta F/F$ (LJ) vs. female norm. integral $\Delta F/F$ (LJ)

G. male behavior speed [mm/s] vs. male norm. integral $\Delta F/F$ (LJ)

H. female behavior speed [mm/s] vs. female norm. integral $\Delta F/F$ (LJ)
**Figure 3: Neuronal tuning in the LJ matches behavioral tuning for pulse stimuli in males and females.**

**A** Anatomy of Dsx+ neurons in the female brain. Max z-projection of a confocal stack of a fly brain in which all Dsx+ are labeled with GFP. 5/8 cell types (pC1, pC2l, pC2m, blue, pMN1, pMN2) project to the lateral junction (LJ), while 3 cell types (pCd1, pCd2, aDN (movie S11)) do not. Yellow and blue arrows point to the processes that connect pC2l and pC2m to the LJ. See also Fig. S5B,C.

**B** Grayscale image (see color bar) of calcium responses (ΔF/F) to a pulse train (IPI 36ms) in an ROI centered around the LJ (red) and the pC2l process (yellow) in a female. Shown are snapshots of the recording at three different time points relative to stimulus onset - before (T=10s), during (T=1.2s), and after (T=20s) the stimulus. Flies express GCaMP6m in all Dsx+ cells. Conspecific-like pulse elicits strong increases in fluorescence in the LJ and the pC2 process.

**C** LJ responses to sine (blue) and pulses (red) stimuli in females (left) and males (right). Individual dots correspond to integral ΔF/F responses for individual stimuli averaged over the 3-12 individuals tested for each stimulus. Many pulse stimuli evoke much stronger responses than the most effective sine stimulus (p=8x10^{-11} for females and p=2x10^{-11} for males, two-sided rank sum comparison of sine and pulse responses).

**D** Comparison of male and female LJ responses to sine (blue) and pulse (red) stimuli. Responses to both song modes are correlated strongly for pulse (r^2=0.74, p=3x10^{-15}) and weakly for sine (r^2=0.31, p=0.002) stimuli. Individual dots correspond to the integral ΔF/F for individual stimuli averaged across animals. Before averaging, the responses of each animal were normalized to compensate for inter-individual differences in calcium levels (see methods for details).

**E** Fluorescence traces from the LJ in females (top, magenta) and males (bottom, grey) for pulse trains with three different IPIs (see legend, average over 6 individuals for each sex). In both sexes, the LJ responds most strongly to the conspecific IPI of 36ms (Fig. 1D). Responses are much weaker for shorter (16ms) and longer (76ms) IPIs. Calcium responses in the LJ are smaller in males than in females (compare C). See also Supp. Movie S3, S4.

**F** Tuning curves of calcium responses in the female (magenta) and the male (gray) LJ for features of pulse and sine song (compare to behavioral tuning in Fig. 2A, B). Lines and error bars correspond to the mean±s.e.m. across flies. Integral ΔF/F normalized as in D. Insets show peak ΔF/F tuning curves for pulse train duration. In contrast to the integral ΔF/F, the peak ΔF/F saturates for long pulse trains, indicating that the LJ stops accumulating calcium for pulse trains not normally produced by males. Apart from this difference, integral ΔF/F and peak ΔF/F are similar (see Fig. S4F, G).

**G, H** Comparison of behavioral and neuronal tuning in males (G) and females (H). Dots correspond to the average normalized integral ΔF/F over individuals, lines indicate linear fits. In males (G), behavioral and neuronal responses are strongly and positively correlated for pulse (red, r=0.55, p=9x10^{-5}) but not for sine stimuli (blue, r=-0.15, p=0.06). In females (H), behavioral and neuronal responses are strongly and negatively correlated for pulse (red, r=-0.62, p=2x10^{-7}) but not for sine stimuli (blue, r=0.27, p=0.30). All ΔF/F values are from flies expressing GCamp6m under the control of Dsx-Gal4. See also Figure S3 and S4, and Movie S3 and S4.
Figure 4

A

B

C

D

E

F

G

H

fraction of ROIs responding

female

male

somatic

F/F (log scale)

pulse

sine

norm.

Δ F/F in the lateral junction

norm. Δ F/F in pC2l process

nom. Δ F/F in pC2l somata

female speed [mm/s]

somatic

pulse

sine

norm. Δ F/F in pC2l process

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**Figure 4 - pC2 neurons are pulse object detectors common to both sexes.**

A Individual Dsx+ neuron types (black) with somas in the female central brain in which we detected calcium responses for pulse or sine song, registered to a common template brain (gray) (see Methods for details). Of the 8 Dsx+ cell types in the central brain, pC2l, pC2m, the single female-only neuron pMN2 and a small number of pC1 neurons (and only in some individuals) respond to courtship sounds. The lateral junction (LJ) is marked in magenta and somata are marked with golden arrow heads. See also Supp. Movie S10, S11.

B Example somatic fluorescence traces from single somata of the pC1, pC2, and pMN2 cells in response to pulse trains (IPI=36ms, single trial responses). Fluorescence trace from the LJ (magenta) shown for comparison. The gray box marks the duration of the sound stimulus. In each panel. Horizontal and vertical scale bars correspond to 6 seconds and 0.25 ΔF/F, respectively. Horizontal black line marks ΔF/F=0.

C Fraction of cells in Dsx+ clusters with detectable somatic calcium responses to pulse or sine song (females, light grey dots; males, dark grey squares). Complete clusters were imaged using volumetric scan for pC1, pC2 and single plane scans for pMN2. We did not distinguish between pC2l/m, since in most flies both groups are spatially intermingled at the level of cell bodies. Note that all flies included showed calcium responses to sound in the LJ, even when we did not detect responses in specific somata.

D Peak somatic ΔF/F for pulse (red) and sine (stimuli). Lines connect responses to pulse (36ms IPI) and sine (150Hz) recorded in the same animal. Note that responses are plotted on a log scale – the average of the ratio between sine and pulse for all cells is ~2.6. 36/38 pC2, 4/5 pC1 and 2/2 pMN2 prefer pulse over sine. See also Supp. Movie S5, S6.

E High resolution confocal scan of a single pC2l neuron (obtained via a stochastic labelling technique, see Methods for details). Only the side ipsilateral to the cell body is shown. The neurites in the lateral junction appear varicose, indicating that they contain pre-synaptic sites.

F Normalized integral ΔF/F values recorded simultaneously in the LJ and the process that connects the LJ with the somata of pC2l (and no other Dsx+ cell type) are highly correlated (r²=0.99, p=6x10⁻³⁹). Each point corresponds to an individual stimulus (pulse or sine) averaged over flies (N=10-24 flies per stimulus). The high correlation means that calcium responses in the LJ reflect responses in pC2l neurons.

G Normalized ΔF/F recorded first in the LJ and then in single pC2l somata in the same fly are highly correlated (r²=0.81, p=2x10⁻¹⁴, N=8 flies per stimulus), demonstrating that calcium responses in the LJ represent the responses of individual pC2l cells, with some variability across individual cells and animals.

H Comparison of calcium responses pC2l process and female speed for the same stimuli. pC2l and behavioral responses are highly correlated for pulse but not for sine stimuli (pulse: r=-0.74, p=3x10⁻⁷, sine: r=0.44, p=0.46), just as for the LJ (compare Fig. 3H). The match between neuronal and behavioral tuning for pulse song indicates that pC2l neurons are pulse song detectors. Each point corresponds to an individual stimulus (Δspeed: N~120 flies per stimulus, ΔF/F: N=10-24 flies per stimulus).

All ΔF/F values correspond to the integral fluorescence and are from flies expressing GCamp6m under the control of Dsx-Gal4. See also Figure S5 and Movie S5 and S6.
Figure 5

(A) Figure showing a sine pulse with a duration of 500 ms.

(B) Graph showing the relationship between LED intensity and speed.

(C) Graph showing the change in rank correlation between male song and female speed.

(D) Graph showing the effect of sound playback on female speed.

(E) Graph showing the change in speed in response to inter-pulse interval.

(F) Graph showing the change in speed in response to sound

(G) Graph showing the change in speed in response to inter-pulse interval.

(H) Graph showing the effect of activating pC2l on female speed.

(I) Graph showing the change in speed in response to LED intensity.

(J) Graph showing the change in speed in response to LED intensity.

(K) Graph showing the change in speed in response to LED intensity.

(L) Scatter plot showing the relationship between first and second principal component scores.

(M) Graph showing the effect of inactivating pC2l on female speed.

(N) Table showing rank correlation between male song and female speed.

(O) Bar chart showing the change in rank correlation.

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Figure 5 – pC2 activation generates sex-specific behaviors.
A Song evoked in males by optogenetic activation (627nm LEDs, intensity 13 mW/cm²) of a driver line that labels pC2l and pCd neurons (R42B01ΔDsx). Top trace shows a song recording marking pulse and sine song in red and blue, respectively. The grey area indicates the duration (4 seconds) of optogenetic activation. Pulse song is evoked during activation while sine song occurs immediately following activation. Bottom plots show pulse rate (red) and sine song probability (blue) averaged over 7 flies (18 stimulation epochs per animal). See also Movie S7.
B, C Average pulse rate (B) and sine song probability (C) evoked in the 6 seconds following LED light onset (LED duration is 4 seconds). Dose-response curves for individuals are shown as thin lines, population averages (mean±s.e.m.) are shown as thick lines with error bars. P-values result from two-sided sign tests and are adjusted for multiple comparisons using Bonferroni’s method. Same data as in A. See also Supp. Movie S7, S8.
D-G Changes in speed in FLYTRAP for pulse trains (duration indicated by grey boxes) with different IPIs (see legend) in the R42B01ΔDsx strain. Female behavioral responses (magenta) were weak with multi-phasic dynamics (D, E). Males (grey) slowed for all pulse stimuli tested and responses outlast the stimulus duration (F, G). Data represent averages over 112 female and 113 male flies.
H-K Optogenetic activation of R42B01ΔDsx using csChrimson evokes locomotor responses with sex-specific dynamics. Changes in speed (grey box) and tuning curves were corrected for intrinsic light responses by subtracting the responses of control flies with the same genotype that were not fed retinal (see Fig. S6E). Females slow for weak and speed for strong activation with multi-phasic dynamics as for sound (H, I, compare D). Males decrease their speed and responses outlast the optogenetic stimulus as for sound (J, K, compare F). See also Supp. Movie S9. See S6E for N flies. The x scales of panels I, K are inverted to match the tuning for sound since the direction of the mapping between LED intensity and IPI is arbitrary.
L Principal component (PC) analysis of male and female locomotor speed traces (12s following stimulus LED or sound onset, traces taken from D, F, H, J). Shown are first and second principal component (PC) scores of females (magenta) and males (grey) for sound (squares) and optogenetic stimulation (circles). Female responses to different LED intensities and to different IPIs spread along the second PC while male responses to sound and optogenetic stimulation largely spread along the first PC.
M Locomotor tuning for IPI during natural courtship obtained from single females that were courted by a wild-type NM91 male. pC2l synaptic output in the females was inhibited using TNT using the R42B01ΔDsx driver. Lines and error bars correspond to the mean±s.e.m speed of N females per genotype tested (pC2l TNT – orange, pC2l control – blue, N=48 females for each genotype, see methods for details on how the tuning curves were computed). pC2l control females (blue) do not change their speed with IPI within the range commonly produced by males (rank correlation 0.02, p=0.59, compare Fig. 1D). pC2l TNT females (orange) accelerate for longer IPIs (rank correlation 0.31, p=3x10⁻³⁰). C
N Rank correlation between female speed and different song features during natural courtship (pC2l control – blue, pC2l TNT – orange).
O Difference between the rank correlations for control (blue) and pC2l TNT (orange) flies in N.
pC2l inactivation specifically changes the correlation between female speed and IPI (dark gray, p=6x10^{-8}). All other changes in correlation are much smaller and not significant (p>0.18). P-values were obtained by fitting an ANCOVA model (see methods for details) and were corrected for multiple comparisons using the Bonferroni method.

See also Figure S6 and Movie S7.
Figure 6

A

\[ \Delta \text{speed [mm/s]} \]

B

\[ \text{normalized peak } \Delta \text{F/F Lj} \]

C

\[ \Delta \text{F/F 36/56 (log scale)} \]

p=0.93

p=0.06

female

male
Figure 6 – Locomotor and pC2 responses are similarly modulated by social experience.

A Changes in speed for pulse trains measured using FLyTRAP with different IPIs in single-housed (solid line) or group-housed (dashed lines) female (left, magenta) and male flies (right, grey). Plots show mean ± s.e.m. across 112 group-housed and 119 single-housed female or male flies. Female IPI tuning is not strongly affected by housing conditions. By contrast, males change their speed more strongly and more selectively when group-housed.

B Calcium responses from the LJ for pulse trains with different IPIs in single-housed (solid line) or group-housed (dashed lines) female (left, magenta) and male flies (right, grey). Plots show mean ± s.e.m. across 5-6 female or male flies in each condition. In females, group housing only weakly suppresses LJ responses for some IPIs. By contrast, male LJ responses are selectively suppressed for long IPIs, which sharpens the IPI tuning.

C Ratio of Calcium responses to 36 and 56 ms IPIs in single-housed or group-housed female (left, magenta) and male flies (right, grey). Individual dots correspond to individual flies, the solid lines connect the population average ratios. P-values were obtained from a two-sided rank sum test.

All ΔF/F values correspond to the integral fluorescence and are from flies expressing GCamp6m under the control of Dsx-Gal4.

See also Figure S7.
Figure S1. Related to Figure 1.

A Locomotor responses of females for pulse trains (36ms IPI stimulus) and sine tones (100Hz tones) of different intensities (color coded, see legend). Intensity is given in mm/s since flies are sensitive to the particle velocity of sound, not sound pressure.

B Speed tuning curves for the traces in A obtained by averaging the speed in the six seconds following stimulus onset. For pulse song, responses are weak for intensities <1mm/s and don’t change much beyond that. While sine responses are weak, there is a tendency for speed to change more for louder sine tones. Lines and error bars indicate mean±s.e.m. over ~120 flies.

C Excluding the transient response component only negligibly affects behavioral tuning curves. Shown are IPI tuning curves for males (gray) and females (magenta) obtained by averaging different epochs of the speed traces. The full response (solid lines) corresponds to the average, base-line subtracted speed in the 6 seconds following sound onset. For the sustained response (dashed lines), we start integration of the speed traces not at sound onset but 500ms into the sound. Tuning curves for the full and sustained phases are very similar – the negative transient response component adds only a weak negative bias to the tuning curves.

D Full vs. sustained responses for all stimuli tested in males (gray) and females (magenta). Both measures yield highly correlated responses ($r^2=1.00$, $p=2x10^{-284}$). The purely negative transient response component in the full responses adds a negligible negative bias of -0.05mm/s.
Figure S2

A. Female and male behavior as a function of pulse carrier frequency, pulse duration, inter-pulse interval, and pulse train duration.

B. Female and male behavior as a function of sine carrier frequency, sine tone duration, number of pulses, and stimulus.

C. Stimulus examples showing different pulse durations and pulse pauses.

D. Tuning curves for pulse duration, pulse pause, and pulse period (IPI) for both female and male behavior.

E. Heatmap showing female behavior responses to pulse duration and pulse pause tuning.

F. Heatmap showing male behavior responses to pulse duration and pulse pause tuning.

G. Compound responses showing sine pulse and linear prediction for both female and male behavior.
Figure S2. Related to Figure 2.

A, B Locomotor response traces for all stimuli in Fig. 2A, B for females (A) and males (B). Stimulus values are color coded (see legends). Gray shaded areas mark the duration of the sound stimulus. Vertical black lines indicate sound onset for stimulus sets with varying durations (pulse train duration, sine tone duration). Lines correspond to the mean over typically ~120 animals (see supp. table 1 for exact values). Error bars are similar to those in 1C and omitted for clarity.

C Pictograms (not to scale) illustrating each song feature examined in A-B. Pulse and sine song features are marked red and blue, respectively.

D Three principal types of tuning are observable when testing behavioral responses for stimuli with different pulse durations and pauses. Black ellipses indicate the range of stimulus parameters that evoke strong behavioral responses, dots correspond to the three (gray) stimuli shown below each tuning field. Horizontal, vertical, and anti-diagonal lines mark stimuli with constant pause, duration and period, respectively. Pulse duration tuning (left) corresponds to high selectivity (narrow tuning) for pulse duration and higher tolerance for pulse pauses. Pause duration tuning (middle), corresponds to high selectivity for pulse pause and high tolerance for pulse pause. Note that for both types, the tuning for pulse duration and pulse pause does not interact – e.g. the preferred pause does not change with pulse duration. Pulse period (a.k.a. inter-pulse interval) tuning (right) corresponds to a preference for stimuli with a constant pulse period, given by the sum of pulse duration and pause. For this type of tuning, pulse duration and pulse pause interact – the preferred pulse pause increases with decreasing pulse duration.

E, F Locomotor responses of females (E) and males (F) for pulse trains with different pulse durations and pulse pauses. Speed values are color coded (see color bar in F). Black dots mark the parameter combinations of the stimuli tested in FLYTRAP. Intermediate values were obtained using interpolation (see methods). Male and female response fields are similar except for the sign – were females tend to slow (blue colors), males tend to accelerate (red colors), and vice versa (compare Fig. 2C). Responses are more selective for pulse duration than for pulse pause and pulse duration tuning is relatively independent of the pulse duration.

G Responses to sequences of sine tones (blue) and pulse trains (red). First and third row correspond to 2s sine followed by 2s pulse for females and males, respectively. For the second and fourth the order is reversed – stimuli start with 2s pulse song and transition into 2s sine song. The top row shows responses to the individual components of the sequence aligned to the onset of the component in the combined stimulus. The bottom row shows responses for both sexes to the compound stimuli (black) and the linear prediction obtained by summing the responses to the individual components (green). The linear prediction matches the measured responses well except at the transition due to transient response to sound onset only present in individual component responses. Lines and shaded areas correspond to the mean ± s.e.m. over 189 female and 217 male flies.
Figure S3

A

B

Δ speed [mm/s]

IPI [ms]

male

female

CM07

CARM03

ZH23

N30

ZW109

CSTully

TZ58

NM91

DsxGCaMP6m

Δ speed [mm/s]

IPI [ms]

male

female

CM07

CARM03

ZH23

N30

ZW109

CSTully

TZ58

NM91

DsxGCaMP6m

Δ speed [mm/s]

IPI [ms]
Figure S3. Related to Figure 3.

A Male (left) and female (right) locomotor tuning for pulse trains with different IPIs for NM91 – the wild type strain used in FLyTRAP – and flies expressing GCaMP6m in all Dsx+ neurons used for calcium imaging (see legend). Dsx-GCaMP6m and NM91 flies exhibit similar and species-typical IPI tuning that also matches the tuning found using alternative assays (see text for details).

B IPI tuning in seven additional wild type strains is diverse and not consistent with the species-typical tuning (male left, female right, see legend for strains used). Note, however, that most strains still respond sex-specifically to pulse song sex, similar to NM91 and Dsx-GCaMP6m. These strains produce similar responses to song in a natural courtship assay, suggesting that these strains require sensory cues that are missing in FLyTRAP for expressing their song preference.

Graphs show mean ± s.e.m. over individuals (100-150 flies per strain and sex).
Figure S4

Best duration & pulse pause

Figure S4A: Best carrier & pulse pause

Figure S4B: Best carrier & pulse pause

Figure S4C: Best carrier & duration

Sine song has no pauses

Sine song has long pulses

Weak responses for sine song due to short pauses and long pulses even for the "right" carrier frequency.

Female

Normalized F/F

Normalized peak F/F (LJ)

Normalized integral F/F (LJ)

Male

Normalized F/F

Normalized peak F/F (LJ)

Normalized integral F/F (LJ)
Figure S4. Related to Figure 3.
A Calcium response of the female LJ as a function of the pulse duration for all stimuli in our data set with a duration of 4 seconds. Blue circles correspond to pulse stimuli and red triangles mark sine stimuli, which by definition do not have pauses and can be thought of as very long pulses. Note that besides pulse duration, all stimuli also differ in pulse pause duration and carrier frequency – for instance all sine stimuli (red triangles) differ in carrier frequency (see C). This explains why stimuli with the same pulse duration evoke diverse calcium responses (integral ΔF/F) - they differ in these other stimulus features. The black line connects stimuli that have the optimal pulse carrier frequency (250 Hz) and pulse pause (20ms). To account for differences in ΔF/F across individuals, values were normalized by the maximal ΔF/F for each individual.
B Same calcium response data as in A but now plotted as a function of pulse pause. Sine stimuli correspond to pulse trains with no pauses – they are by definition continuous oscillations. The black line connects stimuli with the optimal pulse duration (12ms) and pulse carrier frequency (250Hz).
C Same calcium response data as in A but now plotted as a function of pulse carrier frequency. Sine stimuli differed in their carrier frequencies. The black line connects stimuli with the optimal pulse duration (12ms) and pulse pause duration (24 ms).
D, E Calcium responses from the female (D) or male (E) LJ for pulse trains with different combinations of pulse pauses and pulse durations. The stimuli constitute a subset of those measured in the behavior. LJ tuning for pulse trains with different pulse pauses and pulse durations recapitulates the behavioral tuning (compare Fig. S2E, F): LJ responses are more selective for pulse duration and the preferred pulse duration does not change with pause duration.
F, G Comparison of peak and integral ΔF/F values from the LJ for all stimuli tested in females (F) and males (G). Pulse and sine song are marked with red and blue, respectively. The black lines correspond to the best linear fit. Both measures of calcium responses are highly correlated (males: r=0.79, p=4x10^{-26}, female: r=0.80, p=5x10^{-39}).
Figure S5

Individuals

- ▸ response
- ▼ no response

A

- pC2 soma position along normalized lateral-medial axis
- pC2 soma position along normalized anterior-posterior axis
- pC2 soma position along normalized dorsal-ventral axis

B

- Male
- Female
- 10um

D

- pCd2

E

- aDN
- pC2l
Figure S5. Related to Figure 4.
A Locations of pC2 somata in the female brain with (pink) or without (grey) auditory responses along the lateral-medial and anterior-posterior (left) or dorsal-ventral (right) axis. Soma positions were normalized to between 0 and 1 for each animal. Individual symbols (see legends) correspond to somata from different animals. Responsive pC2 somata are concentrated in the lateral proportion of the cell cluster (lower half of each plot) and largely correspond to the pC2l subcluster, although we also observed auditory responses in pC2m somate (see Movie S5).
B, C Max z-projection of baseline fluorescence values from a two-photon volumetric scan of a male (B) and female (C) brain expressing GCaMP6m in all Dsx+ neurons. Each of the three clusters (pC1 - white, pC2m – blue, pC2l - yellow) connects to the LJ (red) via unique processes (arrows). pC2l and pC2m are hard to distinguish by soma location in many flies (mostly females) since they are intermingled.
D A single pCd2 neuron labeled using MCFO (black) registered to a template brain (JFRC2, gray). The lateral junction (LJ) is marked in magenta. pCd1 (not shown, {Kimura:2015ff}) and pCd2 do not project to the LJ.
E Max z-projection of a confocal stack in which aDN (green) and pC2l (red) were labelled with different colors using MCFO. pC2l but not aDN projects to the LJ (blue).
Figure S6

A. Female R42B01/Dsx and male R42B01/Dsx

B. Inter-pulse interval [ms] vs. pulse carrier frequency [Hz]

C. Probability of inter-pulse interval [ms] and pulse carrier frequency [Hz]

D. 5ms pulse

E. Male R42B01/Dsx

F. Female R42B01/Dsx retinal food vs. normal food vs. difference

G. Pulse amount [1/minute] vs. sine amount [1/minute] vs. song amount [1/minute]

H. Correlations between pairs of song features

I. pC2l TNT vs. time [minutes]

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Figure S6. Related to Figure 5.

A  Expression pattern of CsChrimson.mVenus in the intersection of R42B01 and Dsx (green) for females (left) and males (right). Neuropil is labelled with nc82 (magenta). The intersection labels 11/22 female and 22/36 male pC2l neurons, as well as 5-6 pCd1 and 2 pCd2 in either sex.

B  Calcium responses from a female of this line for pulse trains (IPI 36ms) in pC2l somata (blue), the pC2l process (yellow) and the LJ (red). We detected auditory response in all 5 pC2l cells visible in the imaging plane, as well as in the LJ and in the pC2l process.

C, D  Song evoked by 655nm activation of R42B01∩Dsx neurons (black, N=7 flies) resembles the natural song produced by wild type flies (NM91, N=47 flies) during courtship (red). Shown are distributions of IPIs, pulse carrier frequencies, and sine carrier frequencies (C) as well as average pulse shapes (D).

E  Pulse rates and sine song probability upon optogenetic activation of males expressing CsChrimson (red-shifted channel rhodopsin) in R42B01∩Dsx (see A) on food without added retinal (top, solid lines, N=3 flies) or on food with retinal (top, dashed lines, N=7 flies), males expressing CsChrimson in R41A01∩Dsx (labels pCd1) fed retinal food (middle, N=2 flies), and females expressing CsChrimson in R42B01∩Dsx neurons fed retinal food (bottom, N=3 flies). Optogenetic activation of pCd1 in the male (middle) and of pC2 (and pCd) in the female (bottom) does not evoke song, demonstrating that the singing evoked by R42B01∩Dsx activation in males is 1) due to pC2 and not the pCd1 also labeled in this line, and 2) sex-specific. Males expressing CsChrimson in R42B01∩Dsx that were kept on normal food produced some song upon activation, though much less than males fed retinal (see dashed lines). This residual activation likely stems from small amounts of retinal in normal food.

F  Speed traces for female (magenta, top) and male (grey, bottom) flies expressing CsChrimson in R42B01∩Dsx. Shown are responses for flies fed retinal (left), normal food (middle) and the difference between the traces of retinal and normally fed flies (right). Colors correspond to different driving voltages of an array of 655nm LEDs. Lines correspond to averages over 70 females and 83 males fed retinal food, and 29 female and 34 males fed normal food.

G  Statistics of male song do not change when they court pC2l TNT females (using the R42B01∩Dsx driver). Shown are distributions of 11 song parameters from NM91 males courting pC2 control (blue) or pC2l TNT females (orange).

H  Female genotype does not change correlation statistics (correlations between pairs of song parameters) in the song of NM91 males. Shown are all unique pair-wise correlations between the 11 song parameters in F for the song of NM91 males courting pC2l control (x values) or pC2l TNT females (y values).

I  Cumulative fraction of copulated pairs of NM91 males courting pC2l control (blue) or pC2l TNT females (orange). There is weak but statistically not significant effect of female genotype on copulation rates (p=0.19, Cox's proportional hazards regression model).

F-H: Data from 48 pC2 control and 48 pC2l TNT pairs.
Figure S7

A

pulse train duration [ms]

B

sine carrier frequency [Hz]
Figure S7. Related to Figure 6.
A Calcium responses in pC2 neurons (measured via the LJ) for pulse trains with different durations (IPI=36ms) in single- and group-housed (solid and dashed lines) females (left) and males (right). Data from 5-6 flies for each condition (single/group-housed, females/males).
B Same as A, but for sine carrier frequency. Data from 20/5 single/group-housed females and 12/6 single/group-housed males.
Responses to pulse trains with different durations and to sine tones with different carrier frequencies do not change substantially with housing conditions.
Lines and error bars correspond to mean ± s.e.m over flies.
**Movie legends**

**Movie S1** – Female flies (wild type NM91) in FLyTRAP. Track histories are shown as colored trails. Movie speed corresponds to real time.

**Movie S2** – Males extend their wings in response to pulse song in FLyTRAP. In this example, 10 out of 12 flies extended their wings after stimulus onset (4 second pulse train, inter-pulse interval = 36ms). Responding flies are marked with red circles. Movie is slowed down 4X. Some flies extend their wings spontaneously (not triggered by sound), see for example fly 5 in this movie.

**Movie S3** – Two-photon Calcium imaging of the female lateral junction and pC2l process. GCaMP6m is expressed in all the Dsx+ neurons. The response in the lateral junction (LJ) and pC2l process are highly correlated (see also Fig 4F). Three responses are shown for a single fly – 4 second pulse trains with inter-pulse intervals of 16/36/76ms. The text in the corner appears when the sound stimulus is on. Movie speed corresponds to real time.

**Movie S4** - Two-photon Calcium imaging of the male lateral junction and pC2l process (same as in S3).

**Movie S5** – Two-photon Calcium imaging (single plane) of female pC2 cell bodies. The responses to a 4 second pulse train and to a 4 second sine tone (carrier frequency ~ 200Hz) are shown. In most females the pC2l and pC2m cell bodies can not easily be separated into distinct clusters. Movie speed corresponds to real time.

**Movie S6** – Two-photon Calcium imaging (single plane) of male pC2 cell bodies (same stimuli as in movie S5). pC2l and pC2m cell bodies can be spatially organized in two distinct clusters. Movie speed corresponds to real time.

**Movie S7** – Optogenetic activation of pC2l neurons in an isolated male. Three light intensities are shown (1.8, 9, 13 mW/cm2). Sound is recorded using microphones that tile the chamber floor. Video and audio are synced and played at real time.