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Neuron, Vol. 45, 193–200, January 20, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2004.12.031

Drosophila OBP LUSH Is Required for Activity of Pheromone-Sensitive Neurons

Report

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Summary

Odorant binding proteins (OBPs) are extracellular proteins localized to the chemosensory systems of most terrestrial species. OBPs are expressed by nonneuronal cells and secreted into the fluid bathing olfactory neuron dendrites. Several members have been shown to interact directly with odorants, but the significance of this is not clear. We show that the *Drosophila* OBP *lush* is completely devoid of evoked activity to the pheromone 11-*cis* vaccenyl acetate (VA), revealing that this binding protein is absolutely required for activation of pheromone-sensitive chemosensory neurons. *lush* mutants are also defective for pheromone-evoked behavior. Importantly, we identify a genetic interaction between *lush* and spontaneous activity in VA-sensitive neurons in the absence of pheromone. The defects in spontaneous activity and VA sensitivity are reversed by germline transformation with a *lush* transgene or by introducing recombinant LUSH protein into mutant sensilla. These studies directly link pheromone-induced behavior with OBP-dependent activation of a subset of olfactory neurons.

Introduction

The sense of smell mediates important behaviors in insects, including localizing hosts by agricultural pests and human disease vectors. Insects detect volatile odorants with olfactory neurons sequestered in hair-like chemosensory sensilla located primarily on the antenna. Each sensillum is hollow and contains the dendrites of one to four olfactory neurons bathed in sensillum lymph. This anatomic segregation of olfactory neurons within sensilla allows for the independent regulation of the composition of sensillum lymph in different sensilla. Odorant binding proteins (OBPs) are a large, diverse family of proteins, each differentially secreted into the sensillum lymph of specific subsets of sensilla. Therefore, it is likely that OBPs play important, ligand-specific roles in olfaction. Despite our appreciation of this family for more than 20 years, the function of these proteins in olfactory signal transduction remains unclear.

First identified in moths as pheromone binding proteins (Vogt and Riddiford, 1981), a large number of insect OBP members are expressed in olfactory and taste organs (Galindo and Smith, 2001; Kim et al., 1998; Leal et al., 1998; McKenna et al., 1994; Pikielny et al., 1994; Shanbhag et al., 2001; Vogt et al., 1991; Zang et al., 2001). These extracellular proteins have been shown to directly interact with odorant ligands in several insect species (Danty et al., 1999; Du and Prestwich, 1995; Kaissling, 2001; Kruse et al., 2003; Vogt and Riddiford, 1981; Wojtasek et al., 1998), and different insect OBPs within a species bind different odorants (Plettner et al., 2000).

In insects, volatile pheromones are detected by specific subsets of olfactory neurons (reviewed in Vogt, 2003; Kaissling, 2004). Pheromones drive most social behavior in insects, triggering a plethora of responses ranging from mating, recruitment, and aggregation to aggression and dispersal (reviewed in Vander Meer et al., 1998). The molecular basis for pheromone sensitivity and how pheromones elicit innate behavior is poorly understood. However, the initial step in pheromone perception is thought to involve the interaction of pheromone with members of the odorant binding protein family.

The only mutant defective for expression of an OBP is the *Drosophila* mutant, *lush* (*obp76a*; Kim et al., 1998). LUSH is expressed exclusively in the chemosensory system in both males and females and is restricted to the approximately 150 trichoid sensilla located on the ventral-lateral surface of the third antennal segment. Trichoid sensilla are one of three major morphological classes of chemosensory hairs on the antenna (Stocker, 1994). We previously showed that *lush* mutants are defective for avoidance behavior to high concentrations of alcohols (Kim et al., 1998). Electroantennogram recordings of the summed electrical activity of the antenna in response to alcohols revealed no clear differences between wild-type and *lush* mutants. However, subtle differences might be masked by other alcohol-responsive neurons unaffected by loss of LUSH. Therefore, we set out to specifically examine the electrophysiological responses of trichoid sensilla neurons in *lush* mutants using single sensillum recording techniques to gain insight into how LUSH influences olfactory neuron activity. Indeed, we identified defects in LUSH-expressing sensilla to alcohols, consistent with our previous work. However, we report here the surprising finding that *lush* mutants have a complete loss of sensitivity to 11-*cis* vaccenyl acetate, resulting in behavioral insensitivity to this pheromone. We show this defect results directly from loss of LUSH protein in the extracellular space surrounding these olfactory neurons and present genetic evidence that LUSH interacts with unknown receptors responsible for triggering action potentials expressed in a pheromone-sensitive subset of trichoid sensilla. These studies link social behavior and VA sensitivity of T1 neurons with LUSH expression and demon-

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strate that LUSH is an essential component in pheromone signal transduction.

Results

Olfactory Neurons in T1 Sensilla Are Insensitive to VA in *lush* Mutants

Using single sensillum recording techniques (de Bruyne et al., 1999), we assayed the electrical activity of trichoid olfactory neurons from *lush* mutant animals to determine if there were defects in sensitivity to odorants compared to controls. Differences were observed between wild-type and *lush* mutants consistent with previous behavioral studies (see below). However, more stunning was the observation that a subset of trichoid neurons is completely insensitive to 11-*cis* vaccenyl acetate pheromone.

11-*cis* vaccenyl acetate has been implicated as a volatile pheromone in *Drosophila* (Bartelt et al., 1985; Vander Meer et al., 1986; Zawistowski and Richmond, 1986) and can activate a subset of trichoid olfactory neurons (Clyne et al., 1997). VA is a male-specific lipid and has been suggested to function as an aggregation cue or anti-aphrodisiac in *Drosophila melanogaster* (Bartelt et al., 1985; Jallon et al., 1981). Figure 1 shows the electrical responses of trichoid sensilla from wild-type and *lush* mutants in response to VA. Functionally, *Drosophila* trichoid sensilla have been divided into two distinct classes. Type 1 (T1) sensilla contain olfactory neurons that are excited by the *Drosophila* pheromone 11-*cis* vaccenyl acetate, while Type 2 (T2) sensilla contain neurons inhibited by alcohols (Clyne et al., 1997). LUSH is secreted exclusively into the sensillum lymph of all trichoid sensilla (Shanbhag et al., 2001). We identified both T1 and T2 sensilla, and with minor variation, the location of these two types was stereotypical from animal to animal (Figure 1A). Figure 1B shows VA-induced responses of T1 neurons from wild-type flies, *lush* mutants, and *lush* mutants transformed with a wild-type copy of the *lush* transgene. We also created and tested flies expressing the moth *Antherea polyphemus* pheromone binding protein APO3 under control of the *lush* promoter.

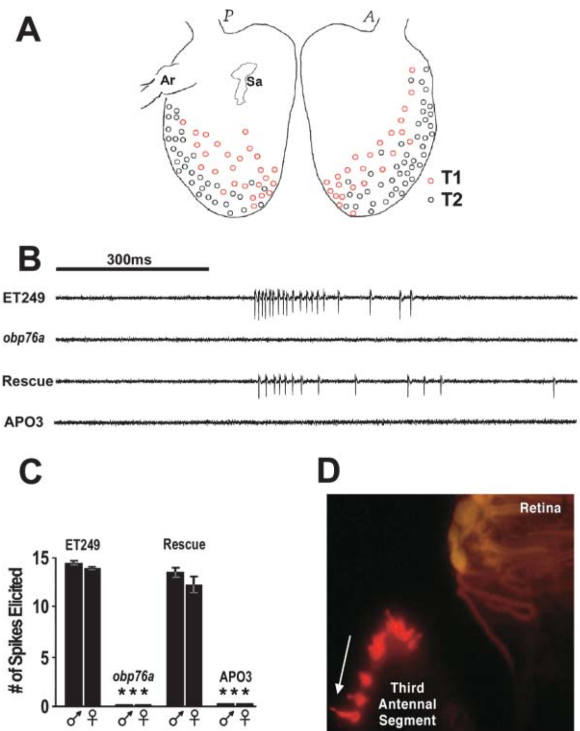

Figure 1. LUSH Is Required for 11-*cis* Vaccenyl Acetate to Evoke Action Potentials from T1 Neurons

(A) Surface representation of the anterior (right) and posterior (left) of the *Drosophila* third antennal segment. T1 sensilla tend to be localized more proximally than T2 sensilla.

(B) T1 neurons from wild-type control animals (ET249) respond to 11-*cis* vaccenyl acetate, but mutants lacking LUSH (*obp76a*) do not. *lush* mutants carrying a wild-type transgenic copy of the *lush* gene have VA responses restored (Rescue). Expression of the moth pheromone binding protein, APO-3, in *lush* mutants fails to restore VA responsiveness (APO3). OBP83a and OBP83b, which are still expressed normally in *lush* mutant trichoid sensilla (Shanbhag et al., 2001), do not compensate for loss of LUSH. Bar above the traces denotes the odor stimulation.

(C) Summary of VA-evoked activity. Graph denotes the mean number of spikes elicited by VA with SEM. No significant differences in VA responses were present between sexes. $n = 18$ for each bar.

(D) Moth pheromone binding protein APO-3 is expressed in trichoid sensilla in transgenic flies. Arrow indicates secretion of APO-3 into the sensillum lymph of a trichoid sensillum. For a schematic of the recording setup, see de Bruyne et al. (1999).

Wild-type T1 sensilla respond to VA with a robust burst of action potentials following a latency of approximately 400 ms. This latency is longer than the 100 ms typically observed for responses of basiconic sensilla neurons to odorants (de Bruyne et al., 1999). T1 sensilla from *lush* mutants are completely defective for VA-evoked action potentials. We tested a range of VA concentrations up to 100% and were unable to elicit any responses from the mutants. To eliminate the remote possibility that a second, unrelated mutation in the *lush* mutant stock was producing VA insensitivity, we introduced a wild-type, transgenic copy of *lush* regulated by its own promoter into the mutant animals. Expression of the *lush* transgene in the mutants restores LUSH expression (Kim et al., 1998) and VA sensitivity (Figures 1B and 1C, "rescue"). The moth pheromone binding protein APO3 failed to restore VA sensitivity in the *lush* mutant background, despite its presence in the sensillum lymph in the transgenic animals (Figure 1D). Furthermore, at least two other endogenous OBPs, OBP83a and OBP83b, are normally secreted into the sensillum lymph of T1 and T2 trichoid sensilla in wild-type and *lush* mutants (Shanbhag et al., 2001). None of these other OBPs, therefore, can functionally compensate for loss of LUSH in T1 sensilla. We conclude that LUSH is specifically required for VA sensitivity of T1 neurons.

LUSH Mediates VA-Induced Aggregation Behavior in *Drosophila melanogaster*

Having established that *lush* mutants are defective for detection of VA by T1 sensilla, we set out to establish whether this deficit influences behavior to VA pheromone. VA is thought to function as an aggregation pheromone (Bartelt et al., 1985). Therefore, we used odorant trap assays to test whether *Drosophila* males and females are attracted to VA-producing males or pure VA

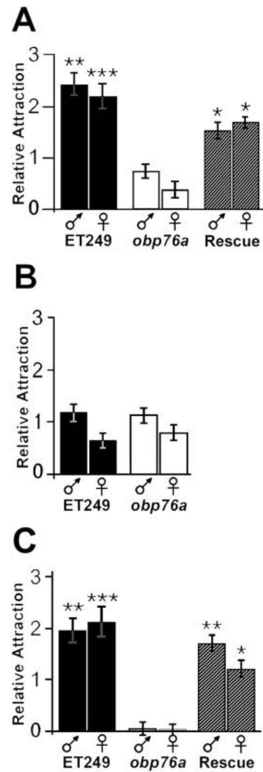

Figure 2. LUSH Mediates Social Aggregation of Male and Female *Drosophila* to Male Flies and 11-*cis* Vaccenyl Acetate

(A) Male and female control flies (ET249) are attracted to traps containing males, but mutants lacking the LUSH protein (*obp76a*) are significantly less attracted. Introduction of a wild-type transgenic copy of the *lush* gene regulated by its own promoter restores aggregation behavior (rescue). The rescue group is not significantly different from wild-type controls.

(B) Female flies in traps do not induce differences in aggregation between control and *lush* mutants.

(C) Wild-type males and females are attracted to the male-specific pheromone 11-*cis* vaccenyl acetate, but mutants lacking LUSH protein are completely defective for attraction to VA. Error bars depict SEM. *, significantly different from *lush* mutants at $p < 0.05$; **$p < 0.005$; ***$p < 0.001$.

and whether *lush* mutants are defective for responses to these cues (Kim et al., 1998; Woodard et al., 1989). Figure 2A shows that wild-type male and female flies are equally attracted to wild-type male flies placed in odor traps, consistent with previous work indicating that VA functions as an aggregation pheromone. Behavioral attraction of *lush* mutant flies to wild-type males is significantly reduced compared to control flies. When female flies are used as bait, we found that male flies showed an increased attraction compared to females, but there was no difference in this attraction between wild-type and *lush* mutants (Figure 2B). This suggests the presence of an unknown female cue preferentially attracting males, but this behavior is independent of LUSH, as no difference is observed in these mutants.

When VA was substituted for male animals to bait the odorant traps, we found even more dramatic differences between wild-type and *lush* mutants (Figure 2C). 1% VA induces attraction in wild-type flies that is similar to that observed for male flies. However, *lush* mutants are completely defective for attraction to VA (Figure 2C). This reveals that T1 neurons are solely responsible for attraction to VA pheromone in adult animals. Residual attraction to live male flies in *lush* mutants compared to pure VA indicates that other attractive cues are produced by live animals that are independent of VA and LUSH (compare Figures 2A and 2C). Mutant flies transformed with a wild-type copy of *lush* respond to VA and male flies (Figures 2A and 2C). We could identify no difference in mating latency, inappropriate mating behavior, or mating stages (Hall, 1994) in *lush* mutants compared to wild-type controls (data not shown), supporting the previous conclusion of Bartelt that VA is a cue mediating social aggregation but is probably not a sex-specific cue (Bartelt et al., 1985; Jallon et al., 1981). Together with the electrophysiology data, we conclude that VA induces attraction of *Drosophila* males and females and this attraction requires LUSH-dependent activation of T1 neurons.

A Genetic Interaction between LUSH and the T1 Neuron Action Potential Generation Machinery

T1 sensilla typically contain a single olfactory neuron with spontaneous activity in the range of 1 action potential per second (1.0 ± 0.24 action potentials per second) under charcoal-filtered air (Clyne et al., 1997). This rate of spontaneous activity is similar to spontaneous rates reported previously for other olfactory neurons in *Drosophila* (de Bruyne et al., 2001; Hallem et al., 2004). T2 sensilla contain multiple olfactory neurons (2 or 3), have higher spontaneous action potential firing rates, and are insensitive to VA (Clyne et al., 1997). We noted in our experiments that the normal amount of basal activity in the T1 neurons present in wild-type flies in the absence of odor stimulation was essentially abolished in the *lush* mutants. Instead of one spike per second, the spontaneous activity in T1 neurons from *lush* mutants is approximately 1 spike every 430 ± 55 s (compare Figures 3A and 3B). This is greater than a 400-fold reduction in spontaneous activity. The fact that we still observe activity shows that the T1 neurons are still present and functional in the *lush* mutants. No difference in spontaneous activity was observed in the T2 neurons of wild-type and *lush* mutants, despite an equivalent loss of LUSH protein from these sensilla (Figure 3). Therefore, there is a specific requirement for LUSH by T1 neurons to produce normal spontaneous activity. These differences are not due to male flies "smelling" themselves, as virgin females, which completely lack VA, showed identical responses as males. Furthermore, we eliminated rig contamination with VA as a source of spontaneous activity, as virgin female flies responded appropriately to genotype both under charcoal-filtered air and in still air (data not shown). Normal spontaneous activity rates are restored to T1 neurons upon introduction of a transgenic, wild-type *lush* gene into the mutants (Figure 3C), clearly establishing that this odorant-independent reduction in neuronal activity is dependent on *lush* expression.

LUSH Is Not Required to Establish Cell Fate or Development of T1 Neurons

Spontaneous activity is reduced in T1 neurons but not in T2 neurons in *lush* mutants, despite loss of LUSH

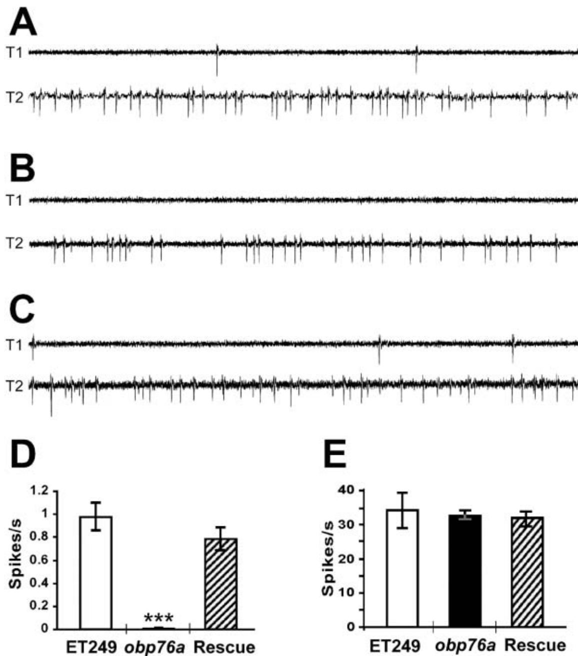

Figure 3. Spontaneous Activity in T1 Neurons Is Severely Diminished in *lush* Mutants

(A) Sample traces showing 1 s recordings of neuronal activity from T1 and T2 sensilla in control ET249 flies.

(B) Traces from T1 and T2 sensilla from *obp76a* mutants.

(C) Traces from T1 and T2 sensilla from *lush* mutants carrying a wild-type transgenic copy of the *lush* gene regulated by its own promoter.

(D) Summary of spontaneous activity depicted as action potentials per second from T1 neurons in wild-type (ET249), *lush* mutants (*obp76a*), and *lush* mutants carrying a rescuing transgenic copy of *lush* (Rescue). ***, significantly different from *lush* mutants at $p < 0.001$. Error bars represent SEM. $n = 18$.

(E) Summary of spontaneous activity from T2 neurons in control, *lush* mutants, and mutants with a rescuing transgene. Spontaneous activity in T2 neurons is not different among the groups. $n = 11$.

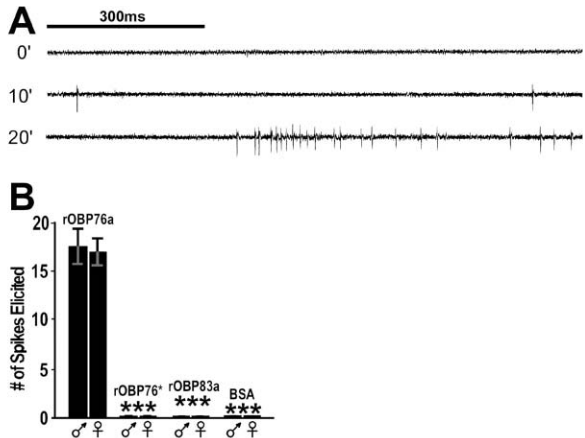

Figure 4. Introduction of Recombinant LUSH Protein through the Recording Pipette Restores Spontaneous Activity and VA Sensitivity to T1 Neurons

(A) Sample traces recorded from *lush* mutant T1 sensilla just after penetration into the sensillum (0′). No spontaneous activity or VA sensitivity is present. After 10 min (10′), spontaneous activity becomes apparent, but VA fails to elicit a response. By 20 min (20′), spontaneous activity is at normal levels and VA sensitivity is restored. Bar above the traces denotes VA stimulation.

(B) Summary of direct OBP introduction studies. Control proteins including bovine serum albumin (BSA), recombinant OBP83a (rOBP83a), or unfolded OBP76a (rOBP76a*) fail to restore spontaneous activity or VA sensitivity when introduced directly into *lush* mutant T1 sensilla. VA sensitivity and spontaneous activity are both restored by introducing refolded recombinant OBP76a (rOBP76a) into the mutant T1 sensilla. ***$p < 0.001$; $n = 4$. VA sensitivity was restored despite the fact that the introduced LUSH concentration was approximately 180 micromolar, while normal OBP concentrations have been estimated to be 10 millimolar in the sensillum (Klein, 1987). This suggests that OBPs may be normally present at levels greater than required for normal function.

from both functional types of trichoid sensilla. One explanation is that LUSH protein directly or indirectly activates receptors expressed by T1 neurons that are not expressed by T2 neurons. However, there are other possible explanations for the observed genetic interaction between LUSH and the T1 neurons. For example, loss of spontaneous activity in T1 neurons in *lush* mutants could result from a developmental requirement for LUSH in conferring T1 cell identity. Alternatively, LUSH could function as an extracellular, neuronal maintenance factor required for normal T1 neuron function.

To address when LUSH is functionally required, we introduced recombinant LUSH protein directly into adult *lush* mutant T1 trichoid sensilla through the recording pipette. Figure 4A shows that immediately following penetration of a recording pipette containing recombinant LUSH protein, we observed no increase in spontaneous firing rate of the T1 chemosensory neurons, and VA fails to elicit responses. However, over a few minutes we observed a gradual increase in the spontaneous firing frequency, and eventually VA was able to induce robust responses from the T1 neurons (Figure 4). In some experiments, VA responses could be elicited in as little as 5 min after penetration of the recording pipette. This time scale was similar to diffusion of dye into the sensilla (data not shown). Introduction of control proteins OBP83a, BSA, or unfolded LUSH protein in the recording pipette failed to restore either spontaneous activity or VA sensitivity to the *lush* mutant T1 neurons (Figure 4B). Therefore, the specific requirement for LUSH protein in the adult sensillum lymph of T1 sensilla for normal spontaneous activity and VA sensitivity likely reflects a role for LUSH as an extracellular signal transduction component for T1 neurons.

A Subset of T2 Neurons Displays Abnormal Responses to Alcohols in *lush* Mutants

In addition to the dramatic effects on T1 neurons in *lush* mutants, we also identified more subtle defects in a subset of T2 neurons that could account for the abnormal chemotactic responses of *lush* mutants to alcohols. We observed at least two functional types of T2 sensilla in wild-type animals. T2A sensilla, previously described by Clyne et al. (1997), contain neurons that are inhibited by 1-hexanol but not butanol and ethanol. We also identified a second functional type, T2B, containing neurons inhibited by high concentrations of ethanol and butanol. We observe that T2B neurons are defective for inhibition by these odorants in *lush* mutants (Figure 5).

In wild-type T2B sensilla, at least one and usually

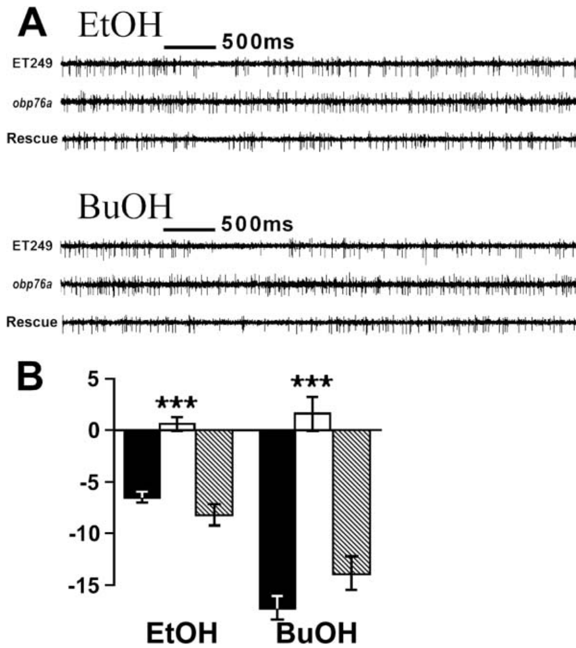

Figure 5. A Subset of T2 Sensilla Are Normally Inhibited by Ethanol and Butanol in Wild-Type but Not in *lush* Mutants

(A) Representative traces of responses from selected T2B sensilla showing inhibition of spontaneous activity by 100% ethanol (EtOH) or 10% 1-butanol (BuOH). *lush* mutants are resistant to this inhibition (*obp76a*), but normal responses are restored by a wild-type *lush* transgene (rescue).

(B) Summary of inhibition by alcohols in T2B neurons. The change in spikes per second was calculated by subtracting the total number of spikes 1 s after the stimulus and the total number of spikes 1 s before the stimulus. This results in a negative number in wild-type T2B (black bars), because spontaneous activity is inhibited. *lush* mutant T2B neurons were insensitive to these odorants, and no inhibition is observed (white bars). Expression of a *lush* transgene in the *lush* mutants restores normal inhibition (striped bars). Error bars represent SEM, n = 10. *** indicates significant at $p < 0.001$.

several olfactory neurons within the sensillum reduce firing rates upon stimulation by concentrated butanol or ethanol. *lush* mutant T2B neurons do not show this normal inhibitory response to these odorants and continue firing at prestimulus rates (Figure 5B). Low concentrations of these odorants have no effect on the activity of these neurons. *lush* mutants expressing the *lush* transgene have inhibitory responses to concentrated alcohols restored (Figure 5, "rescue"). This defect in *lush* mutant T2B responses correlates with the defect in behavior previously observed in *lush* mutants (Kim et al., 1998) and suggests that LUSH mediates both avoidance of concentrated alcohols and attraction to VA pheromone.

Discussion

Mutants lacking LUSH are insensitive to 11-*cis* vaccenyl acetate, both at the level of olfactory neuron activation and at the level of aggregation behavior normally elicited by this pheromone. These defects are due specifically to loss of LUSH expression in T1 sensilla, because transgenic expression of LUSH protein restores function. Other members of the OBP family (OBP83a, or OS-F and OBP83b, or OS-E and moth APO3, or unfolded LUSH) do not functionally compensate for the loss of LUSH. Therefore, LUSH is absolutely and specifically required in the sensillum lymph for VA pheromone signal transduction by T1 neurons, providing a clear demonstration that an OBP member functions in olfaction and mediates activation of olfactory neurons.

lush mutants are also defective for spontaneous activity in T1 neurons. This unexpected pheromone-independent phenotype reveals a genetic interaction between LUSH and T1 neuron activation mechanisms. T1 neurons do not produce LUSH and do not require it for cell fate determination or general health, as direct introduction of recombinant LUSH restores function as fast as the protein can diffuse into the sensillum lymph. This time frame would seem too short for any growth factor-like signal requiring transcription and translation. Individual action potentials have normal shape and kinetics in *lush* mutants (not shown), further suggesting there is no intrinsic defect in the T1 neurons. Even relatively dilute preparations of exogenous LUSH restore spontaneous activity and VA sensitivity, while expression of other OBPs at high levels does not. This eliminates any nonspecific osmotic effects resulting from absence of the abundant LUSH protein in the sensillum lymph producing the observed defects. Finally, VA activates wild-type T1 but not T2 sensilla, though both sensilla types express LUSH. This demonstrates a requirement for both LUSH and a T1 neuron-specific factor. The simplest explanation consistent with these findings is that extracellular LUSH protein can stimulate, directly or indirectly, T1 neurons to produce action potentials through an unknown T1-specific receptor.

Members of the OBP family interact directly with odorant ligands, leading to several proposals for OBP function (reviewed in Vogt, 2003; Kaissling, 2004). For example, OBPs may function by removing or inactivating odorants from the sensillum lymph (Steinbrecht and Mueller, 1971; Vogt and Riddiford, 1981; Ziegelberger, 1995), by solubilizing hydrophobic pheromone ligands (Kaissling et al., 1985; Vogt et al., 1985; Wojtasek and Leal, 1999; Sandler et al., 2000), by concentrating pheromone molecules in the lymph (Pelosi, 1995), by acting as filters to screen out subsets of odorants (Pelosi, 1994), by functioning as buffers to prevent saturation of the responses during high stimulus intensities (Pelosi, 1994), or by transporting odorants to the olfactory neurons or to act as coreceptors with odorants to activate olfactory neurons (Du and Prestwich, 1995; Kaissling, 1986, 2001; Krieger and Breer, 1999; Vogt, 2003). Recent structural studies have led to the proposal that local pH changes near dendrites might induce unloading of pheromone that subsequently proceeds to activate neuronal receptors (Wojtasek and Leal, 1999; Horst et al., 2001). However, there has been little direct in vivo evidence to support or refute these models. Diffusion of antiserum to an OBP in taste sensilla reduced activity, suggesting that the binding protein might facilitate activation of a chemosensory neuron (Ozaki et al., 1995). Pophof implicated a role for binding proteins in the specificity of neuronal activation in moths. This work showed that the wrong pheromone could activate a pheromone-sensitive neuron when prebound to an OBP that nor-

mally binds the activating pheromone (Pophof, 2002). We show that LUSH OBP is absolutely required for activation of T1 neurons by VA. This finding is not consistent with odorant removal as a sole function for LUSH. Similarly, a role in activation of pheromone-sensitive neurons indicates that LUSH is not a buffer or filter for VA. Our data suggest that LUSH activates T1 neuronal surface receptors responsible for action potential generation. Therefore, while LUSH may bind and transport pheromone, it is not a simple carrier or solubilizing factor for pheromone but instead has a more specific role as a signal transduction component. This model would be consistent with the findings of Pophof in the moth system (Pophof, 2002) and may reflect a general mechanism through which OBPs function in insects.

We propose a working model in which LUSH functions as an adaptor to bridge the presence of gaseous pheromone molecules to activation of specific neuronal receptors expressed on T1 olfactory neurons. VA may induce a specific conformational change in LUSH protein that in turn activates T1 receptors. If such a conformational change occurs spontaneously at low frequency, this would explain the observed loss of spontaneous activity in *lush* mutants. Ligand-induced conformational changes have been reported previously in pheromone binding proteins from *Mamestra brassicae* and *Bombyx mori* upon pheromone binding (Campanacci et al., 2001; Horst et al., 2001; Wojtasek and Leal, 1999). An important test of this model will be to show that VA pheromone itself is not a direct activator of T1 olfactory neurons, but triggers neuronal activity indirectly through conformational changes in LUSH. Consistent with this idea, even 100% VA is incapable of producing activity in T1 neurons in *lush* mutants. If LUSH is the ligand for the T1 receptors, this would refute the pH release model that posits that pheromone release from the OBP mediating activation of neuronal receptors. Alternatively, components of both LUSH and an exposed portion of the bound pheromone may activate neuronal receptors (Kaissling, 2001). LUSH could also act indirectly by recruiting other factors in the sensillum lymph that ultimately activate T1 neurons. Solving the X-ray crystal structure of the LUSH-VA complex and identifying the neuronal receptors that mediate VA sensitivity will allow us to corroborate or refute this model. Finally, LUSH also influences the alcohol responses of T2B neurons. *lush* mutants are defective for avoidance of concentrated alcohols, and *lush* mutant T2B neurons fail to show inhibition by concentrated alcohol. Both defects are reversed by expression of a wild-type *lush* transgene, suggesting the LUSH-dependent inhibition of T2B neurons results in behavioral avoidance. This finding would be consistent with our model if a LUSH-alcohol complex resulted in activation of T2B receptors and activation of these receptors inhibits these neurons. Inhibition of *Drosophila* olfactory neurons by odorants has been previously reported (de Bruyne et al., 2001). LUSH binds ethanol (Kruse et al., 2003) and several pthalate compounds in vitro (Zhou et al., 2004). Pthalates, even at full strength, do not influence activity in trichoid neurons in our hands (data not shown). Ethanol influences the responses of T2B neurons but not T2A or T1 neurons, and VA has no effect on T2 neurons. This suggests that ligand binding to a binding protein does not confer universal biological activity in vivo. Instead, different ligands may induce distinct conformations in the binding protein, or perhaps form part of a receptor interaction domain that is discriminated by different neuronal receptors. Structural analysis of these complexes with receptors will allow us to define these interactions.

Social aggregation behavior is induced by activation of T1 neurons, revealing the first stage of a neuronal circuit mediating aggregation in this insect. It is not clear why an aggregation pheromone would be produced only in males. Perhaps this aids roaming flies to identify a safe environment to mate and lay eggs. VA appears to act synergistically with food odorants, consistent with this notion (Bartelt et al., 1985). In *Drosophila*, at least 35 genes encode OBPs (Galindo and Smith, 2001; Graham and Davies, 2002; Hekmat-Scafe et al., 2002) expressed in virtually every *Drosophila* olfactory and gustatory organ (Galindo and Smith, 2001). Recently, a putative taste receptor was implicated in detecting contact pheromone during mating in *Drosophila* (Bray and Amrein, 2003). It will be interesting to determine if OBPs are required for this behavior and if other members of the chemosensory gene family are required for VA sensitivity in T1 neurons. Similarly, it would be of great importance to determine if other OBPs mediate additional behaviors in this animal. In fire ants, the number of egg-laying queens determines the size of the colony. Queen number is determined by workers that kill extra queens depending on the allele of Gp-9 the workers carry. Gp-9 has been identified as a member of the odorant binding protein family (Krieger and Ross, 2002). Therefore, it is possible that binding proteins mediate a diverse array of pheromone-mediated social interactions in insects. It should be feasible to design synthetic ligands capable of interacting and inducing appropriate conformational changes with various binding proteins, permitting the manipulation of these signaling pathways. Such compounds could be used to control any number of insect behaviors including aggregation, mating, and colony size.

Experimental Procedures

Drosophila Stocks

ET249, *obp76a* mutants, and *obp76a* mutant flies with a rescuing transgene were described previously (Kim et al., 1998). ET249 flies served as wild-type controls with a genetic background essentially identical to the *obp76a* mutants and the *obp76a* rescue strains. Flies expressing moth pheromone binding protein were made by transforming a moth cDNA regulated by the *obp76a* promoter into *Drosophila* (Spradling and Rubin, 1982). The cDNA encoding the APO-3 pheromone binding protein from the moth *Anthereae polyphemus* (Raming et al., 1989) was cloned into a rescuing *obp76a* transgene (Kim et al., 1998) such that the APO-3 cDNA replaced the *obp76a* coding sequence. APO-3 protein was detected in trichoid sensilla by anti-APO-1 antiserum (Maida et al., 2000) using the methods described by Kim et al. (1998). No signal was present in wild-type flies incubated with this antiserum (not shown).

Behavior Assays

Aggregation behavior was assayed using a modification of the olfactory trap assay (Woodard et al., 1989). Briefly, 10 1- to 3-day-old virgin male or female flies of a particular sex and genotype were placed in a petri dish containing a thin layer of agarose and two odorant traps. One trap contained the test odorant (either 1% VA or 5 live virgin flies), and the other contained only agarose. A minimum of 30 replicates was performed for each genotype and odorant.

After 48 hr, the flies within the traps were counted and flies in blank traps was subtracted from the number in the test traps to obtain relative attraction values. The means and standard errors were calculated and differences among groups were evaluated using ANOVA.

Recombinant Proteins

cDNAs encoding LUSH and OBP83a were isolated by PCR from antennal cDNA and sequenced. Primers used for *obp76a* were previously described (Kruse et al., 2003). The PCR products were cloned into pET28b as an Nde1-BamH1 fragment, thus introducing a 6-histidine tag at the N terminus that can be cleaved by thrombin. Proteins were expressed, purified, thrombin cleaved, and refolded as described by Kruse et al. (2003). Recombinant proteins were concentrated (Amicon, Beverley, MA), and 100 to 180 μM solutions were prepared in 1× sensillum lymph buffer (Kaissling and Thorson, 1980) and introduced into the sensilla through the glass recording micropipettes. Bovine serum albumin (BSA) was obtained from Roche and diluted to 200 μM in 1× sensillum lymph buffer.

Single Sensillum Recordings

Extracellular electrophysiological recordings were carried out according to de Bruyne et al. (2001). Flies were under a constant stream of charcoal-filtered air to prevent any potential environmental odors from inducing activity during these studies. Air flow rate was 36 cc/s. 11-*cis* vaccenyl acetate was diluted 1:100 in paraffin oil. 1-butanol was diluted 1:10 in paraffin oil, and ethanol was used at full strength. Increased stimulus duration of 1–3 s was used for the ethanol studies. Signals were amplified 1000× (USB-IDAC system; Syntech, Hilversum, the Netherlands) and fed into a computer via a 16-bit ADC and analyzed offline with AUTOSPIKE software (USB-IDAC system; Syntech). Low cutoff filter setting was 200 Hz and the high cutoff was 3 kHz. Analysis of data was performed as previously described (de Bruyne et al., 2001). Each recording was performed from separate sensilla with a maximum of two sensilla recorded from any single fly.

Pheromone Synthesis

11-*cis* vaccenyl acetate was synthesized from 11-*cis* vaccenyl alcohol (Sigma) by reacting with anhydrous acetic anhydride overnight under nitrogen. Purity was confirmed by mass spectrometry and NMR.

Immunostaining Frozen Tissue Sections

Frozen tissue sections were prepared, reacted with APO1 antiserum at a dilution of 1:100, and detected according to Kim et al. (1998).

Acknowledgments

We thank Gunde Zeigelberger for the gift of anti-APO-1 antiserum, J. Krieger and H. Breer for the APO-3 clone, Sylvia Alvarado for protein expression, and Al Gilman, Wynand Naters, and John Carlson for helpful discussions. This study was supported by the NIH and the American Heart Association. We apologize to colleagues whose work was not cited due to space limitations. The authors declare that they have no competing financial interests.

Received: August 27, 2004
Revised: November 10, 2004
Accepted: December 2, 2004
Published: January 19, 2005

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