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Multimodal chemosensory circuits controlling male courtship in *Drosophila*

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Summary

Throughout the animal kingdom, internal states generate long-lasting and self-perpetuating chains of behavior. In *Drosophila*, males instinctively pursue females with a lengthy and elaborate courtship ritual triggered by activation of sexually dimorphic P1 interneurons. Gustatory pheromones are thought to activate P1 neurons but the circuit mechanisms that dictate their sensory responses to gate entry into courtship remain unknown. Here, we use circuit mapping and *in vivo* functional imaging techniques to trace gustatory and olfactory pheromone circuits to their point of convergence onto P1 neurons and reveal how their combined input underlies selective tuning to appropriate sexual partners. We identify inhibition, even in response to courtship-promoting pheromones, as a key circuit element that tunes and tempers P1 neuron activity. Our results suggest a circuit mechanism in which balanced excitation and inhibition underlie discrimination of prospective mates and stringently regulate the transition to courtship in *Drosophila*.

Introduction

Discrete environmental stimuli have long been known to "release" enduring states like aggression or sexual arousal that motivate specific patterns of behavior (Tinbergen, 1951). Entry into arousal states can alter the processing and interpretation of sensory information over both short and long time scales and must therefore be carefully regulated. Consequently, sensory cues that gate arousal are honed by evolution to restrict the expression of innate behavioral programs, like fighting or mating, to appropriate contexts.

In many species, elaborate mating rituals precede copulation (Bastock, 1967). In *Drosophila melanogaster*, this begins with a male's assessment of the desirability of a potential mate—he extends a foreleg and taps the other fly's abdomen to sample gustatory pheromones coating its cuticle (Bastock and Manning, 1955; Spieth, 1974). If the target is a conspecific virgin female, this gustatory evaluation, combined with visual and olfactory information, triggers a long-lasting courtship state during which the male pursues the female, tracking her closely and extending a single wing to produce a species-specific song. The male will eventually contact her ovipositor with his proboscis and mount her to attempt copulation, and courtship can continue for tens of minutes until the pair copulates or the female decamps.

Drosophila males and females are both discriminating in their pursuit and acceptance of sexual partners and courtship offers an opportunity for the mutual assessment of prospective mates. Lengthy courtship rituals are thought to arise because females, who make a large investment in the production of offspring, take time to judge whether the male is suitable (Trivers, 1972). *Drosophila* male courtship is comprised of behaviors that signal his identity and serve as aphrodisiacs to arouse *Drosophila* females (Bastock and Manning, 1955; Connolly and Cook, 1973). It is advantageous for males to court only appropriate targets, given the large metabolic and temporal investment of courtship displays. This has likely contributed to the evolution of male discrimination in mate choice and reliance on multisensory signals to determine the sex, species and mating status of a potential mate (Edward and Chapman, 2011).

Drosophila courtship has been studied for a century as an overt representation of sexual arousal (Sturtevant, 1915) and emerged as a powerful model system to elucidate the genetic and neural basis for innate behaviors. Remarkably, the potential for the entire male courtship ritual in *Drosophila* is largely specified by translation of the male-specific isoforms of the Fruitless (Fru) transcription factor in ~1,500 neurons dispersed throughout the nervous system (Demir and Dickson, 2005; Lee et al., 2000; Manoli et al., 2005; Stockinger et al., 2005). Fru is thought to label sexually dimorphic circuits that control both mate assessment and courtship enactment including sensory afferents that detect female cues, interneurons that initiate and coordinate courtship displays, descending neurons that drive specific behavioral modules, and central pattern generators that shape courtship motor programs. While an anatomic atlas of Fru+ neurons has been assembled and specific interneuron subsets have been implicated in regulating male courtship behaviors (reviewed in Pavlou and Goodwin, 2013; Yamamoto and Koganezawa, 2013), the circuit mechanisms underlying sensory control of courtship entry remain largely undefined.

A population of ~30 male-specific P1 interneurons have been identified as a central node in the Fru+ circuitry that translate sensory input to initiate persistent courtship behaviors (Bath et al., 2014; Inagaki et al., 2014; Kimura et al., 2008; Kohatsu and Yamamoto, 2015; Kohatsu et al., 2011; Pan et al., 2012; von Philipsborn et al., 2011). P1 neurons are excited by female contact pheromones (Kohatsu and Yamamoto, 2015; Kohatsu et al., 2011), and their exogenous activation can drive enduring courtship displays, even in the absence of a target fly. Moreover, recent evidence suggests that P1 neurons are further excited by the male's enactment of courtship (Kohatsu and Yamamoto, 2015), potentially sustaining his

pursuit of a female. To reveal how behavioral state transitions like courtship entry are regulated at a circuit level, we sought to describe the sensory pathways that control P1 neuron activity.

Multisensory signals drive arousal in *D. melanogaster* males; here, we focus on chemosensory pathways due to the essential role of chemical cues in mediating sex and species discrimination to gate courtship behavior (Billeter et al., 2009; Fan et al., 2013; Ferveur, 2005; Krstic et al., 2009) and because the previous identification of pheromone-responsive sensory neurons (Koh et al., 2014; Lu et al., 2012; Miyamoto and Amrein, 2008; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012) provides an inroad to trace these circuits from the periphery into the brain. Using *in vivo* functional imaging in tethered males, we show that the response of P1 neurons to target flies with different pheromone profiles correlates with courtship ardor towards those targets. These observations support a role for P1 neurons in encoding the sexual suitability of a target fly and serving as a checkpoint in the release of male courtship behaviors. We then trace Fru+ gustatory and olfactory pheromone pathways to P1 neurons to reveal how their combined input underlies chemosensory discrimination of potential mates. We show that female gustatory pheromones that promote courtship produce both feed-forward excitation and inhibition of P1 neurons while olfactory pathways provide net inhibition. Our work thus identifies a circuit mechanism in which balanced excitation and inhibition regulate P1 neuron activity to provide stringent control of courtship initiation.

Results

Courtship as a persistent behavioral state

To explore the dynamics of male courtship behavior, we performed single pair courtship assays in large (120 mm diameter) chambers and scored the male's unilateral wing extension as a proxy for singing and his distance to the female until mating occurred (Figure 1A, B). Males' behavior toward females could be divided into two phases (Bastock and Manning, 1955; Eastwood and Burnet, 1977): During an initial "lag" period, males were far from females on average (>30 mm) and did not court despite occasional encounters. Perturbations in sensory detection pathways affect courtship latencies (Krstic et al., 2009), suggesting that during this lag period males assess cues from the other fly to determine whether it is an appropriate mate. Once a male initiated courtship, he remained in close proximity to the female (<6 mm or 2 body lengths away) as he faithfully tracked her movements and sang to her. These observations highlight that male courtship in *Drosophila* represents a temporally sharp transition to an enduring behavioral state.

Persistent courtship behaviors can also be induced by exogenous activation of P1 neurons, even in the complete absence of a target (Bath et al., 2014; Inagaki et al., 2014; Kohatsu and Yamamoto, 2015; Pan et al., 2012; von Philipsborn et al., 2011). We expanded on these observations by providing a male with a motor-actuated, fly-sized magnet that rotates around the behavioral arena at a constant speed (Agrawal et al., 2014). Prior to P1 neuron activation, the rotating magnet was of no interest to the male, as he neither tracked nor sang to it. Brief excitation of a subset of P1 neurons expressing the channelrhodopsin variant ReaChR drove tracking and singing behavior towards the magnet that far outlasted the light

stimulus (Figure 1CD, Figure S1A). Transient P1 neuron activation therefore elicits a discrete change in a male's behavioral state, producing sustained courtship behaviors in the absence of female-specific sensory feedback.

Gustatory pheromones stimulate P1 neurons

Prior to initiating courtship, males tap the abdomen of another fly with their foreleg tarsi to sample cuticular hydrocarbons (Speith 1974). In *D. melanogaster*, these low-volatility molecules are produced differentially by males and females and serve as pheromones that regulate courtship behavior. Males predominantly produce 7-tricosene (7-T), a compound that suppresses male courtship, while females produce primarily 7,11-heptacosadiene (7,11-HD), a chemical that promotes courtship (Billeter et al., 2009). Foreleg tapping of a female is sufficient to excite P1 neurons and initiate sustained visual pursuit of a courtship target (Kohatsu and Yamamoto, 2015), while removal of a male's forelegs encourages him to court unsuitable mates (Fan et al., 2013; Manning, 1959). As P1 neurons can drive the initiation of courtship, we investigated whether their chemosensory tuning might reflect mate discrimination.

We drove specific expression of the calcium indicator GCaMP6s in P1 neurons of tethered males walking on a freely rotating foam ball and presented target flies varying in pheromone profile, allowing males to sample their olfactory and gustatory bouquet (Figure 1E, S1B). Consistent with earlier results, we observed robust increases in the GCaMP fluorescence of P1 neurons when a male fly tapped the abdomen of a virgin *D. melanogaster* female with his foreleg (Figure 1F, H, I, Supplemental Movie). P1 neurons were not stimulated when the male touched a female with his middle or hind leg (data not shown) in accord with the unique behavioral role of the foreleg in detecting pheromone signals that regulate male courtship. Little P1 neuron activity was evoked when a male contacted the abdomen of another male (Figure 1G–I). P1 neurons were also activated by the purified female hydrocarbon 7,11-HD (Figure S1C), confirming the chemosensory origin of this response. Thus P1 neurons are strongly activated by contact pheromones carried on females but not males.

P1 Neuron Activity Correlates with Male Courtship Preferences

The sufficiency of P1 neurons to evoke persistent courtship behaviors suggests their activity should correlate with a male's mate preferences. We assessed the relationship between P1 neuron excitation and courtship ardor by stimulating tethered males with target animals differing in sex, species, or mating status, choosing a panel of targets known to vary in pheromone profile and sexual desirability to *D. melanogaster* males (Figure 1J, Figure S1D–F)(Ferveur, 2005). *D. sechellia* females produce 7,11-HD, the same courtship-promoting cuticular hydrocarbon as *D. melanogaster* females, and likewise both strongly activated P1 neurons. *D. sechellia* females induce robust courtship from *D. melanogaster* males, although these two species rarely meet in the wild (Cobb and Jallon, 1990). In contrast, *D. simulans* and *D. melanogaster* have overlapping habitats, and pheromonal differences between them are thought to prevent interspecies courtship and copulation (Billeter et al., 2009). *D. simulans* females produce 7-T (the same gustatory pheromone as *D. melanogaster* males), elicited little P1 activity in our assay, and evoke little courtship (Cobb and Jallon, 1990; Fan

et al., 2013). Mated *D. melanogaster* females, like *D. melanogaster* males, carry the courtship-inhibiting olfactory pheromone cis-vaccenyl acetate (cVA) (Brieger and Butterworth, 1969; Kurtovic et al., 2007) and evoked low P1 activity.

Thus, we found a remarkable correspondence between the P1 neuron activity evoked by a particular fly stimulus and known courtship ardor towards that target. These observations support the idea that both olfactory and gustatory pheromones contribute to the chemosensory tuning of P1 neurons, such that their activity represents the sexual suitability of a target fly. We therefore wished to trace ascending gustatory and olfactory pathways to P1 neurons in the higher brain to understand how different chemosensory signals generate discriminating courtship towards appropriate targets.

Ascending Pheromone Circuits from the Foreleg

Cuticular pheromones activate gustatory sensory neurons in the foreleg whose axons project to the first thoracic ganglion of the ventral nerve cord (Figure 2A, VNC T1). Multiple classes of pheromone-responsive gustatory sensory neurons on the foreleg have been identified (Koh et al., 2014; Lu et al., 2012; Miyamoto and Amrein, 2008; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012), but little is known about the downstream circuits they activate to regulate male courtship behavior. Using Fruitless expression as a guide, we mapped the spatial arrangement of foreleg projections and revealed that input to the ventral nerve cord is topographically organized, analogous to the spatial ordering of primary sensory neuropils in the brain.

First, we used expression of pox-neuro (Poxn) regulatory elements to label all gustatory sensory neurons (Boll and Noll, 2002), allowing us to differentiate between Fru+ gustatory and mechanosensory foreleg afferents and show their projections are spatially segregated. The majority of these Fru+ gustatory neurons express the degenerin/epithelial Na^+ channel (DEG/ENaC) pickpocket 23 (ppk23). Ppk23+ neurons respond selectively to either male or female gustatory pheromones and extend rich projections to the midline of the first thoracic ganglion (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). A subset of ppk23+ neurons co-expresses the ENaC channel ppk25. The ppk25+ neuronal population is selectively tuned to female pheromones, including 7,11-HD, and necessary for vigorous male courtship (Vijayan et al., 2014). Finally, the axons of these Fru+ pheromone-sensing neurons are segregated from projections of Fru- sugar-sensing neurons (marked by the gustatory receptor Gr5a) and from Fruneurons that detect male pheromones and bitter compounds (expressing the gustatory receptor Gr32a).

We initially focused on tracing ascending circuits from the ppk25+ neuron population because of their clear behavioral valence with respect to male courtship. To identify the downstream targets of ppk25+ sensory neurons, we first targeted their axons for photoactivation in males expressing the photoconvertible fluorophore PA-GFP in all Fru+ neurons. This identified a single class of ascending neurons previously named vAB3 (Figure 2B) (Yu et al., 2010). Intersection of *Abdominal-B*$^{LDN-Gal4}$ (*AbdB*, de Navas et al., 2006) and *Fru*^{LexA} selectively labels vAB3 neurons, revealing a projection pattern in the ventral nerve cord that is remarkably similar to that of Fru+ gustatory sensory neurons (Figure 2C). Notably, vAB3 neurons innervate only the first thoracic ganglion and should receive

selective input only from the foreleg. Within the brain, vAB3 projections bifurcate and terminate in a discrete region of the sub-esophageal zone (SEZ) and in the lateral protocerebral complex, a Fru+ neuropil also innervated by P1 neurons. vAB3 neurons are therefore anatomically poised to transmit female pheromone signals to sexually dimorphic centers in the higher brain.

To identify functional targets of vAB3 neurons, we took advantage of the anatomic segregation of foreleg sensory axons (Figure 2A) to target ppk25+ terminals at the ventral midline of the first thoracic ganglion and locally iontophorese the excitatory neurotransmitter acetylcholine (Figure 2D, see Extended Experimental Procedures). We stimulated while imaging at multiple planes (10–20 planes/experiment, ~5 μm apart; see Experimental Procedures) to capture the projections of activated neurons as they ramified through the brain, generating an anatomic map of functionally responsive neurons. The evoked excitation pattern was remarkably sparse whether GCaMP was expressed only in Fru + neurons or pan-neuronally under the synaptobrevin promoter (Figure 2E,F, Figure S2). Aside from vAB3 itself, only one class of neurons was robustly activated in the brain. We recognized this second neuronal population to be mAL neurons, a previously described cluster (~30 cells/hemisphere) of sexually dimorphic GABA-ergic interneurons (Kimura et al., 2005; Koganezawa et al., 2010). Labeling of vAB3 neurons through precise electroporation of Dextran dye in the ventral nerve cord, combined with photolabeling of mAL neurons with PA-GFP, revealed that the neurites of these two populations intermingle in a sexually-dimorphic compartment of the SEZ, suggesting mAL neurons may be directly postsynaptic to vAB3 neurons (Figure 2G).

Thus vAB3 neurons are positioned to convey sensory signals from ppk25+ axons in the ventral nerve cord to mAL interneurons within the brain. To functionally verify this disynaptic circuit configuration, we selectively expressed the ATP-gated ion channel P2×2 in either vAB3 neurons or ppk25+ sensory neurons and stimulated them by local infusion of ATP at the midline of the first thoracic ganglion (Figure 2H,I, Figure S2). Either stimulation appeared to activate only vAB3 and mAL neurons, reminiscent of the sparse excitation pattern evoked by acetylcholine iontophoresis. These observations suggest that ppk25+ gustatory sensory neurons drive selective, coordinate activation of excitatory vAB3 and inhibitory mAL neurons.

Feed-forward Excitation and Inhibition of P1 Neurons

Both vAB3 and mAL extend axonal projections into the lateral protocerebral complex. Within this sexually-dimorphic neuropil, P1 neuron projections interdigitate with vAB3 and mAL axons and thus are poised to integrate from these two gustatory pathways (Figure 3A–C). We examined this possibility by imaging P1 neuron responses evoked by stimulating the vAB3 pathway (Figure 3D). Both iontophoresis of acetylcholine into the ventral nerve cord and direct activation of vAB3 neurons expressing the $P2X_2$ channel under the *AbdB* promoter elicited only modest excitation of P1 neurons, in contrast to the robust activation of vAB3 and mAL neurons (Figure 2D, Figure S3A–B).

We reasoned that the weak response of P1 neurons might be due to mAL-mediated inhibition countering the excitatory drive from the vAB3 pathway. To assess this possibility,

we used a two-photon laser to precisely sever the highly fasciculated mAL axons and prevent propagation of inhibition to the lateral protocerebral complex. After severing the mAL axon tract, P1 responses to vAB3 stimulation increased substantially (Figure 3D–F). Direct activation of mAL neurons was also able to suppress vAB3-mediated excitation of P1 neurons (Figure S3B–D). Excitation of P1 neurons by the vAB3 ascending pathway is therefore antagonized by inhibition from mAL neurons.

Differential Tuning of vAB3 and mAL Neurons to Contact Pheromones

Together, our anatomic and functional data suggest that P1 neurons receive convergent input from mAL and vAB3 neurons, suggesting that the relative balance of excitatory and inhibitory input to P1 neurons could shape their responses to target flies carrying different pheromones. We therefore examined the functional tuning of vAB3 and mAL neurons by expressing GCaMP in them using $AbdB^{LDN-Gal4}$ or $R25E04-Gal4$ (Figure S4A–D) respectively and imaged responses evoked by *D. melanogaster* male and virgin female stimuli. vAB3 neurons responded robustly to foreleg contact with a female abdomen but only weakly to a male (Figure 4A,C). In contrast, both male and female stimuli activated a largely overlapping subset of mAL neurons (Figure 4B,D, Figure S4E). Intriguingly, this inhibitory neural population was more strongly excited by female stimuli that elicit courtship than by male stimuli that suppress it. As $R25E04-Gal4$ labels only a subset of ~12 mAL neurons, we also imaged all mAL neurons using Fru^{Gal4} or a subset labeled by $9–189^{Gal4}$ and observed similar patterns of excitation (Figure S4F), confirming that the broad tuning of mAL neurons to male and female stimuli is a general feature of this inhibitory population. Both vAB3 and mAL neurons also responded to synthetic 7,11-HD (Figure S4G), in accord with their sensitivity to female pheromones.

The preferential tuning of vAB3 neurons to female stimuli is consistent with anatomic and functional evidence that this ascending pathway receives input from ppk25+ sensory neurons (Figure 2) and inherits their selectivity to female pheromones. mAL neurons, as post-synaptic targets of vAB3 neurons, should likewise be excited by female pheromones. mAL neurons also responded to male stimuli, indicating they likely receive input from an additional sensory pathway. Indeed, mAL neurons are poised to receive input from gustatory sensory neurons in the foreleg that express the Gr32a receptor, respond to the male cuticular hydrocarbon 7-T, and ascend directly to the brain (Figure 2A, 4E) (Fan et al., 2013; Koganezawa et al., 2010; Moon et al., 2009). Consequently, vAB3 and mAL neurons exhibit different functional tuning, likely due to input from distinct sensory populations with distinct terminal fields (Figure 4E).

Proportional Excitation and Inhibition onto P1 Neurons

Interestingly, the level of P1 neuron activation evoked by tasting a female was largely independent of the number or frequency of tapping events, either within individual males or across animals (Figure 4F–G, Figure S4H–I). To examine the role of mAL-mediated inhibition in shaping the responses of P1 neurons, we directly stimulated vAB3 neurons over a range of intensities and compared P1 neuron activity prior to and after severing the mAL axon tract. With inhibition intact, the responses of P1 neurons only marginally increased with progressively more intense vAB3 excitation (Figure 4H). However, after severing mAL

axons, P1 neurons became more sensitive to incremental increases in vAB3 input. We inferred the relative mAL activity at each voltage by calculating the difference between P1 neuron responses before and after mAL axon severing and found that mAL-mediated inhibition scales with vAB3 excitation (Figure 4I). Thus this feed-forward inhibitory circuit assures that excitation of P1 neurons is inextricably accompanied by proportional inhibition, providing a gain control mechanism that produces a relatively consistent gustatory assessment of female pheromone signals.

Olfactory Pheromones Shape P1 Neuron Activity

Gustatory pheromones that promote courtship thus coordinately activate excitatory and feedforward inhibitory pathways to regulate P1 neuron excitation. Olfactory pheromones are also known to regulate male courtship and could allow a male to discriminate chemical features of a target prior to contact. However, we observed no change in P1 fluorescence as targets were brought into close proximity of the male prior to foreleg contact (Figure 5A,B), even when we acutely severed the male's foretarsi and brought the stimulus to within <0.2 mm of his antennae (Figure 5C). Volatile pheromones thus appear to be insufficient to autonomously excite P1 neurons. Olfactory cues might nevertheless guide the selection of an appropriate courtship target by synergistically or antagonistically modulating the responses of P1 neurons to gustatory pheromones.

One well-studied volatile pheromone in *D. melanogaster* is cis-vaccenyl acetate (cVA), a courtship-suppressing chemical transferred from males to females during copulation (Kurtovic et al., 2007); other volatiles have been shown to promote courtship (Dweck et al., 2015; Grosjean et al., 2011). To assess the role of olfactory input in shaping gustatory responses of P1 neurons in an unbiased manner, we examined how P1 responses to contact pheromones were altered in males lacking antennae, preventing odor detection by the vast majority of olfactory sensory neurons (Figure 5D–F). P1 neuron responses to virgin females were similar in males with or without their antennae, confirming female volatiles do not contribute to exciting this neural population. However, the response of P1 neurons to mated females was significantly increased in males lacking their antennae. These olfactory deprivation experiments suggest that olfactory pheromones primarily contribute to suppressing P1 neurons, providing additional chemosensory discrimination among courtship targets.

Olfactory cVA Pathways Project to P1 Neurons

We next took advantage of the relatively well-characterized pathways responsive to cVA to examine how olfactory circuits impinge onto gustatory pathways to shape P1 neuron responses. cVA is detected by sensory neurons in the antenna that express the odorant receptor Or67d and send convergent axons to the DA1 glomerulus within the antennal lobe (Figure 6A) (Datta et al., 2008; Kurtovic et al., 2007). Projection neurons from the DA1 glomerulus terminate in the lateral horn where they synapse onto multiple distinct classes of sexually-dimorphic Fru+ neurons including excitatory DC1 neurons (also termed asp-j or aSP5) and inhibitory LC1 neurons (also named asp-k or aSP8) (Figure 6A) (Cachero et al., 2010; Kohl et al., 2013; Ruta et al., 2010; Yu et al., 2010). Photolabeling of these two lateral horn populations using PA-GFP confirms that both extend projections into the lateral

protocerebral complex where they overlap with P1 neurites (Figure 6B,C). Thus the cVA pathway bifurcates and routes the same pheromone signals through parallel excitatory and inhibitory branches to the lateral protocerebral complex, providing an intriguing structural analogy to the gustatory pheromone pathways described here.

cVA-responsive neurons are anatomically poised to innervate P1 neurons and modulate their responses to gustatory signals from the foreleg. As prior characterizations of the cVA pathway were carried out using high doses of synthetic pheromone (Kohl et al., 2013; Ruta et al., 2010), we first verified that the cVA circuit is activated during our *in vivo* imaging experiments. We monitored the activity of the DA1 glomerulus in a male fly while bringing a target fly into close proximity, without allowing physical contact (Figure 6D, E). In contrast to the temporally discrete stimulation by contact pheromones, the DA1 glomerulus was activated as soon as a male stimulus was brought within 1–2 fly body lengths (3–6mm) of the tethered animal's antennae. Virgin female stimuli, lacking the scent of cVA, produced little DA1 excitation. We confirmed these cVA responses were propagated to lateral horn neurons by imaging the fasciculated processes of DC1 neurons, which are responsive to male but not virgin female stimuli (Figure 6F). While we were unable to visualize GABAergic LC1 neurons in our preparation, LC1 and DC1 neurons are known to be coactivated upon DA1 stimulation (Ruta et al., 2010). Thus endogenous levels of cVA carried on a single fly are sufficient to activate olfactory pathways that innervate P1 neurons.

Our anatomic and functional data suggest that both excitatory and inhibitory branches of the cVA pathway might impinge on P1 neurons but the net balance of this input is inhibition. To examine whether cVA is sufficient to suppress the gustatory responses of P1 neurons, we perfumed virgin females with synthetic cVA and then compared the P1 activity evoked by cVA-scented and unscented virgins in interleaved trials (Figure 6G). P1 responses were strongly reduced when females were perfumed with cVA, consistent with the courtship-suppressing role of this olfactory pheromone. In males mutant for the Or67d odorant receptor, peripheral detection of cVA is impaired (Kurtovic et al., 2007) and the responses of lateral horn neurons to cVA, including DC1 neurons, are abolished (Kohl et al., 2013). Accordingly, we observed that the cVA-mediated inhibition of P1 gustatory responses was reduced in Or67d$^{-/-}$ males. Thus the Or67d+ sensory circuit contributes to cVA-mediated suppression of female contact pheromones.

The residual inhibition by cVA apparent in these olfactory mutants is consistent with evidence that cVA can also be sensed by ppk23+ gustatory sensory neurons in the foreleg responsive to male contact pheromones (Thistle et al., 2012). These data indicate that cVA likely contributes to curbing a male's ardor towards a mated female by suppressing the excitation of P1 neurons by female gustatory pheromones.

Together, these experiments suggest the existence of cross-modal circuit interactions in which olfactory pheromones, acting at a distance, antagonize temporally discrete excitation from contact gustatory cues. To examine the functional interplay between olfactory and gustatory pheromone pathways, we directly activated DA1 projection neurons through iontophoresis of acetylcholine into the glomerular neuropil and imaged P1 neuron responses (Figure 6H). Stimulation of the DA1 glomerulus elicited little excitation of P1 neurons,

whether mAL was intact or severed (data not shown). However, stimulation of the DA1 glomerulus reduced vAB3-mediated activation of P1 neurons.

Olfactory pathways could influence P1 neurons via direct synaptic connectivity, in accord with their anatomic overlap, or through presynaptic modulation of gustatory afferents. However, stimulation of the DA1 glomerulus neither activated vAB3 neurons nor suppressed direct excitation of this ascending pathway, and vAB3 excitation was unaffected by the mating status of a female stimulus (Figure S5A, C). Likewise, stimulation of the DA1 glomerulus revealed no apparent excitation in mAL axon terminals or suppression of vAB3-mediated mAL excitation (Figure S5B). Finally, severing of mAL axons had no impact on vAB3 activity (Figure S5D). Thus our anatomic and functional results are consistent with a model in which olfactory modulation of gustatory responses occurs via parallel convergence onto integrative P1 neurons (Figure 7).

Discussion

In this study, we traced pheromone-processing circuits in the male brain of *D. melanogaster* to understand how their combined input underlies discrimination of potential mates and reveal the circuit logic underlying stringent chemosensory control of a male's courtship behavior. We find that both olfactory and gustatory pheromones activate neural circuits that bifurcate and project excitatory and inhibitory branches into the lateral protocerebral complex. Our data suggest that chemosensory discrimination is achieved through the net balance of these feed-forward excitatory and inhibitory pathways: female pheromones that promote courtship yield net excitation of P1 neurons, while male pheromones that suppress courtship produce net inhibition.

Sensory control of a behavioral state

As a prelude to courtship, *D. melanogaster* males approach and tap the female with a foreleg to sample her pheromones (Bastock et al., 1955; Kohatsu et al., 2011; Spieth, 1974). Activation of P1 neurons, which we demonstrate occurs selectively in response to conspecific virgin female targets, is sufficient to induce males to indiscriminately attend to motion cues for many minutes, leading to tracking and singing behaviors (Kohatsu and Yamamoto, 2015; Kohatsu et al., 2011). Most relevant to the interpretation of our study, recent work suggests that activation of P1 neurons not only releases visual tracking behaviors but also gates visual input onto P1 neurons themselves (Kohatsu and Yamamoto, 2015), creating a recurrent loop that might serve to sustain a male's state of sexual arousal and lead to vigorous and persistent courtship (Figure 1A). Female pheromones thus function as a sign stimulus of classic ethology (Tinbergen, 1951): they activate P1 neurons to trigger the release of courtship behaviors but appear to then be dispensable for their perpetuation.

The potential for a behavioral state to outlive the sensory stimulus that provoked it could produce futile or detrimental actions if not carefully regulated. We speculate that the parallel architecture of pheromone processing circuits, in which excitation is coupled with proportional inhibition, provides precise control of P1 excitation. In the mammalian cortex, coupled excitation and inhibition generated by feed-forward inhibitory circuits shape sensory processing by enabling principle neurons to remain sensitive to weak inputs while

preventing saturation by strong inputs (Isaacson and Scanziani, 2011). Feed-forward inhibition appears to play a similar role in controlling the gain of sensory signaling in *Drosophila* pheromone processing pathways. P1 neurons remain sensitive to weak stimuli and a single fleeting touch of a female can drive males to initiate persistent courtship (Bastock et al., 1955; Kohatsu and Yamamoto, 2015); mAL inhibition ensures that P1 neuron excitation does not saturate in response to strong vAB3 stimulation to produce spurious courtship (Figure 4H). Exogenous activation of P1 neurons, which bypasses mAL neurons, produces sustained courtship behaviors in the absence of a target fly, highlighting the requirement for strict control of P1 excitation.

Decreasing the strength of gustatory signaling closer to the sensory periphery might have provided an alternative mechanism to limit P1 excitation to gustatory pheromones. However, the dynamics of foreleg tapping likely produce inherently variable sensory responses. Males often have only an instant to sample the gustatory pheromones of a moving target fly, in contrast to the potential for temporal integration in their assessment of visual, auditory and olfactory cues emanating from another animal. In addition, the different classes of gustatory sensory neurons that detect pheromones are sparse in the foreleg, implying that only a small number may be stimulated during any tapping event. Moreover, pheromone cues and their receptors are rapidly evolving (McBride et al., 2007; Shirangi et al., 2009) and pheromone-sensing gustatory neurons are not chemically selective but detect an array of different long-chain hydrocarbons (Thistle et al., 2012). Indeed, we observed P1 neurons are weakly excited by a variety of inappropriate mates in our *in vivo* imaging experiments and if P1 neurons integrated weak excitatory events over time, an unsuitable mate might eventually evoke robust P1 activation and inappropriate entry into a reverberant courtship state. Feed-forward inhibition likely serves to prevent temporal integration while sharpening responses to single robust stimulation events. Parallel excitation and inhibition therefore balance two competing needs for mate discrimination: first, to remain sensitive to recognition of correct mates, and second, to avoid persistent courtship of inappropriate mates.

The potential of parallel circuits

This study offers further evidence that the Fru transcription factor marks functionally interconnected sub-circuits in the male brain that selectively process sensory signals relevant to social behaviors (Cachero et al., 2010; Yu et al., 2010). We find that sensory information from the foreleg is topographically mapped in the ventral nerve cord, a spatial ordering that allows Fru+ pheromone-responsive sensory neurons to make select functional connections with Fru+ ascending vAB3 neurons. Fru+ vAB3 and mAL neurons appear to be major pathways for ascending gustatory signals, highlighted even in pan-neuronal multi-plane functional imaging of the brain (Figure 2). Moreover, all the components of this circuit, from the sensory periphery to P1 neurons, are sexually dimorphic, providing a male-specific conduit for pheromone signals that regulate male courtship. Indeed, feminization of P1 or vAB3 arbors alters their connectivity and reduces male courtship (Von Philipsborn et al., 2014). Taken with previous data, vAB3, mAL, DC1, and LC1 are likely to represent central routes to carry contact pheromone and cVA signals to the lateral protocerebral complex. While our work does not demonstrate monosynaptic connectivity between these neural

populations, ascending pheromone pathways functionally converge upon P1 neurons and likely other targets within this dense, sexually-dimorphic neuropil.

Our anatomic and functional data are consistent with a model in which integration of olfactory and gustatory pathways relevant to courtship occurs at P1 neurons and not at earlier nodes in the circuit. One benefit of this convergent circuit architecture is to facilitate flexible assessment of potential mates with distinct pheromonal compositions. In the wild, *Drosophila* mate on food patches where they will frequently encounter flies varying in sex, species, and mating status (Spieth, 1974; Sturtevant, 1915). Our functional imaging suggests that P1 neurons can discriminate among a variety of target flies differing along axes of both odor and taste (Figure 1J). Courtship can be suppressed by either negative contact or olfactory pheromones, and combined inhibition from both pathways, in response to male stimuli, produces the lowest intensity of P1 neuron activity. Furthermore, integration of sensory signals at the level of P1 neurons provides the potential for cross-modal interactions, allowing olfactory signals, like cVA, to suppress the positive gustatory cues from a female to provide an additional level of chemosensory discrimination. Finally, direct inhibition from negative cues may dampen the reverberant activity of P1 neurons or downstream targets in the lateral protocerebral complex and quell continued courtship if a male subsequently encounters an unsuitable mate. Indeed, courting males retain the ability to discriminate among courtship targets (Pan et al., 2011), which could reflect direct suppression by inhibitory populations like mAL and LC1.

The parallel organization of these chemosensory circuits may also permit flexible assessments of pheromone valence, either in different social contexts within an individual animal or in the same social context in different species. For example, the rapid evolution of *Drosophila* pheromones is thought to contribute to establishing a barrier to interspecies courtship and copulation (Billeter et al., 2009; Shirangi et al., 2009). *D. simulans* males have the opposite pheromone preferences relative to *D. melanogaster* males: their courtship is promoted by 7-T and suppressed by 7,11-HD. Reweighting of excitation and inhibition from the parallel branches of pheromone-processing pathways, conserved in *D. simulans* (data not shown), may allow for rapid evolution of pheromone preferences. Likewise, while cVA and 7-T inhibit courtship in *D. melanogaster* males, these pheromones indicate the presence of a possible rival and promote male-male aggression (Wang and Anderson, 2010; Wang et al., 2011). Like courtship, aggression is regulated by Fru+ interneurons that innervate the lateral protocerebral complex (Asahina et al., 2014). The chemosensory control of aggression might be achieved by differential weighting of synaptic inputs from excitatory and inhibitory branches of the pheromone afferents described here onto other neural targets. In this way, the parallel organization of pheromone pathways we have revealed may allow these same circuits to regulate diverse social behaviors and arousal states.

Experimental Procedures

Full details of all methods and fly genotypes can be found in the Extended Experimental Procedures.

Courtship behavioral assays and analysis

Assays in Figure 1A are re-analyzed from Bussell et al., 2014. 4–7 day old males were tested with 1–2 day old virgin females in a modified 120 mm flybowl (Simon and Dickinson, 2010). Interfly-distance tracked using Ctrax (Branson et al., 2009).

Optogenetic stimulation

Male flies were separated from females after eclosion and aged for one day, then transferred to food containing 400uM all-trans-retinal and housed in the dark for 1 or 2 days before assays. The fly was provided with a rotating motorized magnet as in Agrawal et al., 2014. ReaChr stimulation based on Inagaki et al., 2014 was performed by 20 seconds constant illumination at $0.025mW/mm^2$ at 530 nm.

Two-photon microscopy

Imaging experiments were performed on an Ultima two-photon laser scanning microscope (Bruker Nanosystems) equipped with galvanometers driving a Chameleon Ultra II Ti:Sapphire laser. Emitted fluorescence was detected with either photomultiplier-tube or GaAsP photodiode (Hamamatsu) detectors. Images were acquired with an Olympus 60×, 0.9 numerical aperture objective at 512 pixels × 512 pixels resolution.

Flies were prepared for *in vivo* imaging as depicted in Figure 1E and described in Extended Experimental Procedures. Stimuli were presented for ~5 seconds allowing ~10 touches and stimulus bouts were repeated every 30 seconds, 3–6 times. Time courses were collected at 1–3hz.

Antennaectomy or antennal nerve severing was performed on tethered animals prior to imaging using sharp forceps. The two surgeries produced similar results and are combined in Figure 5.

In vitro stimulation and two-photon severing

For *in vitro* imaging, acetylcholine (10mM, iontophoresis) or ATP (2mM, pressure-injection) was locally applied to collagenase-softened CNS explants using a fine glass electrode (~12 MOhm) inserted into the target neuropil under visible light or fluorescent guidance. Negative controls (saline iontophoresis and ATP pressure injection in animals lacking a Gal4 allele) produced no change in GCaMP signals in the higher brain. Time courses were collected at 1–2hz.

For anatomic tracing of functionally responsive neurons, we performed identical stimulations with acetylcholine or ATP while imaging every 2.5 to 10 μm in Z and then combined these to build a volume of the anterior ~100 μm of the brain. To sever mAL axon tracts using the two-photon laser, we used expression of Tomato driven by the Fruitless promoter as a guide. Severing protocol was based on Ruta et al., 2010.

Anatomic tracing and image processing

Photolabeling and dye filling were performed as described in Ruta et al., 2010. For clarity, we masked autofluorescence from the glial sheath and basal fluorescence from out-of-plane

structures in photoactivation experiments and in the Fru^{LexA} expression pattern. To highlight P1 anatomy when using *R71G01-Gal4*, we segmented P1 neurons from the *R71G01-Gal4* expression pattern. Other neurons labeled by the R71G01 driver do not anatomically overlap with P1 neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- P1 neurons are functionally tuned towards appropriate potential mates.

- Gustatory and olfactory pheromone circuits converge on P1 neurons.

- Pheromone signals are carried by parallel excitatory and inhibitory branches.

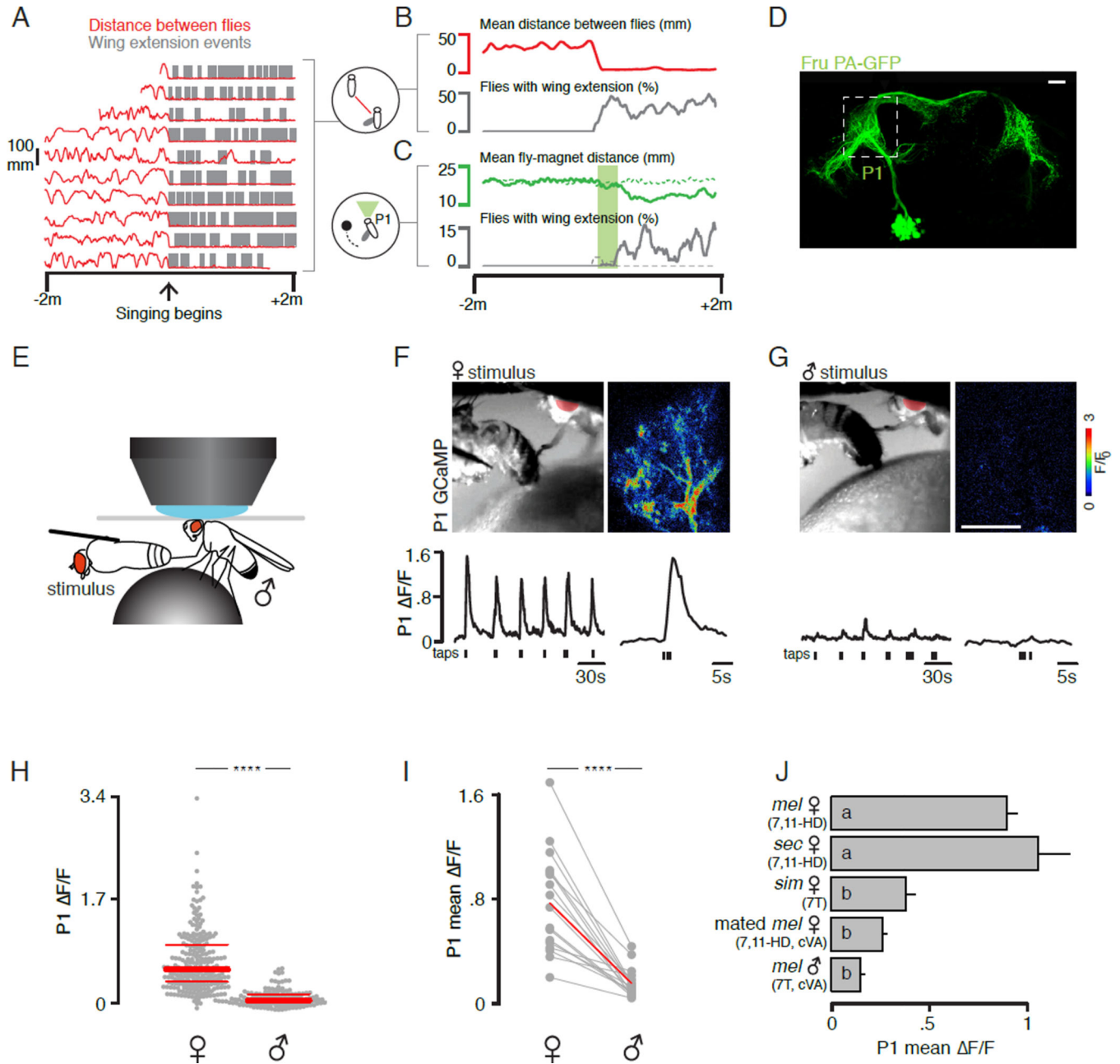- This neural architecture allows stringent and flexible control of courtship behavior.

Figure 1. P1 neuron chemosensory tuning correlates with mate preference

(A–B) Male courtship behavior towards virgin, conspecific females plotted as the male's distance to female (red) and his unilateral wing extension (grey) for 10 individual pairs (A) and population average, smoothed by 10 second sliding window (B). The top 3 individuals have courtship latencies of less than 2 minutes; the last pair mate within two minutes of courtship onset.

(C) Courtship behaviors plotted as in (B) toward moving magnet in males expressing ReAChR in P1 neurons using *P1-Gal4* (solid lines) or no-Gal4 controls (dashed lines), n=7 each genotype. See Figure S1A.

(D) P1 neuron anatomy revealed by photoactivation of PA-GFP expressed under Fru^{Gal4}; box indicates P1 processes in the lateral protocerebral complex imaged in subsequent figures. Scale bar here and throughout 10 μm. All animals imaged in this study are male. Autofluorescence from the glial sheath and basal fluorescence from non-photoactivated structures have been masked.

(E) Schematic of preparation used for *in vivo* functional imaging. See also Figure S1B and Extended Experimental Procedures.

(F–G) Representative P1 neuron GCaMP responses recorded in the same male as he contacts the abdomens of female (F) or male (G) stimulus flies. Top row shows still images from the video used to guide stimulus presentation (illuminated by infrared light, male's eye pseudocolored red) and heat map of fluorescence increase in P1 neurons for a single tapping bout. Bottom row shows normalized fluorescence traces for six bouts of tapping, indicated by tick marks (left), and zoomed in view of a single bout (right). Here and throughout, stimulating females are sexually naïve unless otherwise indicated.

(H–I) Summary of P1 neuron responses to male and female stimuli across repeated stimulus bouts in 17 subject animals. Individual stimulus bouts (H) and paired intra-animal averages (I) are shown. Significance, unpaired (H) or paired (I) t-test. Here and throughout, *: p<.05, **: p<.01, ***: p<.001, ****: p<.0001. Red lines in (H) indicate median and quartiles and in (I), means.

(J) Mean P1 neuron responses to indicated fly stimuli measured by *in vivo* imaging as in F-G. One-way ANOVA with Tukey's correction for multiple comparisons. Here and throughout, different statistical letter groups indicate p<.05. n=40–46 flies for *D. melanogaster* male and virgin female stimuli, n=9–12 flies for other stimuli. See also Figure S1C–F. Error bars here and throughout, SEM.
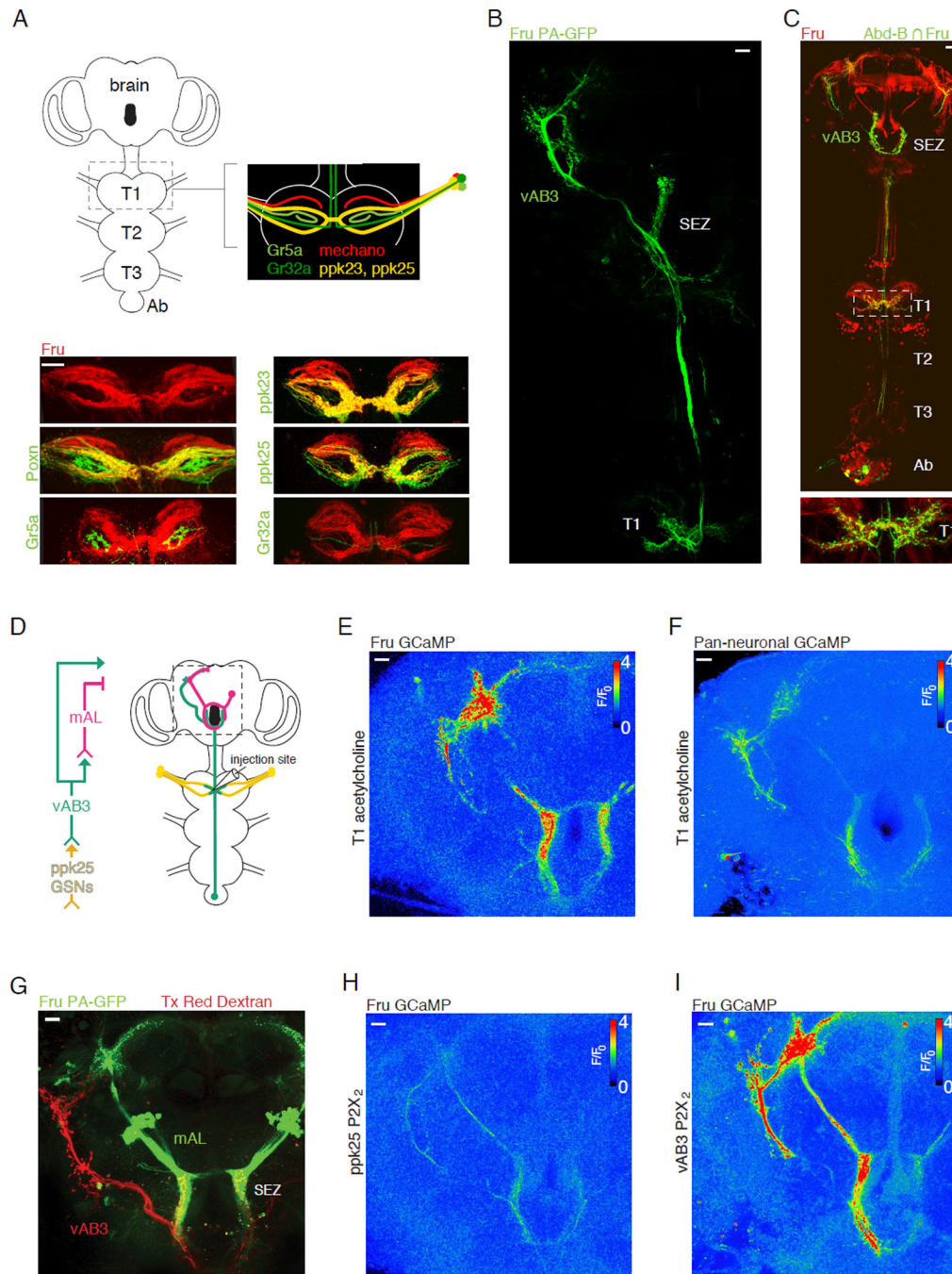
Figure 2. Gustatory pathways transmitting pheromone signals from the foreleg to courtship centers in the higher brain

(A) Schematic (top) and two-photon stacks (bottom) of sensory innervation in the first thoracic ganglion (T1) of the ventral nerve cord (VNC). Second (T2), third (T3) and abdominal ganglia (Ab) denoted.

(B–C) Maximum intensity two-photon stacks with vAB3 neurons labeled by photoactivation of PA-GFP expressed in Fru^{Gal4} (B) and the genetic intersection of $AbdB^{LDN-Gal4}$ and Fru^{LexA} (C). Autofluorescence from the glial sheath, basal fluorescence from non-photoactivated structures, and out-of-plane Fru+ soma have been masked.

(D) Models of pheromone circuit depicted in this figure; boxed area indicates central brain region imaged in subsequent panels.

(E–F) Representative multi-plane imaging of GCaMP responses in Fru+ neurons (E, n=3) or all neurons (F, n=7) in response to acetylcholine iontophoresis onto ppk25+ terminals in the VNC of an explant preparation. Controls and quantification, Figure S2.

(G) vAB3 neurons labeled by electroporation of Texas Red Dextran and mAL neurons labeled by photoactivation of PA-GFP overlap in the sub-esophageal zone (SEZ). Autofluorescence from the glial sheath and basal fluorescence from non-photoactivated structures have been masked.

(H–I) Representative multi-plane imaging of Fru+ neurons expressing GCaMP in response to stimulation of ppk25 gustatory neurons (H, n=7) or vAB3 neurons (I, n=3) expressing the P2X$_2$ channel and activated by local application of ATP in the VNC.
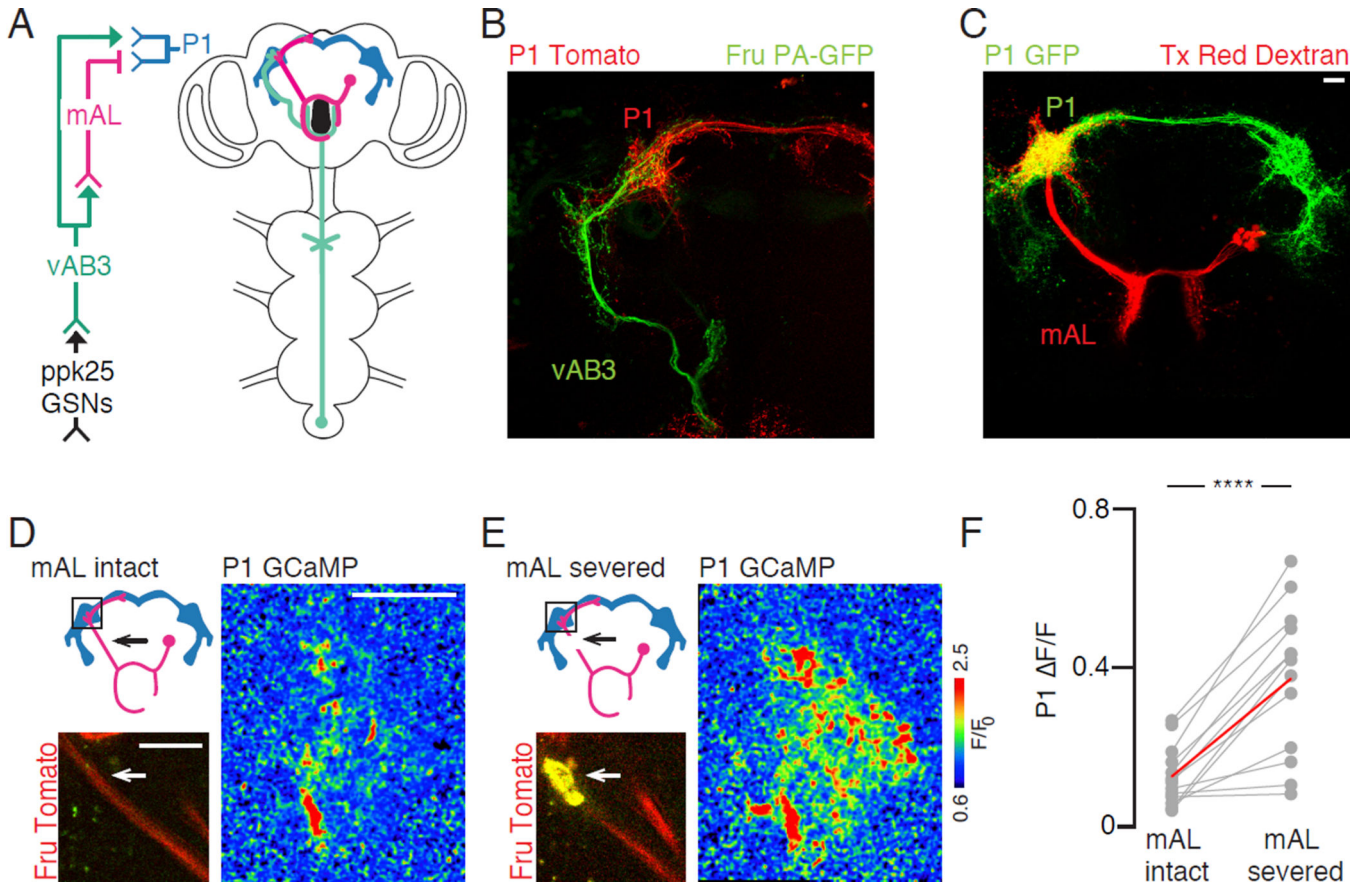
Figure 3. Excitatory vAB3 neurons and inhibitory mAL neurons anatomically and functionally converge onto P1 neurons

(A) Models of pheromone pathways depicted in this figure.

(B–C) P1 projections interdigitate with vAB3 and mAL axons in the lateral protocerebral complex. (B) shows P1 neurons labeled red by *R71G01-Gal4* expressing Tomato and vAB3 projections labeled green by photoactivation using PA-GFP expressed in *Fru^Gal4*. (C) shows P1 neurons labeled green using *P1-Gal4* to express GFP and mAL neurons labeled red by electroporation of Texas Red Dextran into their axonal tract. Autofluoresence from the glial sheath has been masked.

(D–F) Functional imaging of P1 neurons in response to stimulation of vAB3 neurons by acetylcholine iontophoresis, prior to (D) and after (E) severing the mAL axonal tract. P1 neurons express GCaMP under *R71G01-Gal4* and mAL axons were labeled using *Fru^LexA* to express Tomato. Representative GCaMP responses and results of photodamage are shown in (D–E); summary of paired P1 responses in individual animals (n=13) shown in (F). Significance, paired t-test.
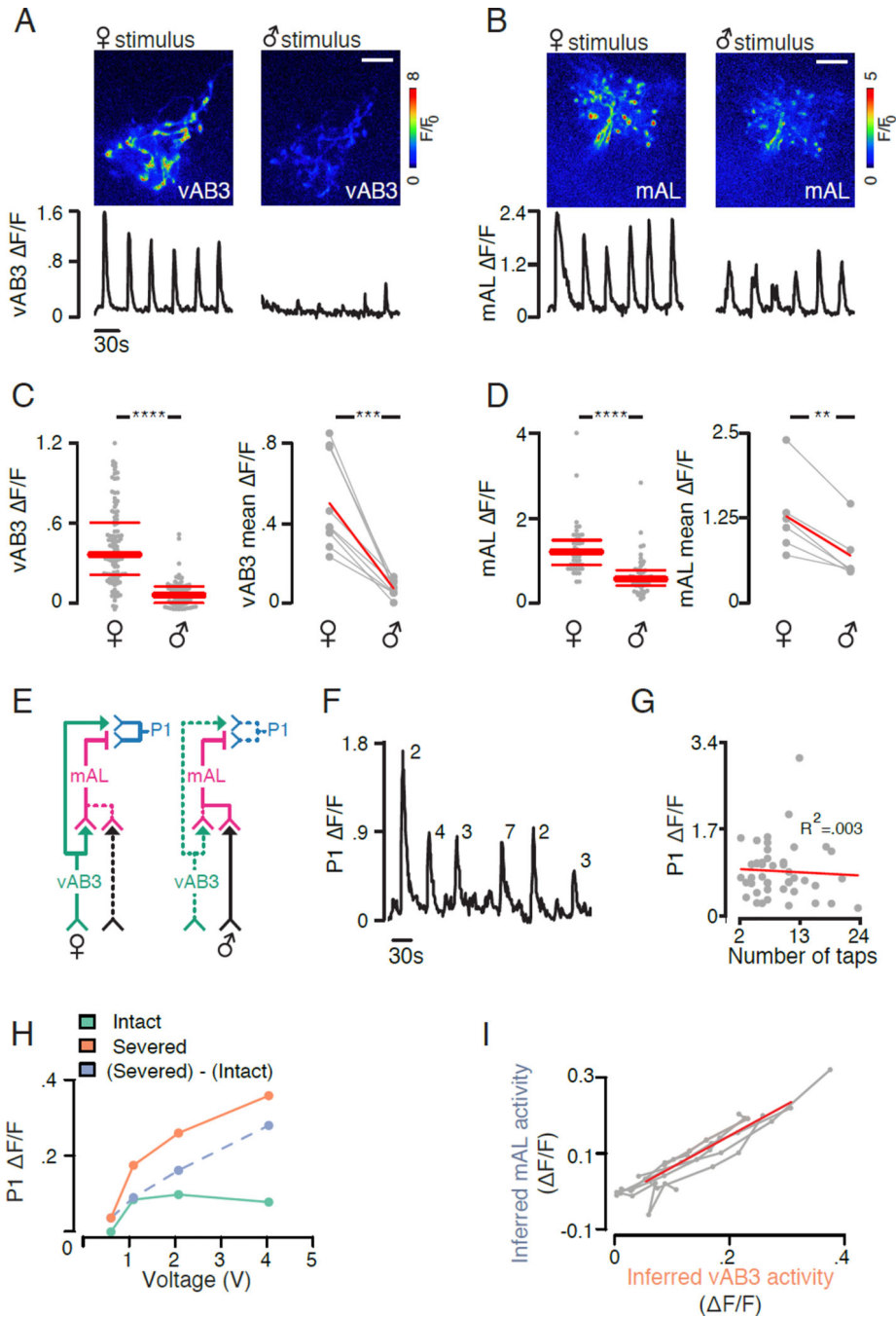
Figure 4. Differential pheromone tuning of vAB3 and mAL neurons

(A–D) Functional responses of vAB3 expressing GCaMP under *AbdB^{LDN-Gal4}* (A, C) or mAL expressing GCaMP under *R25E04-Gal4* (B, D) to foreleg contact with male or female stimuli. (A–B) Representative heat map and fluorescence traces. (C–D) Individual bout responses for all animals (n=46–104 bouts) and paired mean responses (n=10 animals for vAB3, 6 animals for mAL). See also Figure S4E–G.

(E) Circuit models depicting potential routes for pheromone signaling in response to a female stimulus (left) or male stimulus (right).

(F) P1 activity evoked by female stimulation with the number of times the male tapped the female in each bout shown.

(G) P1 responses evoked by foreleg contact to a female stimulus are not correlated with the number of taps within a bout. 45 bouts scored from 8 experiments. Red line indicates linear fit. See also Figure S4H, I.

(H–I) P1 response prior to or after severing of the mAL tract to increasing stimulation of vAB3 by acetylcholine iontophoresis. (H) shows representative experiment. (I) plots inferred relationship between vAB3 and mAL responses (n=7, grey) and linear fit (red). vAB3 activity at each voltage was inferred from the P1 response after mAL severing (orange points, H). mAL activity at each voltage was inferred from P1 response in intact circuit subtracted from the P1 response after mAL severing (blue points, H)
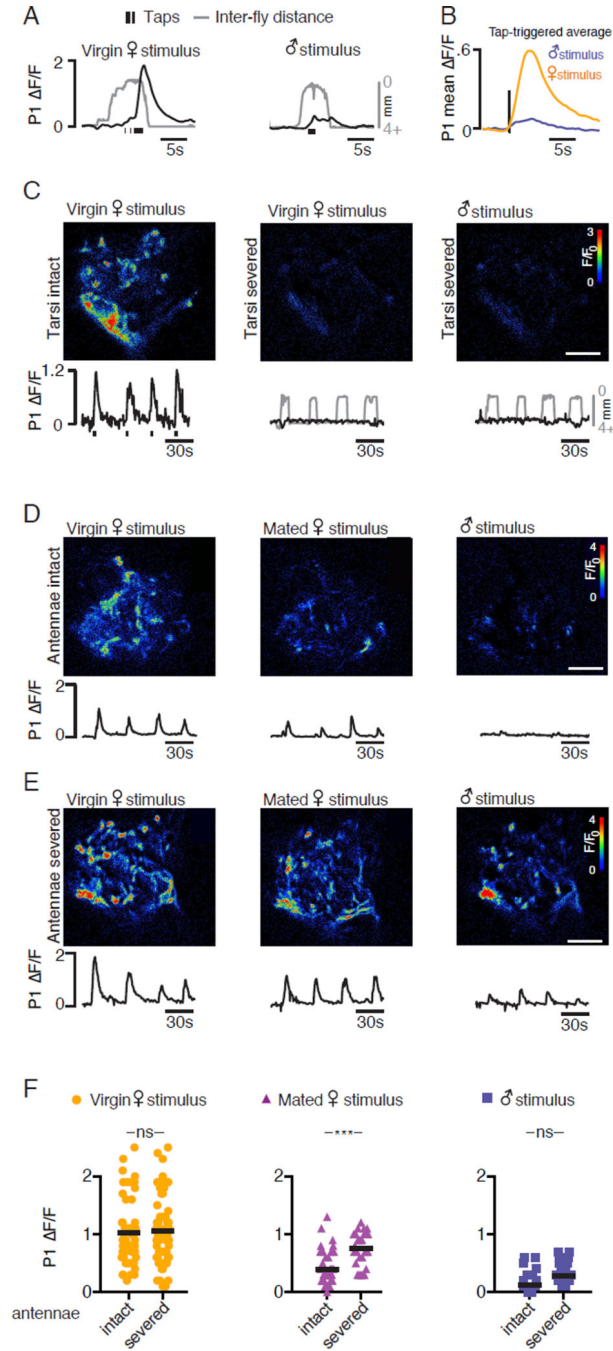
Figure 5. Olfactory signals inhibit but do not excite P1 neurons
(A–B) Representative traces of P1 neurons expressing GCaMP in response to stimulus approach and touch (A) and mean P1 responses aligned to first touch (B, n=38–39 tapping bouts from imaging 7 males). Gray traces here and below define distance between imaged and stimulus flies. (C) Representative responses of P1 neurons to indicated stimuli before and after severing foreleg tarsi.

(D–F) Representative P1 responses to indicated fly stimuli in intact males (D) or males without antennae (E). (F) Pooled P1 responses (n=4 tapping bouts per stimulus in 7–16 imaged males). Two-way ANOVA with Sidak correction for multiple comparisons.
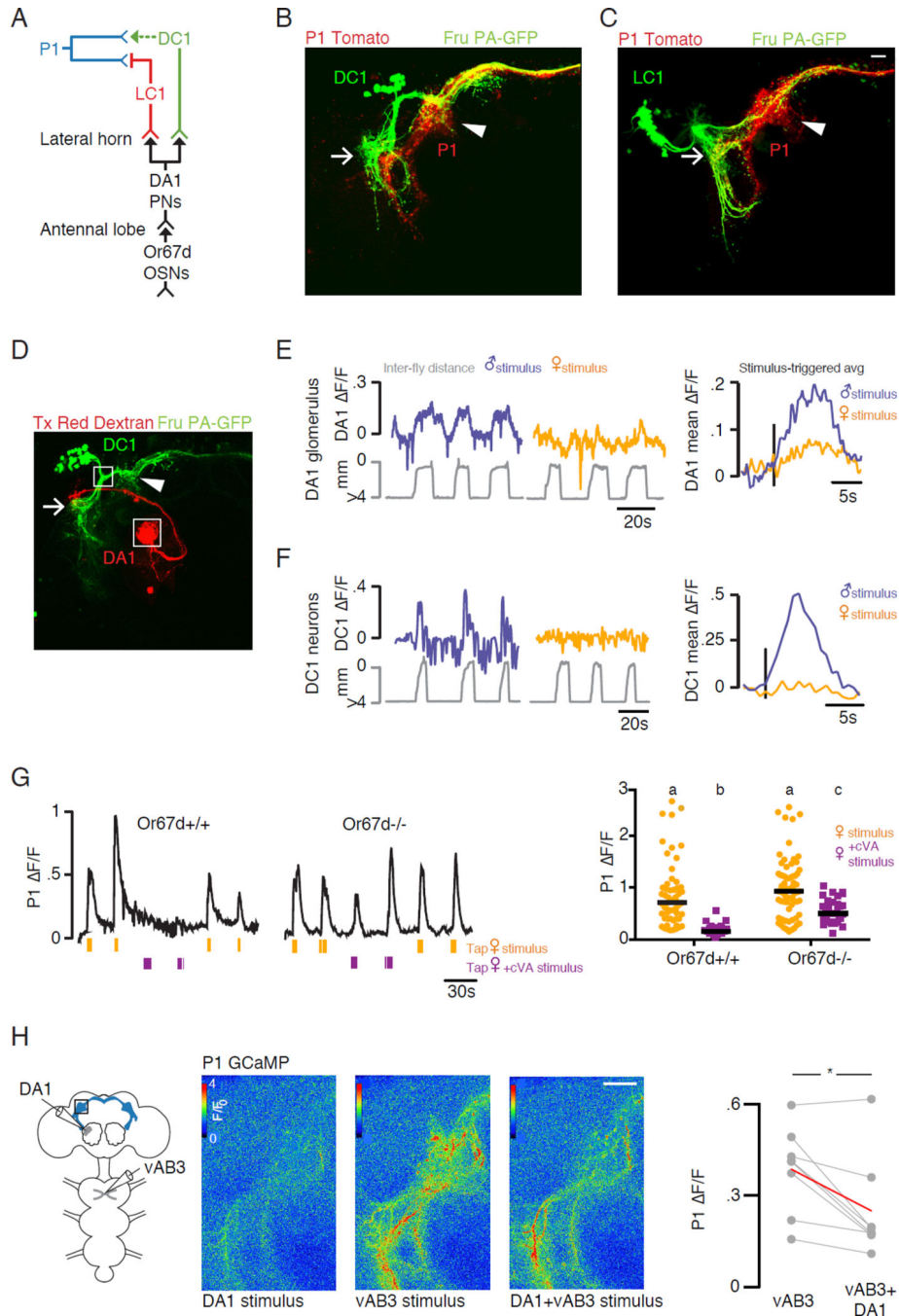
Figure 6. P1 neurons integrate gustatory and olfactory signals to encode mate desirability

(A) Circuit model of cVA pathway to P1 neurons

(B–D) DC1 neurons (green, B, D) or LC1 neurons (green, C) were labeled by photoactivation of PA-GFP expressed under Fru^{LexA}; P1 neurons (red, B, C) were labeled by expression of Tomato using *R71G01-Gal4*. DA1 projection neurons (PNs) were labeled by Texas Red Dextran electroporation (red, D). Arrows indicate lateral horn (site of DA1 projection neuron synapses with DC1 and LC1), arrowheads highlight overlapping neurites

in the lateral protocerebral complex. Boxed areas indicate anatomic sites imaged in (E, F). Fluorescence from nonphotoactivated structures has been masked.

(E–F) Responses of DA1 neurons (E) and DC1 neurons (F) to approach of a single fly. GCaMP was expressed using Fru^{Gal4}. Representative responses (left) and average of ~18 stimuli from 5–7 animals, aligned to when the stimulus is 4mm from the male's antennae (right).

(G) Representative P1 neuron responses to interleaved touch of virgin females or virgin females perfumed with cVA, in males with or without an intact Or67d olfactory receptor (left). Pooled tapping responses (2–4 per animal from 5–6 animals of each genotype) and median response shown at right. Letter groups show results of two-way ANOVA with Tukey's correction.

(H) Imaging of P1 neurons expressing GCaMP in response to stimulation of vAB3 and/or DA1. Representative multi-plane GCaMP image (left, 3 planes, ~10um) and paired comparison across 8 flies (right). mAL was severed to maximize P1 responses to vAB3. Significance: paired t-test.
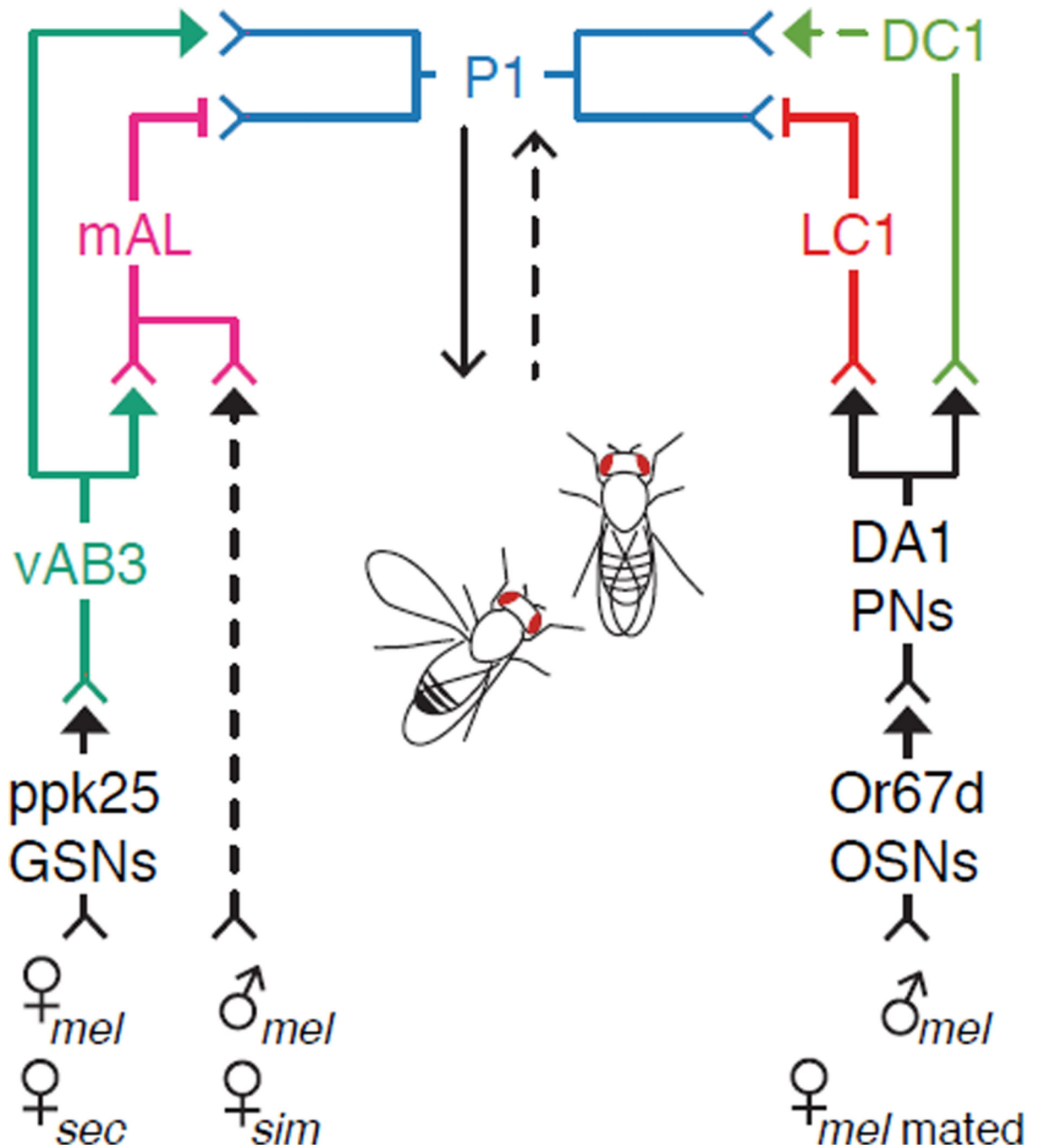
Figure 7. Circuit mechanisms regulating P1 neuron response to social stimuli
Olfactory and gustatory afferents converge functionally on P1 neurons in the lateral protocerebral complex. Dashed lines indicate potential functional connections not explored in this study. P1 neurons drive male courtship and are active during the male's enactment of courtship.