Cell

Activation of Pheromone-Sensitive Neurons Is Mediated by Conformational Activation of Pheromone-Binding Protein

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DOI 10.1016/j.cell.2008.04.046

SUMMARY

Detection of volatile odorants by olfactory neurons is thought to result from direct activation of seven-transmembrane odorant receptors by odor molecules. Here, we show that detection of the *Drosophila* pheromone, 11-*cis* vaccenyl acetate (cVA), is instead mediated by pheromone-induced conformational shifts in the extracellular pheromone-binding protein, LUSH. We show that LUSH undergoes a pheromone-specific conformational change that triggers the firing of pheromone-sensitive neurons. Amino acid substitutions in LUSH that are predicted to reduce or enhance the conformational shift alter sensitivity to cVA as predicted in vivo. One substitution, LUSH^{D118A}, produces a dominant-active LUSH protein that stimulates T1 neurons through the neuronal receptor components Or67d and SNMP in the complete absence of pheromone. Structural analysis of LUSH^{D118A} reveals that it closely resembles cVA-bound LUSH. Therefore, the pheromone-binding protein is an inactive, extracellular ligand converted by pheromone molecules into an activator of pheromone-sensitive neurons and reveals a distinct paradigm for detection of odorants.

INTRODUCTION

The antenna is the principal anatomical substrate for olfactory signaling in *Drosophila*. On the antenna, there are three morphologically distinct types of sensilla that contain odor and pheromone-sensitive receptor neurons: basiconic and coeloconic sensilla house neurons that detect general odorants, while trichoid sensilla are thought to be specialized for pheromone reception (reviewed in Benton, 2007; Smith, 2007; Ebbs and Amrein, 2007; Ejima et al., 2007; Hallem et al., 2006; Vosshall and Stocker, 2007). A subset of trichoid sensilla, the T1 sensilla, contains neurons that are activated specifically by the male-produced pheromone, 11-*cis* vaccenyl acetate (cVA) (Brieger and Butterworth, 1970; Butterworth, 1969; Clyne et al., 1997; Kurtovic et al., 2007; Xu et al., 2005). T1 sensilla are present in both males and females and show similar responses to cVA (Xu et al., 2005). cVA perception mediates a variety of behaviors, including aggregation, mate recognition, and sexual behavior (Ejima et al., 2007; Kurtovic et al., 2007; Vander Meer et al., 1986; Xu et al., 2005).

Like vertebrates, insects express a large family of odorant receptors in the olfactory structures. Each receptor is expressed in a small subset of olfactory neurons that innervate the same glomerulus, the first synaptic relay in the olfactory perception pathway (Buck and Axel, 1991; Clyne et al., 1999; Vosshall et al., 2000). Members of the *Drosophila* odorant receptor family are necessary and sufficient to confer sensitivity to many food odors, as misexpression of individual Or genes in fly olfactory neurons lacking endogenous receptors often confers the odor specificity of the misexpressed receptor (Hallem and Carlson, 2004b, 2006). However, cVA pheromone perception is more complex, requiring a specific odorant receptor, Or67d, and at least two additional gene products, SNMP and LUSH. Or67d is expressed exclusively in T1 neurons and mediates cVA responses (Ha and Smith, 2006; Kurtovic et al., 2007). The CD36 homolog SNMP is expressed in most or all trichoid neurons and is required for sensitivity to cVA (Benton et al., 2007; Jin et al., 2008). SNMP colocalizes with the odorant receptor complex in T1 neuron dendrites (Benton et al., 2007), and antiserum to SNMP infused into the lymph of T1 sensilla phenocopies SNMP loss-of-function mutants, suggesting that SNMP directly mediates pheromone sensitivity (Jin et al., 2008).

Unlike the neuronal products Or67d and SNMP, LUSH is secreted into the sensillum lymph of all trichoid sensilla by non-neuronal support cells where it bathes the dendrites of these neurons. An analysis of mutants lacking LUSH-binding protein revealed that it is required for cVA-induced behavior and normal cVA sensitivity of the T1 neurons (Xu et al., 2005). Indeed, when Or67d is misexpressed in other trichoid neurons that are normally unresponsive to cVA, this receptor confers cVA sensitivity, but only in the presence of LUSH (Ha and Smith, 2006). How

odorant-binding proteins influence pheromone reception is poorly understood, but it is an important question because their essential role in pheromone perception makes them potential targets to manipulate insect behaviors triggered by pheromones (reviewed in Benton, 2007; Smith, 2007). The current model for pheromone-binding protein function is that they act as carriers that transport and release the pheromone molecules at the dendritic surface, and released pheromone binds and activates pheromone receptors on the dendrites (Benton et al., 2007; Horst et al., 2001; Leal, 2005; Lee et al., 2002; Sandler et al., 2000; Wojtasek and Leal, 1999). However, we noted an inconsistency between the phenotype of the *lush*^{1} mutants and this model. In addition to their loss of cVA sensitivity, *lush*^{1} mutants also have a 400-fold reduction in spontaneous neuronal activity that is restricted to the pheromone-sensitive neurons. This reduced basal activity is reversed by introducing a wild-type *lush* transgene or by directly infusing recombinant LUSH protein into the sensillum lymph through the recording pipette (Xu et al., 2005). Loss of an extracellular carrier protein would not be expected to affect the spontaneous activity of these neurons. To account for this activity, we postulated that perhaps the binding protein is actually a component of the ligand that normally activates these neurons. For example, pheromone-bound LUSH might be a specific trigger for T1 neurons, and LUSH itself is a weak agonist. Using a combination of structural analysis together with in vivo pheromone sensitivity assays with engineered binding proteins, we now show that conformational changes in the binding protein are sufficient for activation of the pheromone-sensitive neurons.

RESULTS

LUSH Tunes Pheromone-Sensitive Neurons to Physiologically Relevant Levels of 11-*cis* Vaccenyl Acetate

Insect pheromone detection systems are among the most sensitive chemosensors known, approaching single pheromone molecule sensitivity (Kaissling and Priesner, 1970). Recent work has shown that cVA pheromone can activate Or67d-expressing neurons in the absence of LUSH, but high concentrations are required that are orders of magnitude over the threshold levels that activate T1 neurons (Benton et al., 2007; van der Goes van Naters and Carlson, 2007; and see Figure S5 available online). Therefore, we sought to estimate the contribution of LUSH toward sensitizing the T1 neurons to cVA pheromone. Figure 1 shows the responses of T1 neurons from wild-type, *lush*^{1} mutants, and *lush*^{1} mutants reconstituted with recombinant LUSH protein infused through the recording pipette (Xu et al., 2005) to various levels of cVA pheromone. Wild-type T1 neurons respond to all cVA concentrations over 0.03% (see Experimental Procedures) with a burst of action potentials, and the spike rates correlate well with the cVA concentration (Figure 1A). *lush*^{1} mutants, completely lacking LUSH protein (Kim et al., 1998), fail to detect cVA except for very weak responses to the 100% cVA pulse (Figure 1B, squares). This represents a loss of sensitivity of over 500-fold when LUSH is absent. The loss of cVA sensitivity in *lush*^{1} mutants is clearly due to the absence of LUSH protein, because the sensitivity deficit is completely reversed when recombinant LUSH protein is added to *lush*^{1} mutant T1 sensilla through the recording pipette (Figure 1B, diamonds). Remarkably, LUSH does not sensitize T1 neurons to structurally similar volatiles, including 11-*cis* vaccenyl alcohol or 11-*cis* vaccenic acid (Figures 1A and 1B). Therefore, LUSH protein selectively increases the sensitivity of T1 neurons to cVA.

We attempted to estimate the cVA levels that are encountered between single flies by measuring the action potential rates elicited in virgin female T1 neurons by a single male fly. cVA is secreted onto the cuticle surface of males where it is thought to mediate sexual recognition behavior. Figure 1C shows that action potentials are elicited in T1 neurons from wild-type virgin females (with no previous exposure to cVA pheromone) as a male abdomen is moved into proximity. Significant increases in spikes (above 5 spikes/s) are first observed when the male fly is within 1 cm, suggesting that cVA perception between single animals normally operates over a limited distance. We correlated these spike rates with the distance from the source. The action potential rates increased inversely with distance up to 50 spikes/s at 0.1 mm. Based on these data, action potentials in the 5–50 spikes/s range probably represent levels of cVA stimulation that are physiologically relevant during single fly interactions. These spike rates correlate to cVA stimulation of 0.03% to 10% cVA delivered by our apparatus (see Experimental Procedures). Consistent with our current and previous findings, *lush*^{1} mutant flies are completely insensitive to cVA at these levels (Figures 1B and 1C and Xu et al., 2005). T1 responses were not elicited by female abdomens, confirming that cVA is the active volatile component (Figure 1D).

Structure of the LUSH-cVA Complex

LUSH specifically sensitizes T1 neurons to cVA, but what is the mechanism of this sensitization? Because the loss of an extracellular carrier protein would not be predicted to affect spontaneous activity of neurons, we suspected that a unique LUSH-cVA surface or perhaps a pheromone-induced conformational change in LUSH might mediate T1 neuron activation. Therefore, we solved the X-ray crystal structure of LUSH bound to cVA pheromone and compared it to the previously solved structure of the apoprotein (Thode et al., 2008) and alcohol-bound forms (Kruse et al., 2003). Figure 2A shows the crystal structure of the LUSH-cVA complex solved at a resolution of 1.4 Å. The overall structure is similar to the previously solved structures and has six α helices (α1–α6) surrounding a central ligand-binding cavity (Figures 2A–2C). In the crystal, there are two protein molecules in the asymmetric unit (arbitrarily labeled A and B), and one of these monomers (monomer A) exists in two distinct conformations (described below). The average pairwise backbone root mean squared deviation (RMSD) between these two monomers is 0.55 Å. The average RMSD compared to the LUSH-butanol structure (PDB ID 1OOH) is 0.64 Å and is 0.63 Å compared to the apo-LUSH structure (PDB ID 1T14). In contrast, the RMSD between the crystal structures of the apo-protein and the LUSH-alcohol structures is 0.14 Å, showing that they are essentially identical.

Each monomer of the LUSH-cVA complex contains a single molecule of cVA (Figure 2D) that completely fills the central ligand-binding pocket. The cVA molecule itself is almost

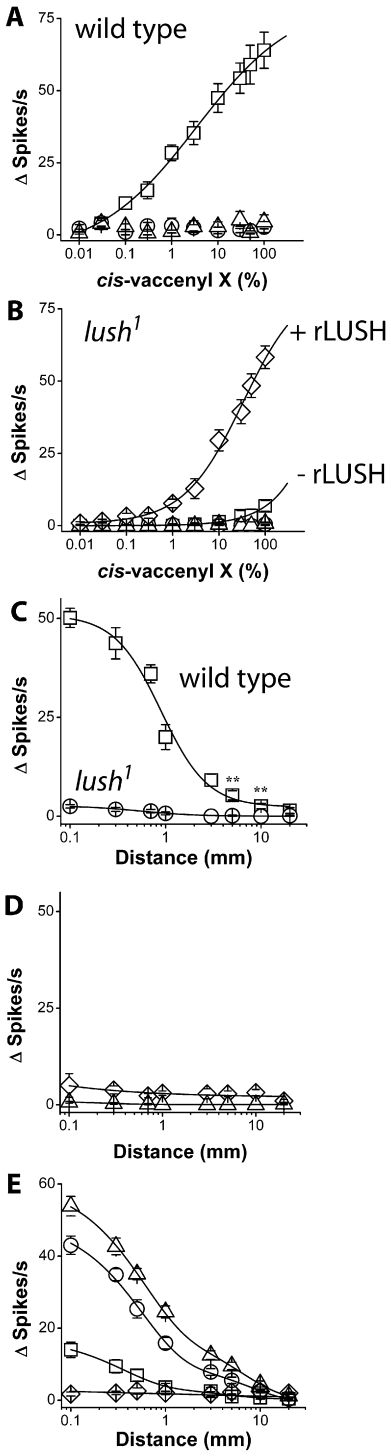

Figure 1. LUSH Selectively Sensitizes T1 Neurons to cVA Pheromone

(A) Comparison of evoked activity in T1 neurons in wild-type flies with *cis*-vaccenyl acetate (squares), *cis*-vaccenyl alcohol (circles), and *cis*-vaccenic acid (triangles).

(B) Comparison of evoked activity in T1 neurons in *lush*^{1} mutant flies with *cis*-vaccenyl acetate (squares), *cis*-vaccenyl alcohol (circles), and *cis*-vaccenic acid (triangles). The T1 sensitivity in *lush*^{1} mutant flies was restored to cVA by infusion of recombinant LUSH protein (+rLUSH, diamonds). Recombinant protein was infused at 3 mg/ml in the recording solution.

(C) T1 neurons from wild-type virgin females (squares) respond to the approach of a wild-type male as a function of distance. T1 sensilla from *lush*^{1} mutant virgin females (circles) do not. The responses of wild-type are significant over *lush*^{1} mutants even out to 10 mm (**p = 0.001).

(D) Wild-type (diamonds) or *lush*^{1} mutant (triangles) T1 neurons show little activity to the approach of a wild-type female.

(E) T1 neurons from *lush*^{1} mutant virgin females were infused with various recombinant LUSH proteins, and the response to the approach of a wild-type male was measured as a function of distance. Triangle symbols denote LUSH^{F121W}; circles, wild-type LUSH; squares, LUSH^{F121A}; and diamonds, LUSH^{D118A}. The means and standard errors were calculated, and differences among groups were evaluated using ANOVA. For Δ spike per second measurements, action potentials were counted 1 s after cVA stimulation, and the numbers of spikes 1 s before the stimulus were subtracted to obtain the Δ spike counts. All recordings were performed from single sensilla from a single fly to avoid any effects from previous cVA exposure. Data are represented as mean ± SEM.

completely enveloped by the protein. There is only a small "dumbbell"-shaped opening to the surface, ~7 Å long and 3.9 Å at its widest point, which narrows to 2.4 Å at the center (Figure S1A). Atoms from residues 1, 9, 13, 54, 55, 75, and 76 form the restriction of this opening (Figure S1B). A small patch of cVA, ~0.5–1 Å, is accessible to solvent through this opening, but there does not appear to be a unique LUSH-cVA surface that might be recognized by a receptor. This is similar to the binding of bombykol to the *Bombyx mori* pheromone-binding protein (Lartigue et al., 2004).

The acetate moiety of cVA forms polar interactions with T57 and S52 at one end of the pocket (Figure 2C). The positioning of the acetate head group is reinforced by interactions with three aromatic residues: F64, F113, and W123 (Figure 2C). In contrast, the remainder of the alkyl chain of cVA adopts different conformations in the two monomers of the crystallographic asymmetric unit such that it coils up inside the cavity in the opposite direction in monomer A compared to monomer B. However, both binding modes form interactions with F121 at the opposite end of the binding pocket from the acetate head group (Figures 2B and 2C). The interaction with F121 appears to mediate pheromone-specific conformational shifts of amino acids in the C-terminal region, including Q120 and D118, and results in the disruption of a salt bridge between D118 and K87 normally present in both the alcohol-bound and apo-LUSH structures (green in Figure 2B), but absent in the LUSH-cVA structure (blue in Figure 2B). These conformational shifts are unique to the cVA-LUSH structure and are significantly different from the apo or alcohol-bound LUSH structures (Kruse et al., 2003; Thode et al., 2008). Therefore, the structural analysis indicates that unique conformational changes occur upon cVA binding that could be important in T1 neuron activation.

The most extensive changes due to the interactions of cVA with F121 are observed in monomer B of the crystal structure (Figure S2, blue structure), where the double bond of cVA is oriented pointing toward the ring of F121. This produces an outward shift of the ring by 2.1 Å (Figure 2B) and a shift in the side chain of Q120 by ~7.6 Å. Other conformational changes are observed in the loop connecting helices 2 and 3, through an interaction mediated by F36, L31, and R30 that ultimately results in the ring of F38 being flipped out into solution. This creates

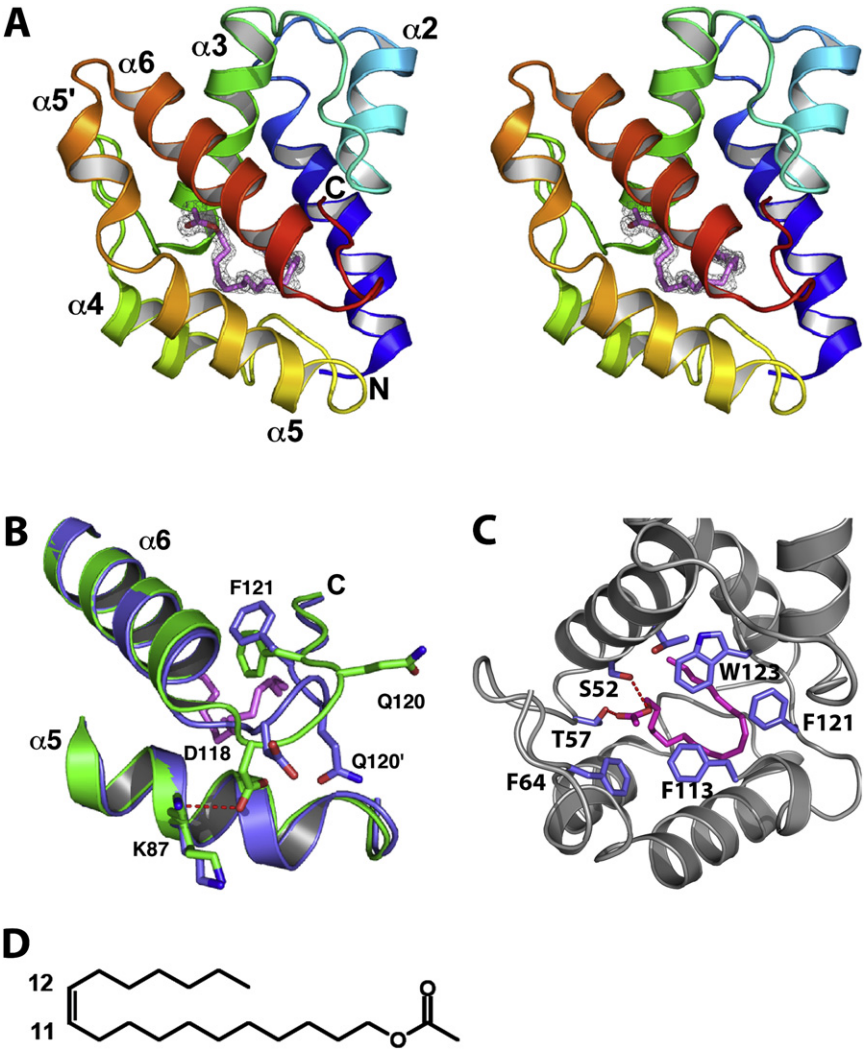

Figure 2. cVA Induces Conformational Changes in LUSH

(A) Stereo diagram of monomer B of the LUSH-cVA structure. cVA (magenta) binds in the hydrophobic cavity in the center of the molecule. The electron density for the cVA in a $2F_o - F_c$ map contoured at 1 σ is shown in gray. The 117–121 loop is shown in red.

(B) Interaction of cVA with F121 induces a conformational change in the loop between residues 117 and 121 compared to previously solved LUSH structures. The LUSH-cVA complex is shown in blue, and the corresponding region of the apo-LUSH structure (PDB ID 1T14) is shown in green. The cVA is shown in magenta.

(C) Cut-away view of ligand-binding pocket. cVA, shown in magenta, completely fills the pocket with the acetate group making polar interactions with T57 and S52 and is held in place by F64, F113, and W123. The double bond of cVA interacts with F121 at the opposite end of the binding pocket.

(D) The chemical structure of 11 *cis*-vaccenyl acetate (cVA).

a small invagination on the surface of the protein of ~150 Å that is open to solvent that could potentially function as a recognition site for binding partners. In monomer A, the double bond of cVA is oriented so that it points away from F121 (Figure S2, orange structure). This produces smaller conformational shifts compared to those seen in monomer B. For example, we do not observe any conformational shift of F36 or F38 in monomer A. In addition, we also observe that the loop between residues 116–121 adopts two different conformations. One of these appears to be a result of partial occupancy of the binding site with cVA, while the other is a consequence of an alternative binding mode of cVA compared to that seen in monomer B. Therefore, there are multiple binding modes of cVA to LUSH that do not result in one unique protein conformation. However, as both monomers alter the structure through F121, we proceeded to generate mutations to evaluate the effect of larger or smaller amino acid side chains at this position.

F121 of LUSH Mediates Olfactory Responses to cVA

Given the central role of F121 in triggering the conformational changes induced by cVA binding, we postulated that if conformational changes in LUSH mediate activation of olfactory neurons in T1 trichoid sensilla, then substitution of F121 with larger amino acids might potentiate these conformational changes while smaller residues at 121 might minimize them. We replaced F121 with alanine ($LUSH^{F121A}$), predicting its smaller side chain would reduce or prevent the cVA-induced conformational shift, but should not significantly alter binding of cVA. Figure 3A shows that recombinant $LUSH^{F121A}$ protein infused through the recording pipette has a similar time course for action potential recovery in *lush*^{1} mutants compared to infusion of wild-type recombinant LUSH at the same concentration. This indicates that both proteins have similar diffusion characteristics. However, $LUSH^{F121A}$ has a greatly reduced capacity to confer cVA sensitivity to the T1 neurons. Dose-response curves reveal that T1 sensilla containing $LUSH^{F121A}$ are ~50-fold less sensitive to cVA compared to the wild-type protein at the same concentrations (Figure 3B). Importantly, $LUSH^{F121A}$ has a similar binding affinity for cVA compared to the wild-type protein (Figure S3). Interestingly, in the absence of cVA, $LUSH^{F121A}$ did not restore spontaneous activity to normal rates (Figure 3E). Therefore, altered conformational changes in $LUSH^{F121A}$ may underlie both the reduced cVA sensitivity and the ability to induce spontaneous activity.

If a smaller residue reduces the ability of LUSH to activate T1 neurons, perhaps a bulkier residue substituted for F121 will enhance the conformational cVA-induced shift in LUSH. We replaced F121 with tryptophan ($LUSH^{F121W}$) to test this possibility. Indeed, sensilla containing $LUSH^{F121W}$ show a 5-fold enhanced sensitivity to cVA compared to wild-type LUSH infused at the same concentration. Spontaneous activity in the absence of

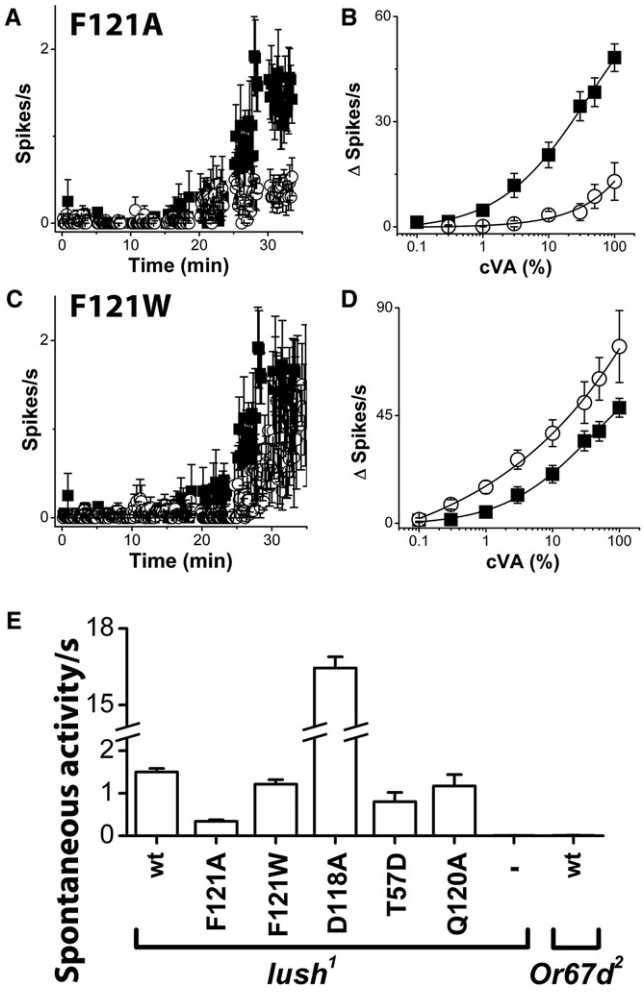

Figure 3. Smaller Amino Acid at Position 121 Reduces cVA Sensitivity In Vivo, While Large Residues Increase Sensitivity

(A) Quantitation of single sensillum electrophysiological recordings comparing the effect of diffusing either wild-type or F121A-substituted LUSH into the sensillum lymph of *lush*^{1} mutant T1 sensilla. The recovery of spontaneous activity is significantly reduced with LUSH^{F121A} (open circles) compared to wild-type LUSH (black squares) when 3 mg/ml of recombinant wild-type LUSH or LUSH^{F121A} protein is introduced through the recording pipette. Ringer solution without recombinant protein fails to restore spontaneous activity (data not shown).

(B) Dose-response curve for cVA with recombinant wild-type (squares) or LUSH^{F121A} (circles) infused into *lush*^{1} mutant T1 sensilla. No significant neuronal activity is observed with LUSH^{F121A} until 50% cVA or higher is applied ($p < 0.001$, ANOVA). Curves are fitted with a sigmoidal function derived from the Hill equation.

(C) Single sensillum recordings comparing the time course for recovery of spontaneous activity when wild-type (black squares) or LUSH^{F121W} (circles) are diffused into lymph of T1 sensilla in *lush*^{1} mutant flies.

(D) Dose-response curves for LUSH^{F121W} (circles) compared to wild-type LUSH protein (black squares). In each case, the protein concentration in the recording pipette was 3 mg/ml. For (B), (D), and (E), sensilla were allowed to equilibrate for 35 min prior to application of the cVA stimulus (from 0.1% to 100% cVA concentration).

(E) Quantitation of spontaneous activity induced by wild-type LUSH or various LUSH mutants. *lush*^{1} mutants and *Or67d*^{2} mutants lack spontaneous activity, but infusion of wild-type, F121W, T57D, or Q120A mutant LUSH proteins into *lush*^{1} mutant T1 sensilla confers normal spontaneous activity, and none are significantly different from each other ($p < 0.01$). F121A induces significantly less spontaneous activity than any of the other proteins ($p < 0.007$). The dominantly activating mutant LUSH^{D118A} triggers robust activity (note the scale break). Data are represented as mean ± SEM.

cVA was restored to normal. Therefore, LUSH^{F121W} functions even better than the wild-type protein in sensitizing T1 neurons to cVA but is equivalent to wild-type LUSH in triggering spontaneous activity (Figures 3C–3E). Essentially identical results were obtained when a male abdomen was used as the stimulus (Figure 1E). We also analyzed the effects of mutating Q120A and T57D, two residues that are shifted in position by cVA. Like F121W, both mutants restored spontaneous activity to normal rates (Figure 3E), but each mutant reduced cVA sensitivity ~50% (Figure S4). Together, these data support the idea that the conformational shifts in LUSH induced by cVA binding could underlie T1 neuron activation.

D118A-Substituted LUSH Activates T1 Neurons in the Absence of Pheromone

cVA binding to LUSH results in the disruption of a salt bridge between residues D118 and K87 (Figure 2B). If this salt bridge maintains LUSH in the inactive state, then disruption of this interaction by mutagenesis of one of the residues might produce an activated conformation. We made recombinant LUSH with residue D118 substituted with alanine (LUSH^{D118A}). Infusion of LUSH^{D118A} into the lymph of T1 sensilla from *lush*^{1} null mutants induced high levels of T1 activation in the absence of cVA (Figures 4A and 4B). LUSH^{D118A} activates these neurons at rates comparable to 1% cVA stimulation in wild-type animals (Figures 4A and 4B). Significantly, the effect of LUSH^{D118A} is specific to T1 neurons, as infusion of this protein into other trichoid sensilla or basiconic sensilla has no effect on the behavior of these neurons (Figures 4D and 4E). Thus, pheromone molecules are not absolutely required for T1 activation. T1 sensilla infused with LUSH^{D118A} show no additional increase in activity when stimulated with cVA (Figure 4C), possibly indicating that the protein has adopted an activated conformation that is not further activated by cVA.

Dominant LUSH^{D118A} Activates T1 Neurons through Or67d and SNMP

LUSH^{D118A} activates T1 neurons, but not basiconic or non-T1 trichoid neurons, suggesting that this activation phenotype is specific to T1 neurons. To further define how LUSH^{D118A} activates T1 neurons, we tested the effect of LUSH^{D118A} infusion in mutants lacking Or67d (Kurtovic et al., 2007). Figure 5 shows that LUSH^{D118A} infusion into *Or67d*^{2} mutant T1 sensilla has no effect on T1 neurons. Likewise, infusion of LUSH^{D118A} into mutants lacking the *Drosophila* CD36 homolog, SNMP (*Snmp*$^{Z3\text{-}0429}$), are not activated by LUSH^{D118A} (Figure 5). Therefore, both SNMP and Or67d are required for T1 activation by LUSH^{D118A} in the absence of cVA. Taken together, these results allow us to make two important conclusions. First, LUSH is the ligand for the pheromone-sensitive neurons, and the role of cVA is to stimulate its conversion into the activating conformation. Second, SNMP and Or67d are required for sensitivity to activated

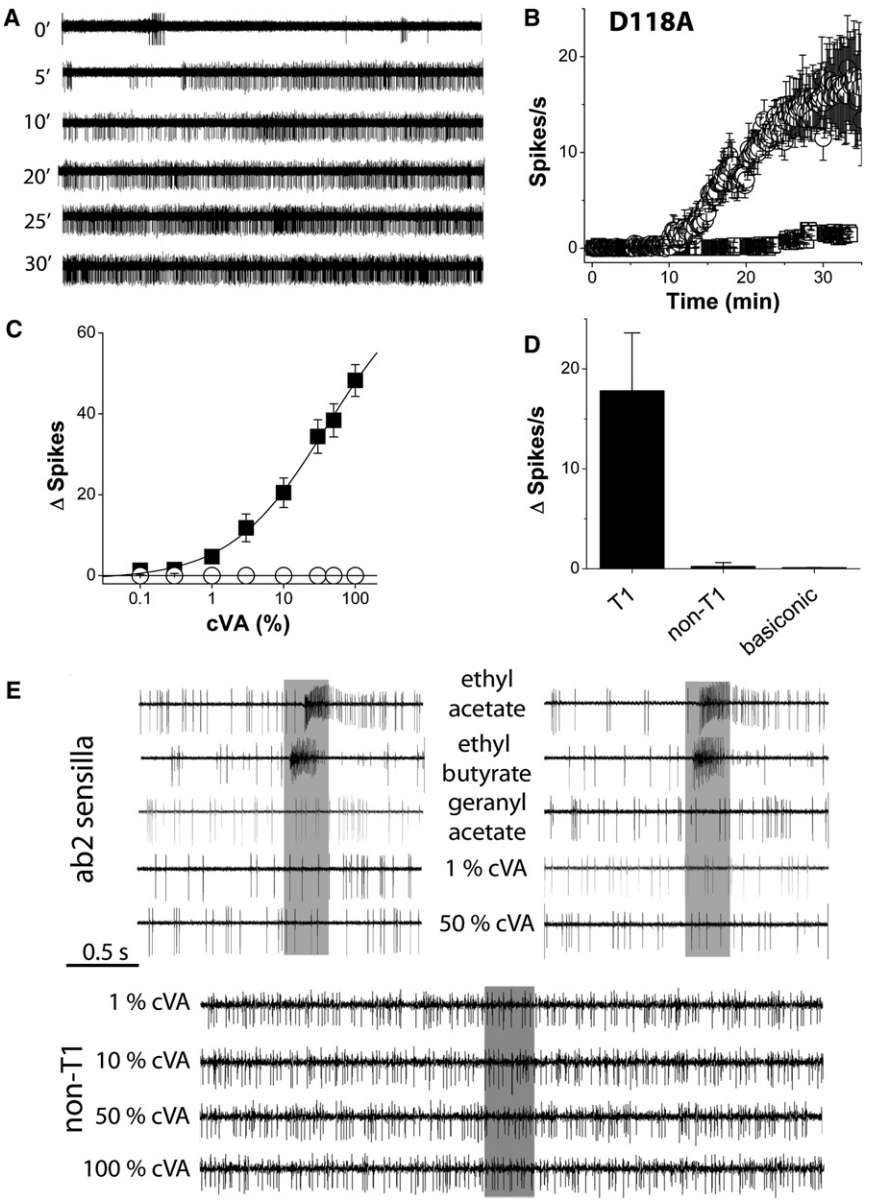

Figure 4. LUSH^{D118A} Mutant Activates T1 Neurons in the Absence of cVA Pheromone

(A) Traces of single sensillum recordings to monitor spontaneous activity induced by LUSH^{D118A} in T1 sensilla in the *lush*^{1} mutant background.

(B) The time course for induced spontaneous activity is shorter for LUSH^{D118A} (circles) than wild-type LUSH (squares) protein. Also, the level of activity produced by LUSH^{D118A} is dramatically higher in LUSH^{D118A} compared to the wild-type LUSH.

(C) *lush*^{1} mutant T1 neurons are activated by cVA when wild-type recombinant LUSH protein is infused through the recording pipette (black squares). However, cVA does not further activate T1 neurons when LUSH^{D118A} is infused (circles).

(D) Recombinant LUSH^{D118A} activates *lush*^{1} mutant T1 neurons in the absence of cVA but does not activate neurons from other sensilla, including non-T1 neurons and basiconic neurons. Bar graphs represent mean responses before and after LUSH^{D118A} infusion ± SEM (n = 10–28). T1 responses were significantly different than the other neuron types ($p < 0.0001$ ANOVA).

(E) Odor-evoked and spontaneous activity from ab2 large basiconic neurons or non-T1 trichoid neurons is not affected by LUSH^{D118A}. Similar results were seen for ab1–ab7 sensilla types (data not shown). Gray bars represent odor stimulus (300 ms).

LUSH and function downstream of LUSH in the T1 activation pathway.

LUSH^{D118A} Mimics the Pheromone-Bound Form of LUSH in the Absence of Pheromone

To determine how the D118A mutation produces a dominant-active LUSH protein, we solved the X-ray crystal structure of the apo form of LUSH^{D118A} to 2.0 Å resolutions (Table 1). We found that disruption of the D118-K87 salt bridge in the D118A mutation results in a conformational shift in the C terminus (cyan in Figure 6) that is virtually indistinguishable from the LUSH-cVA structure (blue in Figures 6A–6C). The average backbone RMSD of the entire protein is 0.51 Å. For the loop between residues 116–121, the RMSD between LUSH^{D118A} and monomer B of the cVA complex is 0.85 Å, but it is 1.29 Å between LUSH^{D118A} and the apo-protein. The only difference we observe is that F121, which normally triggers the conformational change when cVA is bound, is in the same position found in the apo and alcohol-bound protein (yellow in Figure 6B). We postulate that binding of cVA to the wild-type protein induces the conformational change in the 117–121 loop by sterically displacing F121. In contrast, the substitution of D118 works from the other end of the loop by eliminating an interaction that restrains the conformation of the loop but still allows F121 to adopt the same conformation seen in the apo-protein. Ultimately, the effects of the D118A substitution and cVA binding to the wild-type protein produce the same conformational change in the C-terminal loop of the protein (Figures 6A and 6C). Together, these data indicate that conformational changes in LUSH mediate T1 neuron activation through Or67d and SNMP and that LUSH is not acting as a passive carrier of cVA pheromone but is an inactive ligand that is converted by cVA binding into an activator of T1 neurons.

DISCUSSION

LUSH Mediates Activation of T1 Neurons to cVA In Vivo

We have shown that cVA binds to the pheromone-binding protein LUSH and induces conformational changes. Mutations predicted

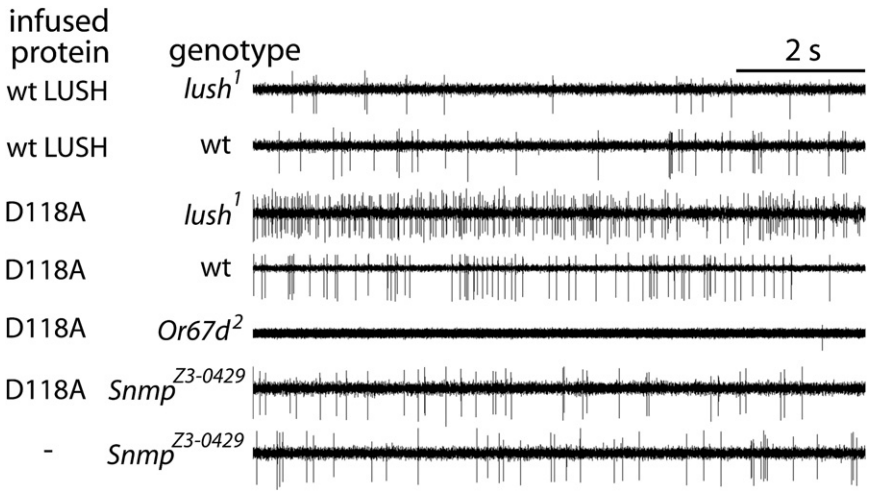

Figure 5. LUSH^{D118A} Activates T1 Neurons through Or67d and SNMP

Recombinant LUSH^{D118A} activates *lush*^{1} mutant and wild-type T1 neurons when infused into the sensillum lymph. Representative raw traces from wild-type, *lush*^{1}, *Or67d*^{2}, or *Snmp*$^{Z3\text{-}0429}$ mutant T1 sensilla infused with recombinant wild-type LUSH or LUSH^{D118A} protein. LUSH^{D118A} activates *lush*^{1} and wild-type T1 neurons but not *Or67d*^{2} mutants or *Snmp*$^{Z3\text{-}0429}$ mutants.

to reduce or enhance the conformational changes also reduce or enhance cVA sensitivity in vivo. One LUSH mutant, LUSH^{D118A}, is dominantly active, triggering robust action potentials in T1 neurons in the absence of pheromone. This effect is specific to T1 neurons, as basiconic and other trichoid olfactory neurons are unaffected by this protein. LUSH^{D118A} activates T1 neurons through the putative cVA-activated neuronal receptor components, Or67d and SNMP, accounting for the specificity of the dominant LUSH. The data reveal that pheromone molecules are not required for activation of T1 neurons and define a novel olfactory signaling paradigm in which the pheromone-induced conformational change in LUSH mediates activation of T1 neurons.

cVA can trigger weak responses in T1 neurons in the absence of LUSH when applied at high concentrations. Direct effects of cVA on Or67d/SNMP receptor complexes may mediate these LUSH-independent responses, as these two components confer marginal cVA sensitivity to the empty neuron preparation (Benton et al., 2007; Figure S5). Alternatively, activated LUSH may normally dimerize with an unknown cofactor that alone can weakly activate T1 receptors in the presence of cVA. However, the sensitivity for cVA in the absence of LUSH is so poor that *lush*^{1} mutants are blind to the pheromone in aggregation assays (Xu et al., 2005). In our proximity experiments, cVA levels emanating from single male flies are below detection limits in the absence of LUSH. Therefore, the LUSH-independent activation of T1 neurons is unlikely to play a role in cVA responses in vivo.

Table 1. Data Collection and Refinement Statistics (Molecular Replacement)

	WT LUSH + cVA	Apo – LUSH^{D118A}
Resolution (Å)	41.2–1.45	35.6–2.0
R_{work}/R_{free}	0.18/0.21	0.21/0.26
Number of atoms	2599	2064
Protein	2156	1975
Ligand/ion	22/5	20
Water	416	69
B-Factors		
Protein	10.9	32.6
Ligand/ion	20.6	60.9
Water	22.5	34.6
RMSD		
Bond lengths (Å)	0.009	0.010
Bond angles (°)	1.289	1.070

LUSH Helps Tune T1 Neurons to cVA

Olfactory neurons are thought to be tuned to odorants exclusively by the odorant receptors they express. Indeed, in *Drosophila melanogaster*, activation of many odorant receptors results from direct binding of food odorants (Hallem and Carlson, 2006; Hallem et al., 2004). Why does cVA reception require a binding protein intermediate? We suggest that the binding protein may enhance sensitivity and specificity in the pheromone detection process. If a pheromone induces a stable, ligand-specific conformational change in a binding protein, single pheromone molecules could be detectable if the neuronal receptor complex is specifically tuned to that conformation. Further, if the conformation of the binding protein that activates the receptors is specific to the pheromone-bound state, other environmental stimuli are less likely to activate the neurons, even if they interact with the binding protein. Consistent with this idea, LUSH increases the sensitivity of T1 neurons to cVA over 500-fold, but, remarkably, does not sensitize the neurons to structurally similar chemicals, such as vaccenyl alcohol or vaccenic acid (Figure 1A). Indeed, LUSH can bind a large array of chemicals (Kruse et al., 2003; Zhou et al., 2004), but only cVA activates T1 neurons. Other OBPs have been shown to bind to a wide range of unnatural compounds, including plasticizers and dyes (Lartigue et al., 2004, 2003; Zhou et al., 2004), and the electrophysiological or behavioral responses to a specific ligand do not correlate with the binding affinity of the OBP for that ligand (Dickens et al., 1997; Grant et al., 1996; Hansen, 1984; Honson et al., 2003). Therefore, binding is clearly not sufficient for sensitization. However, by utilizing a ligand-specific conformational shift in a binding protein, detection of rare pheromone molecules is possible with high fidelity and sensitivity by creating an active binding protein species that diffuses within the sensillum lymph until it contacts and activates a receptor on the dendrites.

Does Activated LUSH Trigger Or67d Receptors Directly?

We attempted to reconstitute the cVA detection pathway in basiconic neurons lacking endogenous receptors (Figure S5;

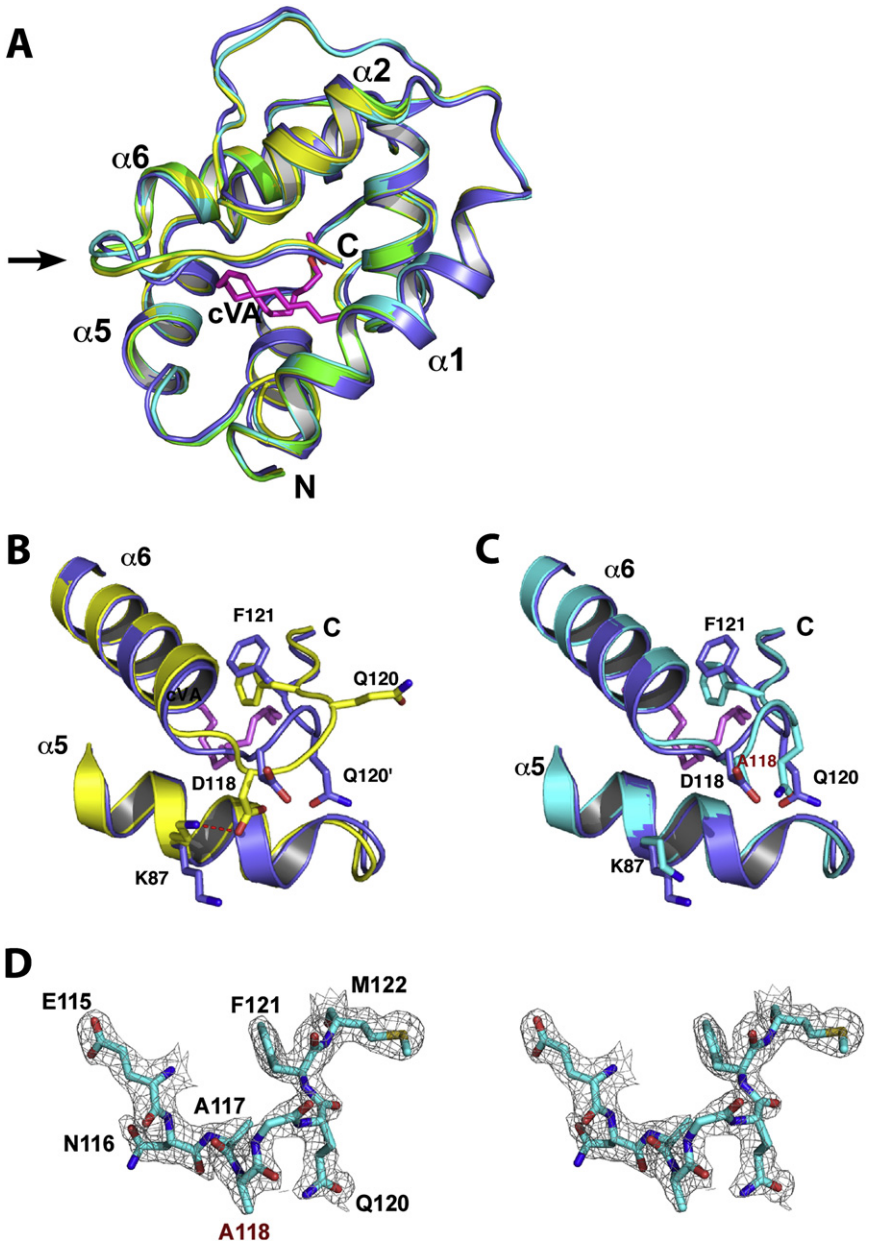

Figure 6. Structure of LUSH^{D118A} Mutant Protein Mimics cVA-Bound LUSH Protein

(A) Ribbon diagram comparing one monomer from the asymmetric unit of the LUSH-cVA complex (blue) with the LUSH^{D118A} (cyan), apo-LUSH (green), and LUSH-butanol complex (yellow). The cVA is shown as a stick model in magenta. Arrow indicates position of structural changes in the C-terminal loop shared by LUSH^{D118A} (cyan) and LUSH-cVA (blue) but distinct from the LUSH-butanol complex (yellow) and apo-LUSH (data not shown).

(B) Comparison of the structures of the 117–121 loop in the LUSH-butanol (yellow) and LUSH-cVA (blue) complexes.

(C) Comparison of the structures of the same regions between LUSH-cVA (blue) and apo-LUSH^{D118A} (cyan). LUSH-cVA and LUSH^{D118A} adopt similar conformations.

(D) Stereo representation of the electron density defining the loop between residues E115–M122 in the LUSH^{D118A} structure solved without cVA. The position of A118 is indicated in red. The electron density is from a $2F_o - F_c$ map contoured at 1 σ.

Hallem and Carlson, 2004a). Expression of SNMP, Or67d, and LUSH together in the empty neuron system failed to recapitulate T1 cVA sensitivity (Figure S5). Or67d alone was unresponsive, but adding LUSH through the recording pipette did sensitize Or67d receptors slightly to cVA in the absence of SNMP, suggesting that LUSH interacts directly with Or67d. Coexpressing SNMP and Or67d enhanced cVA sensitivity, but, surprisingly, adding LUSH failed to further enhance sensitivity. These differences between the empty neuron responses and T1 neurons may reflect reduced levels of one or more components when expressed in basiconic sensilla or, more likely, indicate that additional components are missing. Indeed, in a screen for cVA-insensitive mutants, we have recovered mutations in the known sensitivity factors as well as three additional unknown genes encoding factors that are essential for cVA sensitivity. We expect that, when all of these components are identified and expressed in the basiconic neurons, full cVA sensitivity will be conferred.

OBPs, like LUSH, are a large family of soluble proteins secreted into the lymph fluid surrounding the olfactory neurons. Proposed functions for OBPs include transporting ligands to the ORs (Horst et al., 2001; Leal, 2005; Lee et al., 2002; Sandler et al., 2000; Wojtasek and Leal, 1999), protecting the odor from degradation or deactivation by ODEs (Kaissling, 1996, 1998, 2001; Pophof, 2002), and forming a complex with an odor that either directly activates ORs (Pophof, 2004; Xu et al., 2005) or binds to other accessory proteins (Rogers et al., 1997), which ultimately results in OR activation. In vitro studies of the pheromone-binding protein (PBP) from *Bombyx mori* show that the OBP undergoes a conformational change at low pH that prevents ligand binding, suggesting that OBPs may function primarily as passive carriers and changes in the local pH stimulate pheromone release in the vicinity of the neuronal membranes (Horst et al., 2001; Leal, 2005; Lee et al., 2002; Sandler et al., 2000; Wojtasek and Leal, 1999). Furthermore, previous studies reported that high concentrations of moth pheromones can directly activate cognate pheromone receptors expressed in tissue culture and that DMSO is as effective as the pheromone-binding proteins at sensitizing the neurons to pheromone, leading to the conclusion that the binding proteins are pheromone solubilizers/carriers (Grosse-Wilde et al., 2007). However, similar studies implicate the binding proteins as factors

in receptor specificity (Grosse-Wilde et al., 2006; Pophof, 2002). Our data support the latter view. We note that LUSH homologs in other insects and the 12 *Drosophila* species have conserved the amino acids predicted to form the salt bridge (Figure S6). Only *Drosophila ananassae* (*D. ana*) is predicted to lack the phenylalanine corresponding to F121 in *melanogaster* (replaced by leucine, Figure S6). A similar activation mechanism, therefore, is likely to occur in these species. Recent work in rodents reveals that vertebrate pheromones can be peptides or proteins (Chamero et al., 2007; Kimoto et al., 2005; Sherborne et al., 2007). It will be interesting to determine whether the conformational activation mechanism we have identified for LUSH is conserved in analogous extracellular binding proteins in other species.

EXPERIMENTAL PROCEDURES

Drosophila Stocks

Drosophila melanogaster strains were provided by the Bloomington Stock Center. The wild-type strain used in this study was w^{1118}. $Or67d^{2}$ mutants were generously provided by B.J. Dickson (Kurtovic et al., 2007), and the empty neuron stocks, +/+ ; Δhalo/*Cyo* ; *pOr22a-Gal4*, *pUAS Or67d*/TM3, were provided by J. Carlson. SNMP mutant genotype was $vainsD^{1}$: + *; bw ; st,* $vainsD^{1}$. The genotypes expressed in the empty neuron system were +/+; Δhalo/Δhalo; *pOr22a-Gal4, pUAS Or67d/ pUAS snmp* and +/+; Δhalo/Δhalo; *pOr22a-Gal4, pUAS Or67d.*

Proximity Experiments with Live Animals

We used 2- to 4-hr-old virgin male and female flies raised in isolation for these experiments to avoid crosscontamination among flies. To mimic T1 responses to the proximity of a male, we recorded from virgin female T1 sensilla and introduced a live male fly to various distances using a micromanipulator under still-air conditions. Male flies first induced activity in female T1 neurons at 1 cm and spike rates increased to 50 Hz to 0.1 mm.

Protein Expression, Purification, and Mutagenesis

Expression of LUSH proteins lacking the N-terminal signal peptide was performed as previously described (Bucci et al., 2006; Kruse et al., 2003). The protein was refolded and purified in the presence of 25 mM butanol, concentrated in an Amicon-stirred ultrafiltration cell and dialyzed exhaustively against 20 mM sodium phosphate (pH 6.5) to remove the alcohol. Aliquots of the final protein solution were then flash-frozen in liquid nitrogen and stored at −80°C until further use. Mutations in the $lush^{1}$ gene designed to produce single amino acid substitutions in the proteins were created using the QuikChange site-directed mutagenesis protocol (Stratagene), using the wild-type LUSH gene in the pET13a vector as a template. Mutations were confirmed by DNA sequencing. The protein was expressed and purified in the same manner as wild-type LUSH, and the molecular weight and purity of the protein were confirmed by mass spectrometry.

Protein Crystallization

The LUSH-cVA complex was prepared by adding 1.1 μl of a 10% v/v emulsion of cVA in water to 60 μl of the protein solution to produce a final concentration of ~5 mM cVA, and the resulting solution was equilibrated with gentle mixing on ice overnight. Crystals were grown using the hanging-drop method by mixing 2 ml of the protein solution with 2 ml of well solution containing 100 mM Tris, 30% PEG 4000, pH 8.5 at 18°C. Crystals of the apo $LUSH^{D118A}$ were grown using the hanging-drop method by mixing 4 μl of protein solution in 20 mM sodium phosphate (pH 6.5) in a 2:1 ratio with well solution containing 27% PEG 4000, 100 mM sodium acetate (pH 4.0). Crystals were harvested and then flash-frozen in liquid nitrogen with no other manipulations.

X-Ray Data Collection, Processing, and Model Refinement

Native X-ray diffraction data were collected at Beamline 4.2.2 (Molecular Biology Consortium) at the Advanced Light Source, Lawrence Berkeley Laboratory, Berkeley, CA. Reflection integration and scaling were performed using the program D*TREK (Pflugrath, 1999). Molecular replacement was performed with MOLREP (Vagin and Teplyakov, 1997) within the CCP4 suite of programs (Bailey, 1994), using the coordinates of the protein component of the LUSH-butanol structure (PDB ID 1OOH) (Kruse et al., 2003) as an initial model. The structure was refined by successive rounds of model building in O (Jones et al., 1991) and energy minimization using REFMAC (Murshudov et al., 1997). Water molecules were added using ARP_waters (Lamzin and Wilson, 1993). Model stereochemistry was analyzed during refinement using PROCHECK (Bailey, 1994; Vaguine et al., 1999). Data collection and refinement statistics are given in Table 1. Structure alignments were performed using LSQMAN (Kleywegt, 1996) or SUPERPOSE (Maiti et al., 2004). Visual inspection of the final structures and preparation of figures were performed using PyMOL (DeLano, 2002). Solvent accessibility calculations were performed with SurfRace (Tsodikov et al., 2002). The structures have been deposited with PDB accession codes 2GTE (LUSH-cVA complex) and 2QDI (LUSH-D118A).

Single-Sensillum Recordings and Preparation of cVA

Extracellular electrophysiological recordings were carried out according to de Bruyne et al. (1999). Flies (2- to 7-day-old, males and females) were under a constant stream of charcoal-filtered air (36 ml/min, 22°C–25°C) to prevent any potential environmental odors from inducing activity during these studies. cVA was diluted in paraffin oil, and 1 μl was applied to a filter paper and inserted in a Pasteur pipette, and air was passed over the filter and presented as the stimulus. In our hands, a cVA stimulus that consists of air passed over a filter paper impregnated with 10 nl of cVA (the 1% cVA stimulus) induces robust action potentials from T1 neurons for well over a year. The purity of the cVA was confirmed both by NMR and by mass spectroscopy. Signals were amplified 1000× and fed into a computer via a 16 bit ADC and analyzed offline with AUTOSPIKE software (USB-IDAC system; Syntech, Hilversum, the Netherlands). Low cutoff filter setting was 200 Hz, and the high cutoff was 3 kHz. Action potentials were recorded by inserting a glass electrode in the base of a sensillum. Data analysis was performed according to de Bruyne et al. (2001). Signals were recorded for 20 s or 30 s, starting 10 s before cVA stimulation. Action potentials were counted 1 s before cVA stimulation and for 1 s after cVA stimulation. All recordings were performed from separate sensilla with a maximum of two sensilla recorded from any single fly.

ACCESSION NUMBERS

The structures have been deposited with PDB accession codes 2GTE (LUSH-cVA complex) and 2QDI (LUSH-D118A).

SUPPLEMENTAL DATA

The Supplemental Data for this article, including Supplemental Experimental Procedures and Figures, can be found online at http://www.cell.com/cgi/content/full/133/7/1255/DC1/.

ACKNOWLEDGMENTS

We thank L. Hannigan for production of recombinant proteins for in vivo studies, Drs. E. Westbrook and J. Nix at Advanced Light Source beamline 4.2.2 for assistance with X-ray data collection and processing, Drs. Brian Trippet and Robert Hodges for amino acid analysis, Dr. Schoen Kruse for technical assistance in the early stages of this work, Charles Zuker for access to the "Zuker collection," Barry Dickson for $Or67d^{2}$ mutant flies, and John Carlson for the empty neurons stocks expressing Or67d. X-ray crystallography instruments at UCDHSC were purchased with funds from the Howard Hughes Medical Institute and the National Institute of Health and supported by the University of Colorado Cancer Center Core Grant. Additional support for X-ray facilities at UCDHSC and at the Advanced Light Source was provided by UCDHSC Program in Biomolecular Structure. This work was supported by a grant from the NIH R01DC02539 to D.P.S. and NIH R01 AA013618 to D.N.M.J.

Received: February 13, 2008
Revised: April 17, 2008
Accepted: April 29, 2008
Published: June 26, 2008

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