Neural Evolution of Context-Dependent Fly Song

Highlights

- Genetic reagents target homologous neurons in multiple Drosophila species
- Homologous descending neurons drive distinct fly songs in a similar social context
- Evolutionary changes downstream of the homologous neurons cause song differences
- Courtship song circuit multifunctionality may facilitate rapid fly song evolution

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In Brief

Ding, Lillvis, et al. develop a cross-species transgenic approach to probe the anatomy, physiology, and function of homologous neurons in two fly species. Courting male flies of each species use homologous descending neurons to produce different song types in similar social contexts, due to evolved differences in the ventral nervous system.
Neural Evolution of Context-Dependent Fly Song

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SUMMARY

It is unclear where in the nervous system evolutionary changes tend to occur. To localize the source of neural evolution that has generated divergent behaviors, we developed a new approach to label and functionally manipulate homologous neurons across Drosophila species. We examined homologous descending neurons that drive courtship song in two species that sing divergent song types and localized relevant evolutionary changes in circuit function downstream of the intrinsic physiology of these descending neurons. This evolutionary change causes different species to produce divergent motor patterns in similar social contexts. Artificial stimulation of these descending neurons drives multiple song types, suggesting that multifunctional properties of song circuits may facilitate rapid evolution of song types.

INTRODUCTION

Animals display an extraordinary diversity of behaviors. Although the evolution of behavior in response to natural selection has been studied for many years, the neural differences that underlie behavioral diversity are poorly understood. Given the hierarchical and modular structure of neural networks underlying specific behaviors, it seems possible that some nodes in the nervous system are more evolvable than others [1]. Are evolutionary changes distributed randomly across all neural circuit nodes required to perform a behavior? Or do changes tend to accumulate in specific parts of neural circuits?

Brains of related species consist mainly of homologous neurons—neurons that share the same developmental origin—and behavior evolution may result from changes in the connectivity or physiology of homologous neurons [2]. Several studies have begun to elucidate how changes in homologous neurons have contributed to behavioral evolution [3–12]. But a satisfying answer to the broad evolutionary questions posed above requires a systematic methodology to identify and functionally manipulate homologous neurons.

Historically, study of homologous neurons has been accomplished in only a few taxa [13–15], and this work has required considerable expertise. In most animals, the tiny size and dense packing of neurons precludes ready localization of homologous neurons. Here, we demonstrate that functional comparisons of homologous neurons are achievable, and probably scalable, in Drosophila species, providing a rare opportunity to identify the neuronal differences underlying a wide range of species-specific behaviors.

The approximately 1,500 species of Drosophila display extensive behavioral diversity, even though they share similar overall brain structure [16, 17]. Drosophila neurons adopt identifiable, cell-type-specific morphology, and many cell types consist of only a single bilateral pair of neurons. The extreme stereotypy of Drosophila neurons, together with the development of large collections of genetic reagents that label specific neurons [18], has allowed rapid identification of neurons underlying many specific behaviors in D. melanogaster [19]. The stereotypy of Drosophila neurons further suggests that homologous neurons may be identifiable across Drosophila species by labeling neurons with genetic reagents exported from D. melanogaster.

We have explored this problem in species closely related to D. melanogaster [20]. These species are amenable to genetic manipulation [21], which provides a rare opportunity to perform functional comparisons of homologous neurons in freely moving animals. We demonstrate this approach by investigating the function of homologous neurons required for one aspect of male courtship.

Drosophila species perform courtship rituals that often involve a male chasing the female, dancing around her, singing by vibrating one or both wings, waving their sometimes-spotted wings, licking the female, and other behaviors [22]. Here, we focus on singing, which can be systematically quantified more easily than most courtship behaviors [23]. Females detect courtship song through vibrations of their antennal arista [24, 25], and each species sings a unique song [22].

Species of the D. melanogaster species subgroup sing diverse songs [26, 27] (Figures 1 and S1; Video S1). D. melanogaster males, for example, extend and vibrate a single wing to produce courtship song containing two basic elements: a series of pulse events (pulse song) and continuous hums (sine song; Figure 1A) [28]. In contrast, D. yakuba and D. santomea males produce two song types, both composed of pulses: so-called thud song, produced by unilateral wing extension, and clack song, a higher frequency song type (Figure 1C) produced by vibration of both wings simultaneously without substantial wing extension [29] (Figure 1A). Examination of courtship songs in a phylogenetic context reveals that all species of this group, except D. orena, produce a pulse-like song by unilateral wing vibration (Figure 1B),
Figure 1. Divergence of Courtship Song between D. melanogaster and D. yakuba

(A) Courtship song of D. melanogaster and D. yakuba. Pulse and sine song are generated via unilateral wing extensions and clack song by double-wing vibrations without wing extension.

(B) Almost all species in the D. melanogaster species subgroup produce pulse song via unilateral wing vibration. D. yakuba and D. santomea produce clack song (orange). D. erecta produces an additional song type composed of polycyclic pulses by vibrating one, or occasionally two, wing(s) behind the body (marked by asterisk). See also Figure S1 and Video S1.

(C) Carrier frequency of pulse song types in D. melanogaster (mel) and D. yakuba (yak).

(D) Amplitude of song types in D. melanogaster (mel) and D. yakuba (yak).

(E and F) Probability density of male (E) and female (F) speed when D. yakuba males sing pulse (black) and clack (orange) song. n = 6.

(G) Heatmaps showing the position of male centroids relative to a centered female centroid (0,0) during pulse (top; n = 5; events = 1,202) and clack (bottom; n = 5; events = 1,222) song in D. yakuba. Color map is normalized relative to the unit with highest density.

(H and I) Number of pulse (H) and clack (I) events during the recording session for intact (IntactF) and decapitated (DecapF) females. Mean ± SD shown beside individual data points. One-way ANOVA p values are estimated by permutation. ***p < 0.001. See also Table S2.

Recent work has shown that, although D. melanogaster males produce both pulse and sine song throughout courtship, they bias their song toward pulse song when they are moving rapidly and are relatively far away from the female (see Figure 3 and Extended Data Figure 6 in [32]). The male’s velocity and location with respect to the female correlate with a specific context during courtship. Females avoid and run away from males when they are unreceptive, resulting in males chasing females at relatively high velocity. When females become willing to copulate, they slow down, allowing males to slow down and approach females more closely. Thus, the quantitative bias in the production of pulse versus sine song is correlated with differences in the social context.

D. yakuba biases their song type according to social context in similar ways [29]. D. yakuba males preferentially produce clack song when they are relatively far from females, whereas they increase production of pulse song when they are closer to females [29]. As observed in D. melanogaster, this is not a binary switch, and both song types can be produced in both social contexts. Consistent with these patterns, pulse song is louder than sine song in D. melanogaster [33, 34] and clack song is louder than pulse song in D. yakuba (Figure 1D).

Thus, D. melanogaster and D. yakuba both appear to bias their song type based on the social context, but the match between the song type and the context has switched. D. melanogaster
males preferentially sing pulse song when they are relatively far from females, whereas D. yakuba males preferentially sing pulse song when they are relatively close to females. This species difference may reflect neural changes in the circuit that interprets the social context and transmits this information to conserved song circuitry, changes in how the song circuitry responds to a conserved signal about the social context, or both. Convenitently, in vertebrates and some invertebrates, these two circuit functions are largely separable into neurons in the head that integrate cues and neurons in the thorax—the spinal cord in vertebrates and the ventral nervous system in insects—that pattern specific motor actions. Computations performed in the brain are transmitted to motor patterning centers through descending neurons. Therefore, examining the function of homologous descending neurons required for behaviors that have diverged across species—such as courtship songs—may allow a direct test of the anatomical locus of change underlying behavior evolution.

Here, we examine the function of a homologous courtship song descending neuron in each species. We find that this neuron produces different songs in a similar social context between species. Thus, the neural information encoding this social context appears to be conserved, whereas the circuitry that patterns song types responds differently to conserved descending information.

RESULTS

D. melanogaster Pulse Song and D. yakuba Clack Song Are Produced Primarily in Similar Social Contexts

We first performed a quantitative analysis of the social context during song production in D. yakuba. We measured the relative positions and velocities of males and females during D. yakuba courtship. We found that D. yakuba males preferentially sang clack song at relatively higher velocity and across a wide range of distances and positions relative to females (Figures 1E–1G). They increased production of pulse song at lower velocities and when positioned slightly closer and often directly behind females (Figures 1E–1G). Consistent with these observations, D. yakuba males sang mainly pulse song when we provided them with motionless decapitated females, which allowed a male to remain close to a non-moving female (Figures 1H and 1I).

These results confirm that D. melanogaster and D. yakuba preferentially sing different songs, pulse and clack songs, respectively, when chasing females rapidly. Both species produce a unilateral pulse-like song, but D. melanogaster produces pulses more often at high velocity and far from females, whereas D. yakuba produces pulses preferentially at low velocity, when close to females. We therefore compared the function of the descending neurons that transmit information about social context to neurons that pattern songs to identify where in the nervous system evolutionary changes have caused this change in context-dependent song production.

Neurogenetic Reagents from D. melanogaster Label pIP10 Descending Song Neurons in D. yakuba

Descending neurons in insects can act as command-like pathways that are activated in particular contexts and whose activity is necessary and sufficient for the production of certain behaviors [35]. One such descending neuron, pIP10, is necessary and sufficient for the production of pulse song in D. melanogaster [33, 36–38]. Because unilateral wing extension is time locked with optogenetic activation of pIP10 [38], it has been inferred that pIP10 is excited above threshold in the specific social contexts in which D. melanogaster produces pulse song [32].

D. melanogaster males, but not females, possess a single bilateral pair of pIP10 neurons that project from the brain to the ventral nervous system, where they arborize within the wing neuropil (Figure 2A). We generated new genetic reagents to cleanly target pIP10 in D. melanogaster and transferred genetic reagents from D. melanogaster to D. yakuba to test whether we could label and functionally compare pIP10 homologs.

We first manually screened two large D. melanogaster GAL4 enhancer driver line collections [39, 40] to identify new genetic reagents that labeled pIP10. We then exported these reagents to D. yakuba and D. santomea [21]. GAL4 reagents often drive expression in multiple neurons, and we did not expect to observe identical GAL4 expression patterns in both species because transgenes integrated into different genomic locations often drive slightly different expression patterns [18]. We found that we could identify pIP10 neurons in single GAL4 reagents transferred across species, but as expected, many off-target neurons were also labeled (Figure S2). Therefore, we adopted the split-GAL4 strategy [18] to label pIP10 more cleanly. Because pIP10 expresses the male-specific isoform of the sex-determination transcription-factor-encoding gene fruitless (fru) [36], we reasoned that a fru split-GAL4 reagent may allow production of genetic lines that label pIP10 cleanly. We therefore employed CRISPR/Cas9-mediated homology-dependent repair to replace the first exon of the male-specific fru isoform in D. yakuba with GAL4 or with a transcriptional activation domain or DNA-binding domain compatible with the split-GAL4 system [41] (Figures S3A–S3C).

In D. melanogaster, the split-GAL4 combination VT040556-AD ∩ VT043047-DBD labeled pIP10 with little extraneous expression (Figure S3D). In D. yakuba, we tested seven split-GAL4 combinations and identified a neuron resembling pIP10 in five split-GAL4 lines, including one line, VT040346-AD ∩ VT040556-DBD, that labeled a single bilateral descending neuron with little extraneous expression that was male specific and exhibited pIP10-like morphology (Figure S3D).

Multiple features, including morphology, gene expression, and neurotransmitter expression, allow identification of homologous neurons [42–48]. Here, we found that multiple identifying characteristics are shared between D. melanogaster pIP10 and the putative pIP10 homolog in D. yakuba.

In D. melanogaster, single bilateral neurons often are uniquely identifiable based on morphology alone [49]. The putative pIP10 homologs from D. melanogaster and D. yakuba displayed similar gross morphology (Figures 2A and 2B; Video S2), with quantitative differences in the extent of individual projections (Figure S4). These descending neurons were clearly distinguishable from 78 other single bilateral descending neurons [35]. Moreover, the putative pIP10 homologs were anatomically more similar to each other (mean NBLAST similarity score of 0.64) than to any other neuron in the FlyCircuit database (top mean NBLAST FlyCircuit similarity score of 0.20; Figure 2C) [49, 50]. Thus, the putative pIP10 homologs could be unambiguously distinguished from over 20,000 D. melanogaster neurons.
The putative pIP10 homologs were labeled by the same genetic enhancers (GAL4 reagents) in both species, implying that these neurons share similar patterns of transcription factor expression. Furthermore, both putative pIP10 homologs were immunoreactive for the male-specific fru isoform proteins, FrUM (Figure 2D) [36, 51]. Moreover, both putative pIP10 homologs express choline acetyltransferase, and thus use acetylcholine as a neurotransmitter (Figure 2E), but do not express vesicular glutamate transporter or glutamate decarboxylase 1 (Figures 2F and 2G). Therefore, based on male specificity, similar morphology, and shared gene expression patterns, we conclude that our GAL4 reagents label the pIP10 homolog in D. yakuba, allowing comparisons of pIP10 function across species.

**pIP10 Is Required for Pulse Song in D. melanogaster but Clack Song in D. yakuba**

We tested whether pIP10 function was required for specific song types in each species by inhibiting pIP10 activity during courtship. We found, consistent with a previous report [36], that inhibition of pIP10 in D. melanogaster using our new split-GAL4 line caused almost complete elimination of pulse song and a small reduction in sine song during normal courtship (Figure 3A).

In contrast, inhibition of pIP10 in D. yakuba largely eliminated clack song and quantitatively reduced the amount of pulse song in some treatments (Figures 3B and 3C). We observed this result using two neuronal inhibitors and three separate split-GAL4 combinations, in which pIP10 was the only shared labeled neuron. We show below that artificial stimulation of pIP10 can generate multiple song types in both species, but it is also possible that the reduction of sine song in D. melanogaster and pulse song in D. yakuba in the pIP10 inactivation experiments is an indirect result of largely abolishing pulse and clack song in each species, respectively. For example, males that cannot produce the “fast chasing” song may be less successful at reducing female velocity, which would result in males finding themselves less often in a context to produce their “close, slow, following” song.

Using similar genetic methods, we observed that pIP10 was also necessary for production of clack song in D. santomea (Figure 3D). Thus, the shift in the functional requirement of pIP10 from pulse song to clack song predated the divergence of D. yakuba and D. santomea.

In summary, inhibiting the activity of pIP10 primarily reduced the ability of each species to produce a specific song type, pulse song in D. melanogaster and clack song in D. yakuba. Notably, these songs types are produced preferentially in a similar context, when males rapidly chase females at a distance. These results suggest that the context in which pIP10 is activated is largely conserved across species, whereas the song type that pIP10 elicits has diverged.

**Activation of pIP10 Drives Both Clack(-like) and Pulse Song in an Intensity-Dependent Manner in Both Species**

The silencing experiments suggest that evolutionary changes in pIP10 or downstream circuitry have evolved to allow D. melanogaster and D. yakuba to produce divergent songs in a conserved social context. To further explore the role of pIP10 in driving different song types, we optogenetically activated pIP10 neurons in awake, isolated, freely behaving, intact flies by expressing the red-shifted channelrhodopsin CsChrimson [52] in pIP10 neurons and exposing flies to red light (Figure 4A).
In *D. melanogaster*, optogenetic activation of pIP10 throughout most of the light intensity range drove mainly pulse song with a normal carrier frequency (Figure 4B). Sine song was elicited rarely, and the probability of sine song production increased with increasing light intensities (Figure 4B). These results are consistent with the loss of pulse song and the reduction in sine song observed previously. These results indicate that *D. melanogaster* males possess neural circuitry that is capable of producing clack-like song and that pIP10 can access this circuitry when activated artificially at very low levels.

In *D. yakuba*, optogenetic activation of pIP10 neurons drove clack and pulse song in an intensity-dependent manner (Figures 4C and 4D; Video S6). Low levels of pIP10 activation drove only clack song, but higher levels drove both clack and pulse song. In addition, we observed proportionally more pulse song and less clack song with increasing light intensity. Sine song was never elicited. The inter-clack interval in wild-type *D. yakuba* flies falls between ~115 and ~140 ms [29, 53]. The inter-clack interval of artificially activated song falls within this range at lower levels of light intensity but is abnormally short at higher activation levels (Figure 4C). Overall, in *D. yakuba*, low levels of pIP10 activity drove clack song whereas higher levels of pIP10 activity were required to trigger pulse song. These optogenetic activation results in *D. yakuba* are consistent with the inhibition of pIP10 function in *D. yakuba*, which revealed that pIP10 is required primarily for clack song and perhaps also for quantitatively normal levels of pulse song.

In summary, the results of optogenetic activation of pIP10 in both species are consistent with the effects of silencing pIP10. The primary motor actions driven by pIP10 are pulse song in *D. melanogaster* and clack song in *D. yakuba*. Additionally, pIP10 can activate both clack (or clack-like) song and pulse song in both species, suggesting that the common ancestor of these species possessed neural circuits in the ventral nervous system that could drive both clack and pulse song. Thus, functional clack-like song circuitry likely existed in the *D. melanogaster* species subgroup long before evolutionary changes in the common ancestor of *D. yakuba* and *D. santomea* allowed pIP10 to drive abundant clack song during courtship.

**The Brain Is Not Required for pIP10 to Drive Species-Specific Songs**

The fact that optogenetic activation of pIP10 drives different quantitative patterns of clack-like and pulse song in each species implies that evolutionary changes upstream of pIP10, in the brain, are not required to explain production of different songs by each species in the same social context. We directly tested whether the activation phenotype of pIP10 required participation of neurons in the brain by activating the axons of pIP10 neurons in decapitated *D. yakuba* males. Activation of pIP10 axons in decapitated males drove clack song and pulse song in a light-intensity-dependent manner, like in intact *D. yakuba* males (Figure 4D). Thus, neither signals from the brain nor the connectivity to pIP10 in the brain is required to generate the distinct motor patterns induced via differential pIP10 activation. These results imply that changes either in pIP10 itself or in the circuitry downstream of pIP10 have evolved to allow these species to produce different songs in similar social contexts.
Physiology of pIP10 Does Not Explain the Evolutionary Shift in pIP10 Function

As discussed above, activation of pIP10 neurons generated different patterns of clack-like and pulse song production at different activation levels in the two species (Figures 4A and 4B). We quantified this observation further by measuring the carrier frequency of songs induced by activating pIP10 with light ramped from low- to high-intensity levels in small incremental steps (Figure 4E). We found that activation of pIP10 in D. melanogaster drove predominately the higher frequency clack-like song only at the lowest activation levels. In contrast, activation of pIP10 in D. yakuba drove clack song across a wider range of activation levels (Figure 4F).

One explanation for this difference could be that our artificial manipulations did not excite pIP10 neurons similarly across species. However, the initial onset of song in response to light stimuli was similar in both species (Figure 4F) and we found that red light penetrates the cuticle of both species similarly (Figure S5). Therefore, it is unlikely that these potential technical issues caused the different patterns of song production resulting from pIP10 activation in the two species. Furthermore, the activation results are consistent with the results of inhibiting pIP10 in each species (Figure 3). Thus, our results indicate that the key evolutionary difference in pIP10 function is a shift in the threshold at which the song circuit switches from clack-like to pulse song in response to increasing levels of pIP10 activity.

One hypothesis to explain the evolutionary difference in the response of the song circuit is that the intrinsic excitability of pIP10 has evolved between species. For example, lower pIP10 excitability in vivo or in processes that we could not monitor or in processes that we could not monitor.

To test this hypothesis, we performed whole-cell patch-clamp experiments in brain explants from each species. We found that the electrophysiological properties of pIP10 were similar in both species (Figure 5). Resting membrane potential, spike threshold, spike amplitude, after-hyperpolarization amplitude (Figures 5A–5D), and spike frequency responses to depolarizing current injections (Figures 5E and 5G) were all indistinguishable between species. We expressed CsChrimson in pIP10 and observed no species-specific differences in pIP10 function cannot be explained by differences in electrophysiological properties of

Figure 4. Song Phenotypes from pIP10 Activation

(A) Photostimulation protocol (20 s off and 10 s on at different intensities) for CsChrimson activation experiments in (B)–(D).

(B) Song phenotypes of pIP10-activated D. melanogaster males.

(C) Song phenotypes of pIP10-activated D. yakuba males using the cleanest pIP10 split-GAL4 line (see also Figure S3).

(D) Natural log (ln) of the ratio of number of clacks versus number of pulse events of pIP10-activated males in D. yakuba using three split-GAL4 lines. Data for each animal and mean ± SD are shown.

(E) Heatmaps show the distribution of song carrier frequency (pulse for D. melanogaster; pulse and clack combined for D. yakuba) with ramping irradiance using small incremental steps. Color represents the relative density of song events. For each genotype, mean of eight tested animals is shown. In D. melanogaster, pIP10 activation elicited high-frequency clack-like events (300–600 Hz), mostly at the lowest irradiance levels (red arrow). In D. yakuba, pIP10 activation elicited mostly high-frequency song events (clack song) across all the tested irradiance levels.

See also Videos S4, S5, and S6; Figure S5; and Table S1.
pIP10 itself and that the relevant evolutionary changes reside within the ventral nervous system, downstream of the intrinsic physiology of the descending neuron pIP10.

**D. yakuba Males Require Clack Song for Efficient Mating**

Female choice sexual selection is believed to be the primary driver of novel courtship songs in different *Drosophila* species [54], but critical tests of this hypothesis have been hampered by the challenge of precisely manipulating song in behaving animals [55]. Our neurogenetic reagents allow experimental control of specific components of song and therefore allow a sensitive test of the importance of song components for female choice.

We compared the mating efficiency of *D. yakuba* males with and without the ability to produce clack song. We eliminated production of clack song (and, in some treatments, quantitatively reduced pulse song production) by expressing Kir2.1 in pIP10 neurons using three different split-GAL4 drivers. In all cases, males with inhibited pIP10 neurons had substantially lower copulation success than control males (Figure 6). It is therefore likely that *D. yakuba* females prefer to mate with males that sing abundant clack song and that the abundance of clack song in normal courtship reflects female choice sexual selection for this component of song. However, we cannot rule out the possibility that pIP10 performs an unrecognized function unrelated to song that reduced copulation success of males.

**DISCUSSION**

The study of evolutionary neuroscience, with the goal of identifying the neural changes that cause behavioral evolution, will be facilitated by the ability to monitor and functionally manipulate individual homologous neurons across species. We have demonstrated that this goal is attainable by exporting neurogenetic reagents from the model system *D. melanogaster* to related species. This work has recently become feasible because of the introduction of new genetic reagents in multiple non-*melanogaster Drosophila* species [21]. The efficient introduction of neurogenetic reagents into *Drosophila* species displaying a wide diversity of ecologies and behaviors will likely lead to a renaissance in our understanding of how brain evolution generates behavioral diversity and how the structures of neural networks constrain or facilitate behavior evolution.

Our study illustrates how functional study of homologous neurons can localize the neural changes responsible for an evolutionary change in behavior. Judicious choice of key neurons—neurons that provide a “bottleneck” in the flow of neural information—facilitates these studies, because they separate the contributions of different regions of the nervous system to behavior evolution. In this study, functional manipulation of the descending interneuron pIP10 revealed that changes in the ventral nervous system, downstream of pIP10 activity, are required to explain the production of a different song type in a similar social context, when males are chasing females (Figure 7). Although we do not know whether the inputs into pIP10 are identical between the species, they are sufficiently conserved to...
drive pIP10 in similar social contexts. This observation indicates that it may be useful to explore whether the brain circuitry encoding social context imposes a constraint on how inputs to descending neurons can evolve. Such a constraint could make the ventral nervous system circuitry relatively more evolvable than brain circuitry.

**Descending Neuron Multifunctionality within and between Species**

Descending neurons in *Drosophila* are often characterized as transmitting a single kind of information from the brain to the ventral nervous system, although descending neurons can drive more than one motor output in some conditions [33, 56, 57]. We found that artificial activation of pIP10 can trigger clack(-like) and pulse song, at low and high pIP10 activation levels, respectively, in both *D. melanogaster* and *D. yakuba*. Thus, pIP10 is multifunctional. Similarly, stimulation of cervical connective filaments (plausibly single-axon stimulations) at different rates in crickets can elicit three types of song—calling, aggressive, and courtship-like song [58].

A recent study has reported that *D. melanogaster* males produce two modes of pulse song that differ in average carrier frequency and wing angle and that activation of pIP10 can drive both of these pulse song modes [33]. Additionally, they found that the pulse mode with the higher carrier frequency is produced with a smaller wing angle. Our studies revealed that wild-type *D. melanogaster* males also sing a clack-like song, which has a higher carrier frequency and is associated with movement of both wings without obvious wing extension. These observations raise the possibility that clack-like song might represent an extreme form of pulse-like singing, both with respect to carrier frequency and wing angle. Further analysis is required to test this hypothesis.

Multifunctionality of pIP10 may have facilitated the production of a new song type in a conserved behavioral context, because novel connections between descending neurons and song-patterning circuits would not be required. Instead, the evolution of abundant clack song in *D. yakuba* could have resulted from the accumulation of small changes in the quantitative threshold at which the clack circuit was activated. Thus, the evolution of an abundance of what at first appears to be a novel song type did not necessarily require a discontinuous change in circuit function. Similar kinds of quantitative shifts in the threshold output of a system have been observed in the *cis*-regulatory enhancers that have driven morphological evolution between species [59, 60].

**The Presence of Functional Neural Circuitry to Elicit a Rarely Produced Song Type**

The fact that artificial activation of pIP10 can drive clack(-like) song in both species, together with our observation of rare clack-like song production during natural *D. melanogaster* song, indicates that the neural circuitry required to produce clack song is present in both species and, applying evolutionary parsimony, that this circuitry was likely present in the common ancestor of the two species and closely related species in the *D. melanogaster* species subgroup.

The existence of functional circuitry for clack song in both species allows us to escape the perpetual quagmire in evolutionary inference of determining whether a trait is absent from a species because of developmental constraints or because of...
the absence of appropriate selective pressures. In the case of clack song, it is probable that most species of the *D. melanogaster* species subgroup produce little or no clack song not because they lack the neural circuitry to produce clack song but instead because they have not experienced sexual selection for abundant clack song. We found that *D. yakuba* females currently impose sexual selection for clack song production, suggesting that the shifting preferences of females selected for abundant clack song in the common ancestor of *D. yakuba* and *D. santomea*.

Our finding that artificial activation of a neuron can robustly elicit a behavior that mimics a newly evolved behavior in a different species may have general implications for how circuit organization can influence behavior evolution. Behaviors that appear novel in some species may sometimes reflect the co- 

Conclusions

Comparisons of individually labeled homologous neurons between species can provide novel insights into neural circuit function and evolution. Many future studies will be necessary to determine whether neural changes underlying the evolution of behavior tend to be biased toward particular categories. But the scalable experimental approach to label and functionally manipulate homologous neurons in closely related species that we developed here will greatly facilitate these comparative experiments in *Drosophila* [12]. Our approach therefore provides a new opportunity to dissect the contributions of single identified neurons and circuits to the evolution of behavior and to reveal constraints on the evolution of nervous systems.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found with this article online at https://doi.org/10.1016/j.cub.2019.02.019.

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AUTHOR CONTRIBUTIONS

Y.D., J.L.L., and D.L.S., designed the study and wrote the manuscript. Y.D. generated the *D. yakuba* and *D. santomea* genetic reagents with contributions from J.C. and D.L.S. J.L.L. and B.J.D. generated the *D. melanogaster* split-GAL4 lines. Y.D. and J.L.L. performed behavior experiments. J.L.L. performed immunohistochemistry, imaging, neuroanatomical analyses, and electrophysiological experiments. X.L. performed in situ hybridization experiments. B.J.A. built the behavior rig, and G.J.B. developed the k-means-based song classification algorithm. M.X. performed fly husbandry and manual annotation of courtship. B.J.D. contributed to project discussion and manuscript editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| rabbit anti-GFP     | ThermoFisher Scientific | Cat# A-11122; RRID:AB_221569 |
| chicken anti-GFP    | Abcam | Cat# ab13970; RRID:AB_300798 |
| mouse anti-bruchpilot | Developmental Studies Hybridoma Bank | Cat# nc82; RRID:AB_2314866 |
| rabbit anti-DsRed   | Clontech Laboratories | Cat# 632496; RRID:AB_10013483 |
| rabbit anti-HA      | Cell Signaling Technology | Cat# 3724; RRID:AB_1549585 |
| rabbit anti-Fru     | [51]   | N/A        |
| Alexa Fluor 488-conjugated goat anti-rabbit | ThermoFisher Scientific | Cat# A-11034; RRID:AB_2576217 |
| Alexa Fluor 488-conjugated goat anti-chicken | ThermoFisher Scientific | Cat# A-11039; RRID:AB_2534096 |
| Alexa Fluor 568-conjugated goat anti-mouse | ThermoFisher Scientific | Cat# A-11031; RRID:AB_144696 |
| Alexa Fluor 568-conjugated goat anti-rabbit | ThermoFisher Scientific | Cat# A-11011; RRID:AB_143157 |
| ATTO 647N-conjugated goat anti-mouse | Active Motif | Cat# 15048 |
| Cy3-conjugated goat anti-rabbit | Jackson ImmunoResearch Labs | Cat# 111-165-144; RRID:AB_2338006 |
| Cy2-conjugated goat anti-mouse | Jackson ImmunoResearch Labs | Cat# 115-225-166; RRID:AB_2338746 |
| Cy5-conjugated goat anti-rabbit | Jackson ImmunoResearch Labs | Cat# 111-175-144; RRID:AB_2338013 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| DyLight 550 NHS Ester | ThermoFisher Scientific | Cat# 62262 |
| Cy5 Mono NHS Ester | Sigma-Aldrich | Cat# GEPA5101 |
| Deposited Data      |        |            |
| Raw confocal image stacks | This paper; https://doi.org/10.6084/m9.figshare.7627145.v1; https://doi.org/10.6084/m9.figshare.7624601.v1; https://doi.org/10.6084/m9.figshare.7626680.v1 | N/A |
| Experimental Models: Organisms/Strains |        |            |
| D. melanogaster: Oregon-R (wild-type) | N/A | |
| D. simulans: sim5 (wild-type) | N/A | |
| D. mauritiana: mau29 (wild-type) | N/A | |
| D. sechellia (wild-type) | UCSD | Stock # 14021-0248.07 |
| D. yakuba (wild-type) | UCSD | Stock # 14021-0261.02 |
| D. santomea (wild-type) | UCSD | Stock # 14021-0271.00 |
| D. teissieri (wild-type) | UCSD | Stock # 14021-0257.01 |
| D. orena (wild-type) | UCSD | Stock # 14021-0245.01 |
| D. erecta (wild-type) | UCSD | Stock # 14021-0224.01 |
| D. melanogaster: VT040556-p65ADZp@attP40 | [39] | N/A |
| D. melanogaster: VT040347-ZpGDBD@attP2 | [39] | N/A |
| D. melanogaster: UAS-Kir2.1(pSW921)@260b | [67] | N/A |
| D. melanogaster: 20XUAS-CsChrimson-mVenus@attP18 | Vivek Jayaraman [52] | N/A |
| D. melanogaster: 10XUAS-IVS-mCD8::GFP| Gerry Rubin [18] | N/A |
| D. yakuba: yakw; myr-GFP | This paper | N/A |
| D. yakuba: yakw++; Kir2.1 | This paper | N/A |
| D. yakuba: 20XUAS-CsChrimson-ttdTomato@yak1730 | This paper | N/A |
| D. yakuba: 10XUAS-EGFP::Kir2.1(pJFRC49)@yak2180 | This paper | N/A |

(Continued on next page)
| REAGENT or RESOURCE SOURCE | IDENTIFIER |
|----------------------------|------------|
| D. yakuba: 5XUAS-TNT(pJFRC34)@yak2180 | This paper |
| D. santomea: 20XUAS-CsChrimson-tdTomato@san1504 | This paper |
| D. santomea: 10XUAS-EGFP::Kir2.1 (pJFRC49)@san2174 | This paper |
| D. yakuba: VT040346-GAL4@yak1664 | This paper |
| D. yakuba: VT040346-GAL4@yak1694 | This paper |
| D. santomea: VT040346-GAL4@san2092 | This paper |
| D. yakuba: VT040347-GAL4@yak1694 | This paper |
| D. santomea: VT040556-GAL4@san2150 | This paper |
| D. santomea: VT040556-GAL4@san2151 | This paper |
| D. yakuba: VT040346-p65ADZp@yak2177 | This paper |
| D. yakuba: VT040346-ZpGAL4DBD@yak2180 | This paper |
| D. yakuba: VT040347-p65ADZp@yak2180 | This paper |
| D. yakuba: VT040556-ZpGAL4DBD@yak2177 | This paper |
| D. yakuba: ykw; fru-GAL4 | This paper |
| D. yakuba: ykw; fru-AD | This paper |
| D. yakuba: ykw; fru-DBD | This paper |

Oligonucleotides

| Name | Source |
|------|--------|
| in situ probes for ChAT | [68] |
| in situ probes for vGlut | [68] |
| in situ probes for Gad1 | [68] |
| in situ probes for GFP | This paper (see Table S3) |

Software and Algorithms

| Software and Algorithms | Link |
|-------------------------|------|
| ZEN Digital Imaging for Light Microscopy | Zeiss RRID:SCR_013672 |
| Adobe Illustrator | Adobe Systems RRID:SCR_010279 |
| Computational Morphometry Toolkit | [69] https://www.nitrc.org/projects/cmtk/ and https://github.com/jeffersi/cmtk-gui RRID:SCR_002234 |
| VVD Viewer | Hideo Otsuna [70, 71] https://github.com/takashi310/VVD_Viewer/blob/master/README.md N/A |
| Fiji | [72] http://fiji.sc/ RRID:SCR_002285 |
| NBLAST | [49] http://flybrain.mrc-lmb.cam.ac.uk/s/nblast/www/ RRID:SCR_015884 |
| MATLAB | MathWorks RRID:SCR_001622 |
| FlySongSegmenter: MATLAB program to analyze D. melanogaster, D. simulans, and D. mauritiana courtship songs | [23, 73, 74] https://github.com/FlyCourtship/FlySongSegmenter N/A |
| FlySongClusterSegment: MATLAB program to analyze D. yakuba and D. santomea courtship song | This paper https://github.com/gordonberman/FlySongClusterSegment N/A |
| omnivore: MATLAB program to record audio, video, and light stimulus data | This paper https://github.com/bjarthur/omnivore N/A |
| Tempo: MATLAB program to visualize behavior data and export movies | This paper https://github.com/JaneliaSciComp/tempo N/A |
| ilastik | [75] https://www.ilastik.org/ RRID:SCR_015246 |
| Spike2 | Cambridge Electronic Design RRID:SCR_000903 |
| SigmaPlot | Systat Software RRID:SCR_003210 |
**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David L. Stern (dstern@janelia.hhmi.org).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

Unless noted otherwise, flies were raised on standard cornmeal-agar based medium on a 12:12 light/dark cycle at 23-25°C. Detailed information on fly crosses, genotypes, housing, and age for each experiment are indicated in the relevant Method Details sections and figure legends.

**METHOD DETAILS**

### Transgenic fly stock generation

In total, we generated 20 transgenic lines, including 7 UAS effector reagents, 6 GAL4 reagents, 4 split-GAL4 reagents, and 3 CRISPR/Cas9-mediated knock-in reagents in *D. yakuba* and *D. santomea*. Details are described in the Key Resources Table and Table S1. All transgenic injections were performed by Rainbow Transgenic Flies, Inc. using standard protocols. Most of the plasmids were provided by Gerry Rubin.

The fru knock-in alleles were generated by precisely replacing the first exon of the male-specific fru isoform with GAL4, AD, or DBD via CRISPR/Cas9-mediated HDR. The GAL4, AD (p65ADZp), and DBD (ZpGAL4DBD) fragments were amplified from the plasmids pBPGuW, pBPp65ADZpUw, and pBPZpGAL4DBDUw (Addgene), respectively. The donor plasmids were constructed by concatenating a 1.2 kb left homology arm, the insertion fragment (GAL4, AD or DBD), a 3XP3::DsRed marker (in inverted orientation), a 1.1 kb right arm, and a 1.8 kb backbone using Gibson Assembly [76]. A pair of guide RNAs (5’-GCCAGTCACAGGATTATTT-3’ and 5’-GGAGGCTTACCTAGGGGATG-3’) was cloned into the PCFD4 plasmid [77]. The donor plasmid, the PCFD4 plasmid, and in vitro transcribed *D. melanogaster* codon optimized Cas9 mRNA were co-injected into the embryos as described previously [73].

### Song recording apparatus

The song recording apparatus was described previously [23]. A CMOS camera (Point Grey Flea3 1.3 MP Mono USB3) with F1.4 25mm lens (Navitar) was placed 22 cm above the behavioral chamber for video recording. Synchronization between audio and video was achieved by triggering each frame with a pulse generated by the same data acquisition board (National Instruments) that digitized the audio.

For CsChrimson stimulation, red LEDs (635 nm) were placed 6 cm above the behavioral chamber to provide the light stimulus. Pulse-width modulation with a 100 kHz frequency was used to adjust the light intensity with a custom-built high-side switching LED controller. A data acquisition board (National Instruments) was used to generate the timing signal for the light stimulus. The synchronization of this stimulus was achieved by recording the output of the controller on the same data acquisition board that digitized the audio. Infrared LEDs (850 nm) were used to provide illumination for video recording, and the lens was attached with an 800 nm long pass filter (Thorlabs) to remove light produced by the red LEDs.

The audio, video, and light stimulus data were captured using the custom MATLAB program omnivore (https://github.com/JaneliaSciComp/omnivore). Data were visualized and movies were exported using the custom MATLAB program Tempo (https://github.com/JaneliaSciComp/tempo).

### Courtship song analysis

The *D. melanogaster* song was segmented using FlySongSegmenter [23] with modified parameters [74] to capture higher frequency pulse events. The *D. simulans* and *D. mauritiana* song was also segmented using FlySongSegmenter with different parameters [73]. For *D. yakuba* and *D. santomea* song, we developed an approach (https://github.com/gordonberman/FlySongClusterSegment) to automatically classify pulse and clack song. In brief, we sampled a subset of putative song events that are above noise threshold from a training set of *D. yakuba* and *D. santomea* wild-type songs, performed k-means clustering, and manually defined each cluster as template for pulse, clack, or noise. Models were constructed from each of these templates, such that a new event could be assigned a likelihood (\(p(\text{event} | \text{template})\)). Data from subsequent songs were assigned to one of the song templates by finding the maximum likelihood template. Template assignments for the training set were manually checked to ensure accuracy. For each song type, we used a combination of “good” templates created from multiple training subsets and added additional templates until we were satisfied with the resulting assignments. All the *D. yakuba* and *D. santomea* songs were analyzed using the same set of templates. Clack-like song in wild-type *D. melanogaster* was manually annotated as trains of pulses (\(n > 2\)) when males vibrated both wings behind the body.

Song parameters were measured using BatchSongAnalysis (https://github.com/dstern/BatchSongAnalysis) with modifications to analyze *D. yakuba* and *D. santomea* data. We characterized pulse carrier frequency in the following way. Individual pulse events represent acceleration of the wing from a stationary position to a maximum velocity and then deceleration of the wing to a stationary position again. Consequently, power of an individual pulse event can be distributed across a wide frequency range. We observed that the Fast Fourier Transform of individual pulses sometimes had a single strong peak at a single frequency and sometimes had power.
distributed across a range of frequencies. Thus, there does not appear to be an obviously single best way to measure the “carrier frequency” of a pulse event. We therefore measured pulse carrier frequency in two different ways and compared the results. First, we identified the dominant peak in the spectrogram. Second, we calculated the spectral centroid (“center of mass”) of the spectrogram, as recommended by Clemens et al. [78]. The results from both methods produced qualitatively similar patterns and we show the results from analysis of the major peak of the Fast Fourier Transform in the figures.

**Video analysis**

All videos were recorded at a frame rate of 30 Hz. Automatic fly tracking was achieved using the pixel classification and animal tracking functions of *ilastik* [75] and manually checked afterward. The male and female speed during singing were measured as the distance of fly centers over a 200 ms time window centered by each song event. The relative positions of males to females were manually annotated by measuring the distance between their thoracic centers and the angle between their body axes. Frames were excluded from analysis if the male and female were positioned on opposite sides of the chamber or if one or both flies were positioned on a wall. Wing angle was manually annotated by measuring the angle between the thoracic center-wing distal end axis and the body axis. To establish the relationship between wing angle and pulse carrier frequency, we randomly measured the wing angle of one event among all the pulse events with the same frequency (closest integer) within the defined range (*D. melanogaster*: 150 - 500 Hz; *D. simulans* and *D. mauritiana*: 150 - 650 Hz), and four animals were scored for each species.

**Behavior experiments**

To characterize song types of the *D. melanogaster* species subgroup (results shown in Figures 1D and S1), we used a relatively large chamber (2 cm X 4 cm) to capture a wider range of courtship dynamics than are normally observed in smaller chambers. A 4-10 day old virgin male (single housed) and a 3-5 day old virgin female (group housed) were placed into the chamber and recorded for 20-30 minutes. *D. sechellia*, *D. teissieri*, and *D. orena* males did not court much under this setting, so they were further recorded using a smaller round chamber (1 cm diameter) to collect more courtship events. For each species, At least 10 recordings with abundant song were collected. The strain information and the sample size for each species are described in Table S2. All other song recordings in this study employed 1 cm diameter chambers.

For piP10 inactivation in *D. melanogaster* and *D. yakuba*, experimental flies were obtained by crossing split-GAL4 males with females carrying a UAS line for a neuronal inhibitor (UAS-Kir2.1 or UAS-TNT), and control flies were obtained by crossing neuronal inhibitors to the corresponding AD or DBD lines alone. For the *D. melanogaster* split VT040556-AD ∩ VT040347-DBD, males were crossed to Kir2.1 females (w*; UAS-Kir2.1). For the *D. yakuba* splits VT040346-AD; VT040556-DBD and VT040346-DBD, males were crossed with females carrying 3XP3::DsRed marked Kir2.1 in a wild-type background (yakw*; Kir2.1). This is not applicable for the split fru-AD; VT040346-DBD, because fru-AD is also marked with a 3XP3::DsRed and could not be maintained as homozygotes; therefore, males of this split were crossed to females carrying the neuronal inhibitor in a white mutant background (yak2180_Kir2.1 and yak2180_TNT). For piP10 inactivation in *D. santomea*, experimental flies were obtained by crossing the GAL4 males (san2150_VT040556) with Kir2.1 females (san2174_Kir2.1), and crossing the GAL4 and Kir2.1 males with the *D. santomea* white females (with the same genetic background as the GAL4 and Kir2.1 flies) to generate the controls. For song recording, a 5-7 day old virgin male (single housed) and a < 1 day old virgin female (group housed) were placed into a 1 cm diameter chamber and recorded for 30 minutes. To measure copulation latency, a 4-7 day old virgin male (single housed) and a 4-7 day old virgin female (group housed) were placed in 1 cm diameter chambers and video recordings were collected for 60 minutes. Copulation time was scored manually. The control and experimental groups were always recorded simultaneously.

For CsChrimson experiments, the split-GAL4 males were crossed to females carrying UAS-CsChrimson (D. melanogaster: 20XCsChrimson-mVenus; D. yakuba: 20XCsChrimson-tdTomato). Males were collected 1-2 days after eclosion and group-housed in the dark for 6-7 days on standard media containing 0.5 mM trans-retinal (Sigma-Aldrich). Single isolated males were used for the experiments. Constant red light was applied for stimulation, and the specific protocols for each experiment are described in the following paragraph.

To activate piP10 neurons, a stimulation cycle consisted of 20 s OFF and 10 s ON at the following light intensities from low to high: 1.2 μW/mm², 2.5 μW/mm², 5.3 μW/mm², 8.0 μW/mm², 10.8 μW/mm², and 15.6 μW/mm². This cycle was repeated ten times. piP10 activation elicits courtship song acutely, so each cycle included stimulation with a ramping intensity. For activating piP10 neurons with a ramping intensity using small incremental steps, a stimulation cycle consisted of 10 s OFF and 5 s ON at intensities from 0.5 μW/mm² to 5.3 μW/mm² with incremental steps of ~0.25 μW/mm². This cycle was repeated four times.

During analysis of song phenotypes, outliers were systematically excluded in our song analysis pipeline using the Grubbs test with α = 0.05 (http://www.mathworks.com/matlabcentral/fileexchange/3961-deleteoutliers). P values for ANOVAs were estimated with 10,000 permutations (http://www.mathworks.com/matlabcentral/fileexchange/44307-randanova1). For testing copulation latency, P values were calculated via a logrank test.

**Immunostaining and imaging**

The dissections, immunohistochemistry, and imaging of fly central nervous systems were done as described previously [79]. In brief, brains and ventral nervous systems were dissected in Schneider’s insect medium (Sigma-Aldrich S0146) and fixed in 2% paraformaldehyde (diluted in the same medium) at room temperature for 55 min. Tissues were washed in PBT (0.5% Triton X-100 in...
phosphate buffered saline) and blocked using 5% normal goat serum before incubation with antibodies. Tissues expressing GFP were stained with rabbit anti-GFP (ThermoFisher Scientific A-1122; 1:1000) or chicken anti-GFP (Abcam ab13970; 1:1200) and mouse anti-BRP75a (nC82, Developmental Studies Hybridoma Bank, Univ. Iowa; 1:30), followed by Alexa Fluor® 488-conjugated goat anti-rabbit or goat anti-chicken (ThermoFisher Scientific A-11034 and A-11039, respectively; 1:500) and Alexa Fluor® 568-conjugated goat anti-mouse (ThermoFisher Scientific A-11031; 1:500) or ATTO 647-conjugated goat anti-mouse (15048, Active Motif; 1:300) antibodies, respectively. For FrU-staining, tissues expressing GFP in pIP10 were stained with chicken anti-GFP (see above) and rabbit anti-FruM [51] (1:1000) followed by Alexa Fluor® 488-conjugated goat anti-chicken (see above) and Alexa Fluor® 568-conjugated goat anti-rabbit (ThermoFisher Scientific A11011; 1:500) antibodies. Tissues expressing tdTomato were stained with rabbit anti-DsRed (Clontech 632496; 1:1000) and nC82 (see above), followed by Cy3-conjugated goat anti-rabbit and Cy2-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch 111-165-144 and 115-225-166; 1:500), respectively. For polarity staining, tissues expressing GFP and SYN::HA in pIP10, driven by the D. melanogaster split-GAL4 line VT040556-AD ∩ VT040347-DBD, were stained with chicken anti-GFP (see above), rabbit anti-HA (Cell Signaling Technology #3724; 1:1000), and nC82 (see above), followed by Alexa Fluor® 488-conjugated goat anti-chicken (see above), Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch 111-175-144; 1:500), and Alexa Fluor® 568-conjugated goat anti-mouse (see above), respectively. After staining and post-fixation in 4% paraformaldehyde, tissues were mounted on poly-L-lysine-coated coverslips, cleared in xylene, and embedded in DPX. After the tissue cured for 24 hours, image Z stacks were collected at 1 µm intervals using an LSM710 or LSM880 confocal microscope (Zeiss, Germany) fitted with a Plan-Apochromat 20x/0.8 M27 objective. Parent GAL4 images of D. melanogaster are from Jennet et al., 2012 [80]. Images of VT040346, VT040347, and VT040556 and all male split-GAL4, D. yakuba, and D. santomea images were generated by the FlyLight project team.

**in situ hybridization**

The fluorescence **in situ** hybridization (FISH) method, probe design, and dye labeling procedures for ChAT, vGlut and Gad1 were described previously in Long et al., 2017 [68]. The FISH probes used to detect GFP transcript expression in pIP10 are listed in **Table S3**. Briefly, ChAT, vGlut and Gad1 FISH probes were labeled with DyLight™550 (ThermoFisher Scientific 62626) and GFP FISH probes were labeled with Cy5 (Sigma-Aldrich GEPA15101). The brains of 3-5 day old adult flies expressing GFP in pIP10 were dissected in Schneider’s insect medium and fixed in 2% paraformaldehyde diluted in Schneider’s medium at room temperature for 55 minutes. Brain tissues were washed in 0.5% PBT, dehydrated, and stored in 100% ethanol for two days at 4°C. After exposure to 5% acetic acid at 4°C for 5 minutes, the tissues were fixed in 2% paraformaldehyde in 1X PBS for 55 minutes at 25°C. The tissues were then washed in 1 × PBS with 1% of NaBH4 at 4°C for 30 minutes. Following a 2 hour incubation in prehybridization buffer (15% formamide, 2 × SSC, 0.1% Triton X-100) at 50°C, the brains were incubated in hybridization buffer (10% formamide, 2 × SSC, 5 × Denhardt’s solution, 1 mg/ml yeast tRNA, 100 µg/ml salmon sperm DNA, 0.1% SDS) containing FISH probes at 50°C for 10 hours and then at 37°C for an additional 10 hours. After a series of wash steps, the brains were dehydrated, cleared in xylene, and mounted in DPX. Image Z stacks were collected using an LSM880 confocal microscope fitted with an LD LCI Plan-Apochromat 25x/0.8 oil or Plan-Apochromat 63x/1.4 oil objective after the tissue cured for 24 hours.

**pIP10 segmentation and quantification**

pIP10 split-GAL4 lines (D. melanogaster: VT040556-AD ∩ VT040347-DBD, D. yakuba: VT040436-AD ∩ VT040556-DBD) were registered using the Computational Morphometry Toolkit (http://nitrc.org/projects/cmtk) [69] to the JFRC 2010 brain template and a newly generated VNS template. pIP10 neurons were segmented by extracting the pIP10 signal from non-target neuron signals. pIP10 segmentation and quantification plan-Apochromat 63x/1.4 oil objective after the tissue cured for 24 hours.

**in situ hybridization**

The fluorescence **in situ** hybridization (FISH) method, probe design, and dye labeling procedures for ChAT, vGlut and Gad1 were described previously in Long et al., 2017 [68]. The FISH probes used to detect GFP transcript expression in pIP10 are listed in **Table S3**. Briefly, ChAT, vGlut and Gad1 FISH probes were labeled with DyLight™550 (ThermoFisher Scientific 62626) and GFP FISH probes were labeled with Cy5 (Sigma-Aldrich GEPA15101). The brains of 3-5 day old adult flies expressing GFP in pIP10 were dissected in Schneider’s insect medium and fixed in 2% paraformaldehyde diluted in Schneider’s medium at room temperature for 55 minutes. Brain tissues were washed in 0.5% PBT, dehydrated, and stored in 100% ethanol for two days at 4°C. After exposure to 5% acetic acid at 4°C for 5 minutes, the tissues were fixed in 2% paraformaldehyde in 1X PBS for 55 minutes at 25°C. The tissues were then washed in 1 × PBS with 1% of NaBH4 at 4°C for 30 minutes. Following a 2 hour incubation in prehybridization buffer (15% formamide, 2 × SSC, 0.1% Triton X-100) at 50°C, the brains were incubated in hybridization buffer (10% formamide, 2 × SSC, 5 × Denhardt’s solution, 1 mg/ml yeast tRNA, 100 µg/ml salmon sperm DNA, 0.1% SDS) containing FISH probes at 50°C for 10 hours and then at 37°C for an additional 10 hours. After a series of wash steps, the brains were dehydrated, cleared in xylene, and mounted in DPX. Image Z stacks were collected using an LSM880 confocal microscope fitted with an LD LCI Plan-Apochromat 25x/0.8 oil or Plan-Apochromat 63x/1.4 oil objective after the tissue cured for 24 hours.

**pIP10 segmentation and quantification**

pIP10 split-GAL4 lines (D. melanogaster: VT040556-AD ∩ VT040347-DBD, D. yakuba: VT040436-AD ∩ VT040556-DBD) were registered using the Computational Morphometry Toolkit (http://nitrc.org/projects/cmtk) [69] to the JFRC 2010 brain template and a newly generated VNS template. pIP10 neurons were segmented by extracting the pIP10 signal from non-target neuron expression using VVD Viewer (https://github.com/takashi310/VVD_Viewer/blob/master/README.md) [70, 71]. Individual pIP10 arbors were then further segmented into unambiguous compartments to compare arbor volume across species (Figure S4A). Similar arbors from the left and right of pIP10 neurons were combined in each individual. In the brain, the medial arbors (red region 2) were defined as those extending from the medial, horizontal branch that is the most proximal projection to the soma, the dorsal arbors (cyan region 1) as those extending from the branch projecting dorsally from the medial branch, the ventral arbors (green region 3) as those extending from the branch projecting ventrally from the medial branch, the ventral-posterior arbors (magenta region 7, found only in D. melanogaster) as those extending from the branch projecting posteriorly from the ventral branch, the dorsal subesophageal zone (SEZ) arbors (yellow region 4) as those extending from the two dorsal-most branches projecting from the descending projection into the SEZ, the ventral SEZ arbors (blue region 5, only found in D. yakuba) as those extending from the one ventral-most branch projecting from the descending projection into the SEZ, and the soma arbors (orange region 6, only found in D. yakuba) as those extending from the smaller, secondary branch proximal to the soma. In the ventral nervous system, the anterior triangle arbors (cyan region 8) were defined as those extending from the anterior most portion of the mesothoracic triangle projections, the medial triangle arbors (red region 9) as those medial to the descending projection at the base of the mesothoracic triangle projections, the lateral triangle arbors (green region 10) as those lateral to the descending projection at the base of the mesothoracic triangle projections, the T2 descending arbors (yellow region 11, only present in 1/5 D. melanogaster and 3/5 D. yakuba) as those extending from the descending projection in the T2 neuropil, and the T3 descending arbors (blue region 12 and magenta region 13) as those extending from the descending projection in the T3 neuropil. VVD Viewer was used to calculate the voxel volume of each of these compartments. Significance was determined via two-tailed t tests of each compartment across species.
Cuticle light penetrance

An optical fiber (Thorlabs, FG105LVA) connected (S151C, Thorlabs) to a ThorLabs PM100D Compact Power and Energy Meter was positioned in front of a 3 mm LED fiber connected to a CoolLED pE4000 so that the LED would illuminate the front of the bare fiber (the only portion of the fiber that responded to light). Male flies were decapitated and the bare fiber was inserted into the head via the neck connective. 470, 525, 580, 595, and 635 nm constant on light pulses were presented in a quasi-random order. Light power for each wavelength was recorded once the measurement stabilized. The head was then carefully removed from the fiber and light was presented to the fiber again for measurement. Penetrance was calculated as the light power measurement in the head divided by the measurement without the head present. Measurements were similar to a previous D. melanogaster report [38].

Irradiance calculation for behavior and electrophysiology experiments

Irradiance was measured using a ThorLabs PM100D Compact Power and Energy Meter with a Console S130C Slim Photodiode Power Sensor. For behavior experiments, the sensor (diameter, 9.5 mm) was positioned in the same location as the arena (diameter, 10.5 mm) directly over the recording chamber microphone. Irradiance was calculated as the raw light power measured divided by the area of the sensor (70.88 mm²).

For electrophysiology experiments, the 635 nm LED stimulus (pE4000, CoolLED) was delivered (with stacked 2.0 and 1.0 neutral density filters in the beam path) through a Zeiss Examiner Z1 with a W N-Achroplan 40X/0.75 water objective. The patched pIP10 soma was positioned in the center of the objective, and thus, in the center of the focused LED beam. The LED beam size was calculated using a beam profiler (WinCamD-UCD12, DataRay) with the sensor placed at approximately the same distance from the objective as the sample during experiments (2 mm). This yielded a 1/e² beam area of 0.95 mm². Light power was also measured with the sensor placed 2 mm away from the center of the objective. In an effort to measure the light power of the focused beam and reduce the amount of unfocused or reflected light from being measured by the 70.88 mm² sensor, a painted black foil sheath was placed over the sensor with an opening for the objective to deliver light. Irradiance was calculated as the raw light power measured divided by the 0.95 mm² focused beam area.

Electrophysiology

Male flies were collected shortly after eclosion and housed in isolation. 1-3 day old and 6-8 day old flies were tested. Because no age-related differences were found in any electrophysiological properties measured, flies within species were pooled. Individual flies were anesthetized by cooling. The brain and ventral nervous system were removed from the animal and placed into external saline composed of (in mM) 103 NaCl, 3 KCl, 5 N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, 10 trehalose dihydrate, 10 glucose, 26 NaHCO₃, 1 NaH₂PO₄, 4 MgCl₂, 3 KCl, 2 sucrose, and 1.5 CaCl₂ (280-290 mOsm, pH 7.3; components from Sigma Aldrich). The connective tissue and sheath were removed using fine forceps and the brain and ventral nervous system were transferred to a chamber (Series 20 Chamber, Warner Instruments) superfused with external saline (carboxygenated with 95%O₂ and 5%CO₂) and held into place via a custom holder.

Fluorophore expressing pIP10 neurons were visualized using a Zeiss Examiner Z1 with a W N-Achroplan 40X/0.75 water objective, 470 nm or 580 nm LED illumination (pE-4000, CoolLED), and an IR-1000 infrared CCD monochrome video camera (Dage-MTI). The pIP10 soma was clearly identifiable as the only fluorescent expressing neuron in the region. Whole-cell recordings were obtained using glass patch electrodes filled with an internal solution composed of (in mM) 140 K-gluconate, 10 HEPES, 1 KCl, 4 MgATP, 0.5 Na₂GTP, and 1 EGTA (270-280 mM, pH 7.3, components from Sigma Aldrich) connected to an Axopatch 700B amplifier (Molecular Devices) and digitized (10 kHz) with a Micro 1401-3 using Spike2 software (Cambridge Electronic Design). Glass electrodes were made using a P-1000 micropipette puller (Sutter) from borosilicate glass (Sutter; 1.2 mm outer diameter, 0.69 mm inner diameter). The pipette tip opening was less than one micron with a resistance between 5 and 15 MΩ.

pIP10 neurons were recorded in current clamp mode. The input resistance of pIP10 was tested intermittently throughout the recording and was above 600 MΩ for all data here. All data except for light dose-response recordings were obtained in pIP10 neurons expressing either 10XUAS-IVS-mCD8::GFP (D. melanogaster) or UAS-myr-GFP (D. yakuba). Light dose-response recordings were obtained in pIP10 neurons expressing 20XCsChrimson-mVenus (D. melanogaster) or 20XCaChrimson-tdTomato (D. yakuba). The spike threshold was determined as the lowest membrane potential at which pIP10 fired action potentials. Spike amplitude and after-hyperpolarization amplitude were measured for 5 spikes fired near threshold and averaged. pIP10 rested ~5 mV below spike threshold in both species and was held ~5 mV below spike threshold while light dose-response experiments were conducted. pIP10 was excited by a constant-on 5 s depolarizing current steps or 635 nm light pulses delivered every 30 s through the objective while recording from the soma which was positioned in the center of the field of view. The light stimuli were similar to those used in behavior experiments (both constant on stimuli; similar range of irradiance based on cuticle penetrance calculations). Light irradiance and current amplitude presentation order was varied from experiment to experiment. There was no indication that the order of intensity presentation affected the pIP10 response. Spikes were identified and counted using Spike2 scripts, and verified via manual inspection. Spike frequency (total spikes/5 s) was plotted versus current step amplitude or light stimulus irradiance (SigmaPlot 12.5).
QUANTIFICATION AND STATISTICAL ANALYSIS

Behavior data were analyzed in MATLAB, neuroanatomical data were analyzed in VVD Viewer and SigmaPlot, and electrophysiology data were analyzed in Spike2 and SigmaPlot. Detailed statistical information relating to each experiment is provided in the relevant Method Details or figure legends.

DATA AND SOFTWARE AVAILABILITY

The confocal stack images are accessible at figshare.com. (https://doi.org/10.6084/m9.figshare.7627145.v1, https://doi.org/10.6084/m9.figshare.7624601.v1, and https://doi.org/10.6084/m9.figshare.7626680.v1).

Other data may be obtained upon request. Software is listed in the Key Resources Table.