Habituation of an odorant-induced startle response in *Drosophila*

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Habituation is a fundamental form of behavioral plasticity that permits organisms to ignore inconsequential stimuli. Here we describe the habituation of a locomotor response to ethanol and other odorants in *Drosophila*, measured by an automated high-throughput locomotor tracking system. Flies exhibit an immediate and transient startle response upon exposure to a novel odor. Surgical removal of the antennae, the fly's major olfactory organs, abolishes this startle response. With repeated discrete exposures to ethanol vapor, the startle response habituates. Habituation is reversible by a mechanical stimulus and is not due to the accumulation of ethanol in the organism, nor to non-specific mechanisms. Ablation or inactivation of the mushroom bodies, central brain structures involved in olfactory and courtship conditioning, results in decreased olfactory habituation. In addition, olfactory habituation to ethanol generalizes to odorants that activate separate olfactory receptors. Finally, habituation is impaired in *rutabaga*, an adenylyl cyclase mutant isolated based on a defect in olfactory associative learning. These data demonstrate that olfactory habituation operates, at least in part, through central mechanisms. This novel model of olfactory habituation in freely moving *Drosophila* provides a scalable method for studying the molecular and neural bases of this simple and ubiquitous form of learning.

Animals modify their behavior based on information from their environment, previous experiences and genetic composition. Habituation, an elementary form of behavioral modification, is classically defined as a decrease in response magnitude as a result of repeated stimulation (Groves & Thompson 1970; Harris 1943). By this type of nonassociative learning, organisms can ignore inconsequential stimuli, presumably to focus attention towards more salient environmental features. Habituation is found throughout nature and has been studied in many organisms. The most thoroughly studied model organism is the mollusk *Aplysia californica*, where a gill withdrawal reflex habituates to intermittent mechanical stimulation (Pinsker *et al.* 1970). Synapses between mechanosensory neurons and their targets become functionally depressed during habituation. This depression is linked to modulation of presynaptic calcium currents (Edmonds *et al.* 1990; Klein *et al.* 1980) and reduced availability of synaptic vesicles at the active zone (Bailey & Chen 1988). However, the regulatory mechanisms are incompletely understood.

Several reasons led us to study odor-induced habituation in the genetically tractable model organism, *Drosophila melanogaster*, using a high-throughput locomotor analysis system (Wolf *et al.* 2002). First, *Drosophila* demonstrates habituation to several sensory stimuli. For example, flies extend their proboscis when sucrose is applied to chemoreceptors on their legs; this feeding reflex attenuates after repeated sucrose application (Duerr & Quinn 1982). A cleaning reflex habituates to repeated mechanical stimulation of thoracic bristles (Corfas & Dudai 1989), and the jump-and-flight escape response, mediated by the giant fiber pathway, also habituates (Engel & Wu 1996; Engel & Wu 1998; Engel *et al.* 2000). Second, the neural circuitry of the olfactory system is well established, from primary olfactory neurons to the mushroom bodies (Jefferis *et al.* 2002; Vosshall 2000). Finally, our high-throughput assay is compatible with large-scale genetic screens for mutations that alter habituation, an approach that will lead to novel insights into the molecular bases of this form of behavioral plasticity.

In this article we describe a novel paradigm for quantifying attenuation of an olfactory-mediated locomotor response in a population of freely moving *Drosophila*. Upon exposure to ethanol vapor, an odor abundantly found in this organism's environment, flies exhibit a rapid and transient increase in walking speed. This olfactory mediated 'startle' response attenuates upon intermittent odorant exposure and displays typical characteristics of classically defined habituation, including reversibility and generalization to similar stimuli. Flies lacking the mushroom bodies, brain structures involved in olfactory associative learning and other higher order brain functions (Jefferis *et al.* 2002; Roman & Davis 2001; Vosshall 2000), show reduced habituation. In addition,

rutabaga flies, originally discovered because of a defect in olfactory associative learning, exhibit impaired habituation in our assay. Thus, odorant-induced startle habituation involves the central brain and exhibits molecular similarities with associative learning.

Materials and methods

Fly strains and genetics

Fly stocks were grown and maintained at $25\,°C$ and 70% humidity in constant light. Male *Berlin* flies carrying the w^{1118} mutation were used in the initial description of odorant-induced startle attenuation and subsequent parametric studies. Learning/memory mutant strains (amn^{chpd}, rut^{2080} and rut^{769}) contain a PZ [ry +] element in a *Canton S* ry^{506} background. A strain with a PZ [ry +] element on the X chromosome in the same background was used as a control for these mutants (Han *et al.* 1992; Moore *et al.* 1998). The mutants and their control were back-crossed to parental ry^{506} for at least eight generations. To minimize the effect of autosomal genetic modifiers, males from control and mutant stocks were crossed to attached X (X^X/Y) females. For behavioral assays, male progeny hemizygous for the father's X chromosome and heterozygous for parental autosomes were collected 1–3 days following eclosion (day 12 after egg-laying). To eliminate any effects of CO_2 anesthesia, flies were kept an additional 2 days prior to testing (day 14).

Habituation assay

The video tracking apparatus is described in detail elsewhere (Wolf *et al.* 2002). The acrylic observation chamber, with one inlet for vapor delivery and one outlet for exhaust, was placed on a transparent acrylic stage 2 cm above a fluorescent light box. Delivery of ethanol vapor or humidified air was as described previously (Moore *et al.* 1998; Wolf *et al.* 2002). Briefly, air flow is split into two parallel streams; one stream is bubbled through distilled water to produce humidified air, and the other stream is bubbled through 95% ethanol to produce ethanol vapor. These two streams are combined to produce an ethanol/humidified air mixture by adjusting the flow rates through these parallel streams. Arbitrary flow units are calibrated to flux by Cole Parmer; a flow of 30 units is equivalent to 1.1 l/min; a flow of 35 units is equivalent to 1.3 l/min. Twenty male flies were tapped into the observation chamber and acclimated to humidified air flow for 9 min. Flies were then exposed to a series of 30-second pulses of an ethanol/air (E/A) mixture. Between pulses, flies were exposed to humidified air. The flow rate of E/A during pulse delivery was equal to the flow rate of the humidified air during rest periods to eliminate the possibility of inducing startle by flux changes. In all figures, *n* corresponds to the number of experiments performed on an independent group of 20 flies.

Surgery and mushroom body ablation

The third antennal segments of male flies were removed by the procedure described previously (Wolf *et al.* 2002). Hydroxyurea treatment was performed according to de Belle and Heisenberg (1994). Eggs were collected on apple juice plates at $25\,°C$ in one-hour intervals, and kept at $25\,°C$ for 23.5 h. Newly hatched first instar larvae were transferred to a microcentrifuge tube containing a paste of heat-killed yeast with or without 50 mg/ml hydroxyurea (Sigma, St. Louis, MO) for 4 h at $25\,°C$. Larvae were washed and transferred to regular food. Adult males were collected and maintained in constant light with food at $25\,°C$, 70% humidity for two days prior to behavioral testing.

Calculations and statistics

The total movement during each odor exposure was calculated as the area under the curve, i.e. summing the velocities measured during the 30-second exposure, at 5-second intervals, and multiplying the sum by 5 seconds. The habituation index was calculated by dividing the total movement traveled during the fourth pulse of odor by the total movement traveled during the first pulse. In all experiments *n* represents groups of 20 flies. Significance was established by two-tailed Student's *t*-tests assuming unequal variance or by single factor ANOVA with *post hoc* Newman-Keuls comparisons. Error bars in all experiments represent $\pm$ SEM.

Results

Exposure to ethanol vapor produces a characteristic locomotor response

Continuous exposure to a moderate dose of ethanol vapor elicited a characteristic change in average velocity (Fig. 1a). Within a few seconds of ethanol exposure, flies exhibited a rapid increase in walking velocity that subsided after approximately one minute, even in the continued presence of ethanol. This transient hyperactive period was followed by a second longer lasting period of increased activity. The latter likely reflects the pharmacological effects of ethanol on the central nervous system (Wolf *et al.* 2002). In this study we focus on the initial hyperactive phase, which we term 'olfactory startle' as it is a short latency behavioral pattern elicited by an abrupt and unexpected stimulus (Bullock 1984), and, as shown below, is mediated by the olfactory system. The startle response is not caused by a sudden change in air flow since sudden exposure to humidified air only (0/65 relative flow rates of ethanol/humidified air), did not produce any increase in walking velocity (data not shown).

Bilateral removal of the third antennal segments, the major olfactory organs of *Drosophila* (Carlson 1996), eliminated the startle phase (Fig. 1b). The second phase of hyperactivity, while shifted to earlier times, remained intact in these antennectomized flies (Wolf *et al.* 2002). Removal of the aristae, putative sound and humidity sensors in flies (Eberl *et al.*
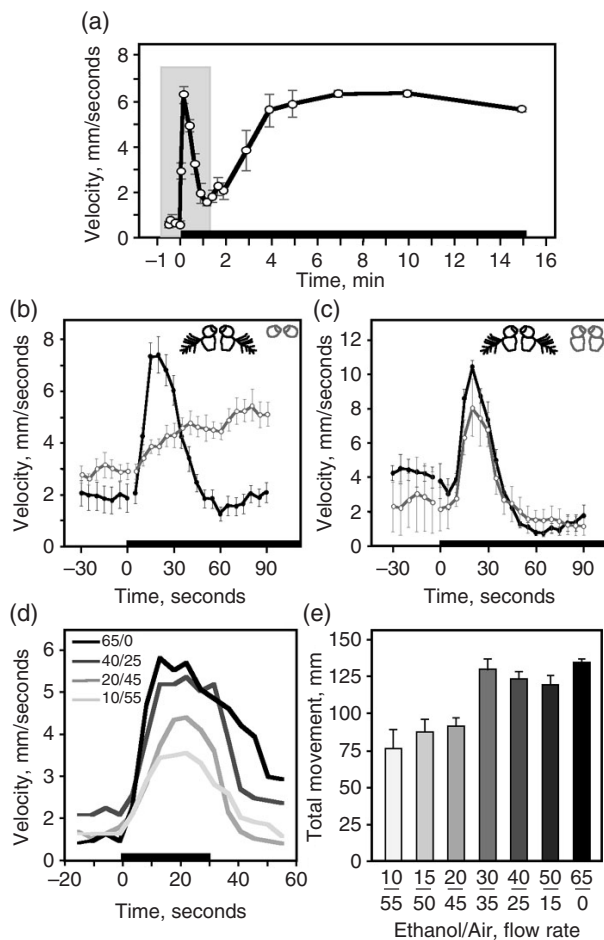
Figure 1: Flies respond to the smell of ethanol by a transient increase in locomotion. (a) Locomotor velocity profile of control flies exposed to ethanol vapor (30/35 relative flow rates of ethanol/air). Continuous ethanol exposure (starting at time 0 min and indicated by the black bar along the horizontal axis) elicits a transient 'startle' response (shaded), which is followed by a second more sustained period of hyperactivity. $n = 4$ experiments using 20 flies in each experiment. (b, c) Antennae are required for the startle response. The period of ethanol exposure (30/35 relative flow rates of ethanol/air) is indicated by the black bar along the horizontal axis. Filled circles depict the startle response in unoperated flies; open circles depict the response of flies with bilateral removal of the antennae (b, inset) or aristae (c, inset). Data correspond to means ± SEM. For data in panel b, $n = 7$ and $n = 11$ for the surgical and control groups, respectively. For data in panel c, $n = 4$ for both groups. (d, e) Startle response of flies exposed to various concentrations of ethanol vapor, achieved by mixing ethanol vapor with humidified air at various flow rates. Panel (d) depicts the locomotor velocity profiles and panel (e) the total movement during the 30-second exposure period (see _Materials and methods_). Maximal velocity and total movement show a saturable response to increasing ethanol doses. Data points represent mean values in panel (d) and means ± SEM in panel (e), $n = 8–10$.

1997; Sayeed & Benzer 1996) that emerge from the third antennal segment (and are thus removed during antennectomy), had no significant effect on the startle response (Fig. 1c). The basal activity of antennectomized flies was somewhat higher than that of unoperated flies; the reason for this is unclear. The magnitude of the olfactory startle depended on the concentration of ethanol vapor (Fig. 1d, e). Higher concentrations of ethanol increased both the peak velocity and total movement; i.e. area under the curve during ethanol exposure. This dose effect was saturable, reaching a plateau at relative flow rates of 30/35 (ethanol vapor/humidified air).

In summary, flies show a complex locomotor response upon exposure to ethanol vapor. An initial phase of increased activity is a dose-dependent response to the smell of ethanol (startle), while a second longer-lasting hyperactive phase is probably caused by direct action of ethanol on the central nervous system (Wolf et al. 2002).

The startle response attenuates with repeated intermittent ethanol exposure

Figure 2(a) depicts a representative experiment in which flies were exposed to eight 30-second pulses of ethanol vapor delivered every six minutes. The maximal velocity decreased with each pulse (Fig. 2a). Similar attenuation kinetics were observed when startle responses were analyzed by calculating the total movement during ethanol exposure (Fig. 2b). Finally, the onset of each startle peak was progressively delayed (Fig. 2c). After four to five pulses of ethanol, flies achieved a near maximal state of attenuation; additional ethanol exposures caused little or no further decrease in maximal velocity and total movement.

Habituation is classically distinguished from adaptation and fatigue by reversibility of the attenuated state, either by a disruptive stimulus or by spontaneous mechanisms (Thompson & Spencer 1966). To determine whether the attenuated state was reversible, we administered a mechanical stimulus (physically shaking the exposure chamber) between the fifth and sixth pulse of ethanol vapor (Fig. 3a). This mechanical stimulus induced a state of increased locomotion that subsided within 2–3 min, and flies returned to baseline locomotion before the sixth pulse of ethanol was delivered. The response to the sixth ethanol pulse was essentially equivalent to that induced by the first exposure with respect to maximal velocity (Fig. 3a). However, the total movement was not completely restored to the naïve response (Fig. 3b), probably due to the fact that the startle latency was not completely reversed (Fig. 3c). It is also possible that the mechanical stimulus was not strong enough to produce complete recovery. Alternatively, attenuation of the startle response may involve several processes that show different sensitivities to the mechanical stimulus. Some habituation paradigms observe enhanced habituation in subsequent habituation trials (Thompson & Spencer 1966). We did not consistently observe this phenomenon in our assay. To determine if the disruptive mechanical stimulus specifically reversed the
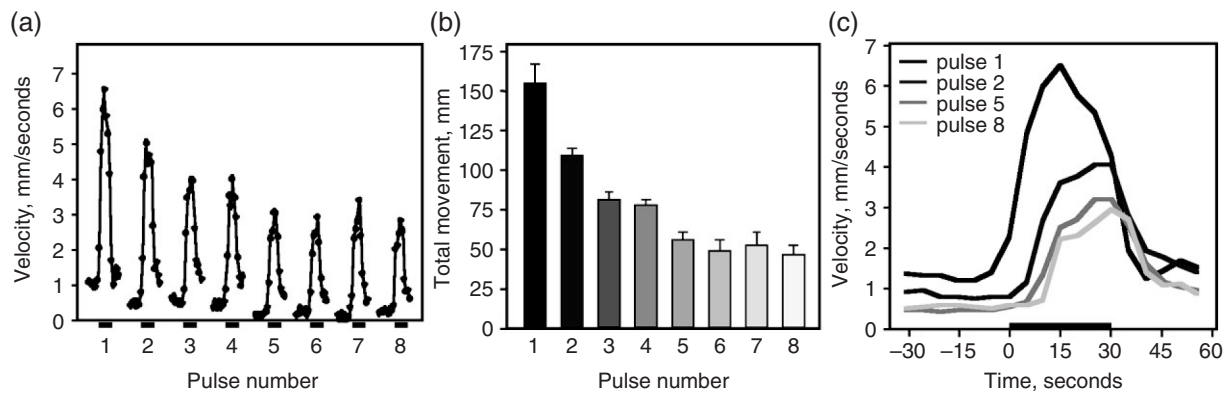
Figure 2: The startle response attenuates with repeated exposures to ethanol vapor. Flies were exposed to ethanol (30/35 relative flow rates of ethanol/air) for 30-second pulses (indicated by the black bars along the horizontal axis) delivered every six minutes. (a, b) The startle response attenuates with successive exposures, reaching nearly maximal attenuation after four to five ethanol pulses. Panel (c) shows a horizontal expansion of a subset of the data shown in panel (a); the startle responses were superimposed to demonstrate a progressive latency in the onset of the responses. (b) The total movement (area under the curve in (a)) decays with repeated exposures to ethanol. The kinetics of total movement decay is similar to that of maximal velocity decay depicted in (a). Data in panels (a) and (c) correspond to mean values, data in (b) to means $\pm$ SEM, $n = 4$.

attenuated state or, alternatively, produced a non-specific sensitization of the startle response, the mechanical stimulus was delivered prior to the first ethanol exposure (Fig. 3d). The mechanical stimulus did not increase the naive startle response. The experiment was performed at a lower dose of ethanol vapor to rule out an ethanol dose ceiling effect. Again, the mechanical stimulus did not sensitize the naïve startle response (Fig. 3e).

The attenuated state recovered spontaneously. Flies were allowed to achieve an attenuated state by delivering four 30-second pulses of ethanol vapor as in previous experiments, but given a 15-minute or 30-minute rest period prior to the fifth ethanol pulse (Fig. 4a). The total movement demonstrated a partial spontaneous recovery after 15 min and a full recovery after 30 min.

Other phenomena that could account for startle attenuation include accumulation of ethanol in the flies or a non-specific deterioration of the startle response with time. To address the first of these possibilities, we measured ethanol levels in the flies immediately before the fourth pulse of ethanol. If startle attenuation were due to ethanol accumulation, we would expect to find a significant accumulation of ethanol by this time. We found ethanol concentrations in whole fly homogenates of ethanol-exposed flies to be indistinguishable from baseline levels measured in unexposed flies. To address the possibility of non-specific deterioration, we measured the startle response of flies placed in the observation chamber for various amounts of time before exposure to ethanol. With time, there was no reduction of the naïve startle response (Fig. 4b). Therefore, it is unlikely that the response attenuation is caused by acclimation to the test conditions or by non-specific response deterioration.

In summary, the attenuation of the olfactory startle response is a function of repeated exposures to ethanol

and is not caused by accumulation of ethanol in the flies. Sensory adaptation and motor fatigue are unlikely to be primary mechanisms of startle attenuation because the attenuated state was reversed by a novel stimulus.

Effects of ethanol concentration and interstimulus interval on olfactory habituation

Flies were exposed to varying concentrations of ethanol vapor (30-second pulses every six minutes). As shown in Fig. 5(a), the kinetics of habituation were similar at all doses tested. Additionally, all concentrations produced a habituated state after four pulses. Finally, the habituated state could be reversed by mechanical stimulation at all ethanol concentrations used (data not shown). The observation that olfactory startle habituation was insensitive to ethanol dose was somewhat surprising. Classical studies of habituation have reported that strong stimuli (i.e. noxious stimuli) may fail to produce response attenuation (Harris 1943; Thompson & Spencer 1966). It is possible that a brief exposure to even pure ethanol vapor is not particularly noxious to *Drosophila*.

The rate of habituation is often related to the frequency of stimulus delivery, such that higher frequency stimulations augment habituation (Davis 1970; Rankin & Broster 1992; Thompson & Spencer 1966). We therefore varied the interstimulus interval (ISI) in our assay from three to 18 min (Fig. 5b). At all ISIs, habituation was well established after four exposures to ethanol vapor. However, the degree of attenuation was greater at shorter ISIs. The 18-minute ISI produced approximately 30% response attenuation, whereas the 3-minute ISI produced approximately 50% attenuation. It is likely that the decreased habituation observed at the 18-minute ISI is partly due to spontaneous recovery between stimuli (Fig. 4a).
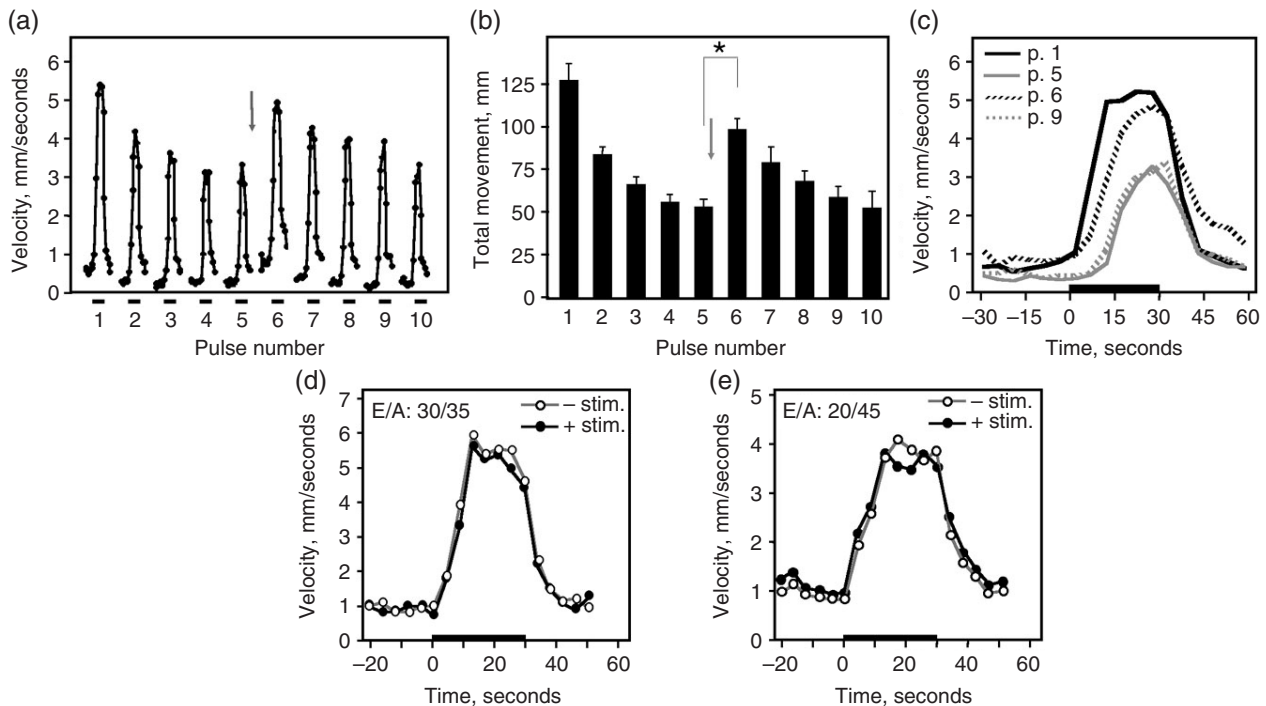
Figure 3: Attenuation of the startle response is reversed by mechanical stimulation. (a, b) The startle response attenuates with successive exposures, reaching nearly maximal attenuation after four 30-second ethanol pulses (30/35 ethanol/air) delivered every six minutes. The startle responses are presented as average velocity in (a) and total movement in (b). Between the fifth and sixth pulses of ethanol, flies were delivered a sudden mechanical stimulus (vertical arrow) exactly 90 seconds after the termination of the fifth ethanol pulse. Statistical analysis by ANOVA was performed on the total movement in (b) (overall significant effect of pulse number $P < 1 \times 10^{-6}$). *Post hoc* analysis revealed a significant effect of mechanical stimulation between pulses 5 and 6 ($P < 0.00005$, $n = 8$). The total movement did not recover completely because there was a significant difference between pulses 1 and 6 ($P = 0.005$). (c) Selected startle responses are superimposed. The increased latency of the attenuated startle response did not fully recover after mechanical disruption. Data points represent mean values, $n = 8$. (d, e) Mechanical stimulation does not cause sensitization of olfactory startle. Flies were exposed to mechanical stimulation four minutes prior to the first exposure to ethanol at 30/35 (panel (d)) or 20/45 (panel (e)) relative flow rates of ethanol/air. There was no significant effect of mechanical stimulation on maximal velocity or in total movement.

### Olfactory habituation generalizes among several odorants

Habituation to one stimulus often generalizes to other related stimuli. For example, habituation is conserved for acoustic stimuli that are similar in frequency (Corman 1967; Rudell 1983), for mechanical stimuli that are delivered in close proximity (Burrell & Sahley 1998; Stopfer *et al.* 1996) and for odorants with structural similarities (Cleland *et al.* 2002; Wilson 2000). To determine if olfactory habituation is unique to ethanol, we measured the startle response to other volatilized odors, including ethyl acetate (EA) and isoamyl alcohol (IA). As shown in Fig. 6, these odorants produced a transient increase in walking velocity, similar to that seen with ethanol. Repeated exposures (30 seconds every six minutes) produced habituation that was reversible by mechanical stimulation (Fig. 6a, b). To test for stimulus generalization, or cross-habituation, flies were habituated to one odorant and tested for continued habituation to a second odorant. For example, flies were exposed to three pulses of EA, followed by one pulse (fourth pulse) of ethanol, and finally, to a fifth pulse of the original odorant, EA (Fig. 6c). The ethanol-induced startle that followed three pulses of EA (fourth pulse) was significantly smaller than that induced by ethanol in naïve flies tested at the same time point. Closer analysis of the shapes of the startle responses revealed that the ethanol-induced startle preceded by three EA pulses showed a delayed onset relative to the naïve startle induced by ethanol (data not shown); this is similar to the modification of startle onset produced by previous ethanol exposure (Fig. 2c). Of note, exposure to a pulse of ethanol vapor does not reverse the habituation to EA because the fifth pulse continues to be depressed (Fig. 6c). Similar results were obtained when cross-habituation was tested in all possible combinations of the three odorants: ethanol, EA and IA (Fig. 6d, e and data not shown).
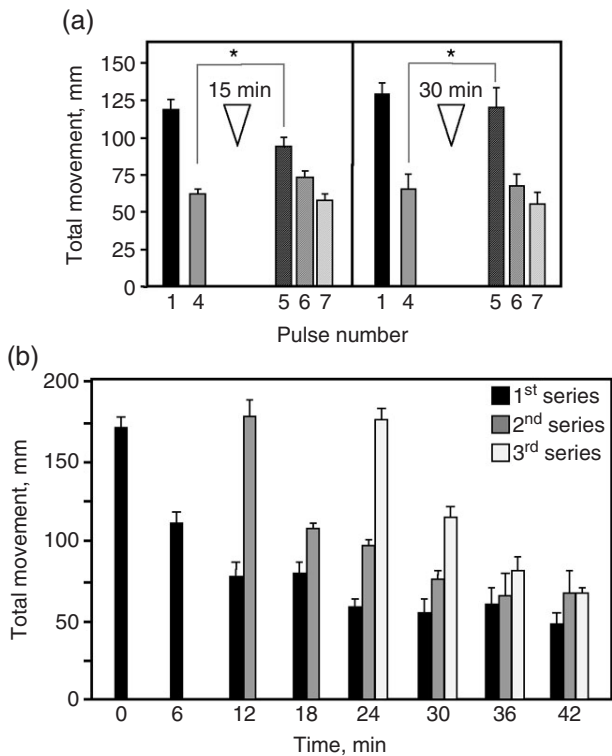
Figure 4: The attenuated state recovers spontaneously and is not due to non-specific mechanisms. (a) Flies were exposed to four pulses of ethanol vapor (30/35 relative flow units of ethanol/air), followed by another three ethanol pulses delivered after a 15-minute (left panel) or 30-minute (right panel) rest period. Total movement spontaneously recovered partially after 15 min of rest and recovered fully after 30 min of rest. One-way ANOVA revealed a significant effect of ethanol pulse in both conditions ($P < 0.0001$ for 15- and 30-minute rest conditions). *Post hoc* Newman-Keuls comparisons for 15-minute rest conditions revealed a significant effect of spontaneous recovery between pulses 4 and 5 ($P < 0.0006$) and a significant difference between pulses 1 and 5 ($P < 0.001$). *Post hoc* Newman-Keuls comparisons for 30-min rest conditions revealed a significant effect of spontaneous recovery between pulses 4 and 5 ($P < 0.002$) and no significant difference between pulses 1 and 5 ($P = 0.53$). Bars represent means $\pm$ SEM, $n = 6$ for 15-minute recovery, $n = 8$ for 30-minute recovery. (b) Flies were placed into the observation chamber for various amounts of time prior to the first exposure to ethanol vapor. Thirty-second ethanol pulses (30/35 relative flow rates of ethanol/air) were delivered every six minutes. The first series (black bars) followed the standard protocol: flies were allowed to acclimate to the chamber for 9 min, during which time they received only humidified air prior to the first ethanol exposure. In the second series (gray bars), flies were allowed to acclimate to the chamber for an additional 12 min (21 min total acclimation), prior to the first ethanol exposure. In the third series (white bars), flies were allowed to acclimate for an additional 24 min (33 min total acclimation) prior to the first ethanol exposure. Bars represent means $\pm$ SEM, $n = 4$ for each series. No differences were observed between the first peaks of each series of exposures.
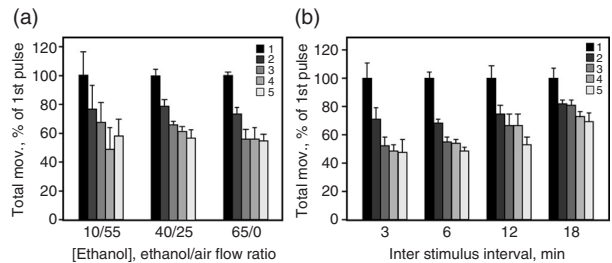
Figure 5: Effects of ethanol dose and interstimulus interval on olfactory habituation. (a) Olfactory startle attenuation was tested at various ethanol concentrations (10/55, 20/45, 30/35, 40/25, 50/15 and 65/0 relative flow rates of ethanol/air); only three concentrations are shown. Thirty-second pulses were delivered every six minutes. Pulse numbers are shaded from black (first pulse) to light gray (fifth pulse) as indicated. While the initial startle response showed a clear dose dependency (see Fig. 1d, e), all concentrations tested produced maximal attenuation to approximately 50% of the initial startle response after four to five pulses of ethanol. Bars represent means $\pm$ SEM, $n = 8$–10. (b) Olfactory startle attenuation was tested at various interstimulus intervals (ISI) (30-second pulses, 30/35 relative flow rates of ethanol/air). Maximal attenuation was achieved after approximately four to five pulses regardless of interstimulus interval. For statistical comparisons, an habituation index was calculated for each experiment by calculating the ratio of the total movement during pulse 4 to the total movement during pulse 1. A one-way ANOVA of the habituation indexes revealed an overall significant effect of interstimulus interval ($P = 0.004$). *Post hoc* comparisons by Newman-Keuls revealed significance at 12-minute and 18-minute ISIs when compared to the 3-minute ISI ($P = 0.040$ and $P = 0.0037$, respectively), and significance at the 18-minute ISI when compared to the 6-minute ISI ($P = 0.018$).

In summary, these data demonstrate that the olfactory startle response is not unique to ethanol, but can also be elicited by odorants such as EA and IA. These odorants produce habituation in a manner that is similar to that seen with ethanol. While the primary olfactory neurons that detect ethanol are unknown, those that detect EA and IA have been identified and are distinct (de Bruyne *et al.* 2001). Thus, cross-habituation between EA and IA is probably not caused by adaptation of common primary olfactory neurons and suggests a central mechanism for habituation.

Mushroom bodies are involved in olfactory habituation

The mushroom bodies (MBs) are large, bilaterally symmetric structures in the insect brain that serve several behavioral functions (Roman & Davis 2001; Zars 2000). We tested the role of the MBs in olfactory habituation by either chemical ablation or inactivation by tetanus toxin. Treatment of newly hatched larvae with the DNA-synthesis inhibitor hydroxyurea (HU) leads to preferential ablation of the mushroom bodies (MBs) (de Belle & Heisenberg 1994; Stocker *et al.* 1997). HU
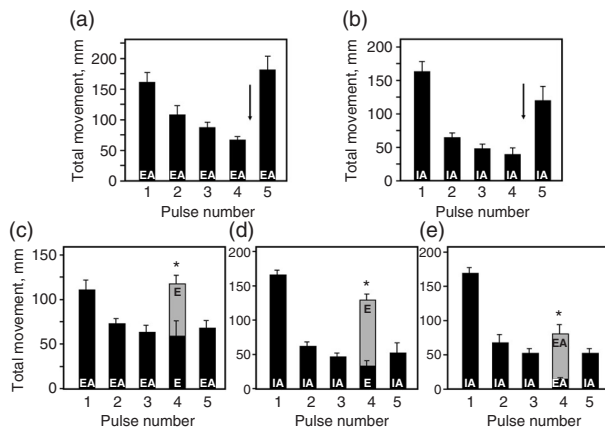
Figure 6: Cross-habituation between ethanol, ethyl acetate and isoamyl alcohol. (a, b) Flies were exposed to ethyl acetate (EA) vapor (10/55 flow rates of EA/air) or isoamyl alcohol (IA) vapor (30/35 flow rates of IA/air) for 30-second pulses every six minutes. Flies were given a mechanical stimulus between the 4th and 5th pulses (vertical arrow). In a manner similar to that seen with ethanol, flies startle upon exposure to EA and IA; this startle habituates upon repeated exposures, and habituation can be reversed by a mechanical stimulus (vertical arrow). (c, d) Flies were exposed to an initial odorant of either EA (10/55 relative flow rates of EA/air; panel (c)) or IA (30/35 relative flow rates of IA/air; panel (d)) for three pulses delivered every six minutes. Ethanol (E) (30/35 relative flow rates) was delivered during the fourth pulse instead of the initial odorant, and the initial odorant was again delivered during the fifth pulse. The lightly shaded bar represents a naïve startle response to ethanol (30/35 relative flow rates) delivered at an equivalent time point. A significant reduction in the ethanol-induced startle is seen when previously habituated with EA ($P < 0.006$ by two-tailed Students' *t*-test assuming unequal variances) or IA ($P < 0.0001$ by two-tailed Students' *t*-test assuming unequal variances). Similar results were obtained when the order of odorant administration was reversed (data not shown). (e) Flies show cross-habituation between IA and EA. Flies were exposed to IA (30/35 relative flow rates of IA/air) for three 30-second pulses, followed by a fourth pulse of EA (10/55 relative flow rates of EA/air) and a fifth pulse of IA. The lightly shaded bar corresponds to the response of naïve flies exposed to EA at the equivalent time point. The difference between the naïve and habituated EA pulses is significant, $P < 0.015$ by two-tailed *t*-test assuming unequal variances. Similar results were obtained when the order of odorant administration was reversed (data not shown). Bars represent means $\pm$ SEM, $n = 6$. Asterisks denote statistically significant differences of relevance.

treatment impairs olfactory associative learning and courtship conditioning in *Drosophila* (de Belle & Heisenberg 1994; McBride *et al*. 1999). As shown in Fig. 7 (a, b), HU treatment significantly decreased the efficacy of habituation to ethanol. MB ablation did not affect the ability of the flies to sense and respond to ethanol, as the naïve startle response was slightly greater in the HU-treated than in mock-treated

flies (data not shown). To determine the degree of MB ablation, we treated, in parallel, flies carrying a P[lacZ] enhancer trap insertion in the *rutabaga* gene (*rut*[769]), which show strong β-galactosidase expression in the MBs (Fig. 7d, –HU panels) (Han *et al*. 1992). Of 12 HU-treated fly brains analyzed, eight exhibited complete (or nearly complete) ablation (Fig. 7d, right panels) and four exhibited partial ablation (Fig. 7d, lower middle panel).

To further assess the role of MBs in olfactory habituation, we expressed the light chain of tetanus toxin (TeTx) specifically in the MBs to block evoked neurotransmitter release (Sweeney *et al*. 1995). This was achieved by the GAL4/UAS binary expression system (Brand & Perrimon 1993), utilizing the MB-specific GAL4 enhancer trap line 17d (Zars *et al*. 2000) to drive expression of a UAS-TeTx transgene. Tetanus toxin expression in the MBs significantly disrupted habituation (Fig. 7c). The presence of the toxin transgene alone produced partial disruption of habituation, perhaps due to leaky expression of the toxin. Thus, two independent methods that manipulate MB function disrupted olfactory habituation.

Rutabaga mutants exhibit reduced olfactory habituation

Several olfactory associative learning mutants, including *rutabaga* (*rut*) and *amnesiac* (*amn*), exhibit abnormal habituation in several reflexes, including a proboscis extension reflex and a visually evoked landing reflex (Duerr & Quinn 1982; Rees & Spatz 1989). We therefore tested two P-element-induced alleles of *rut*, *rut*[769] and *rut*[2080] (Han *et al*. 1992), and one allele of *amn*, *amn*[chpd] (Moore *et al*. 1998) for olfactory habituation. Both *rut* mutants exhibited reduced habituation (Fig. 8), and the extent of impairment correlated with their defect in olfactory learning and memory; *rut*[2080] has been shown to produce a greater reduction in initial learning and in 60-minute retention than that caused by the *rut*[769] mutation (Han *et al*. 1992). The *amn* mutant showed a trend toward decreased habituation that did not achieve statistical significance (Fig. 8). Of note, the *amn*[chpd] allele was isolated due to its increased sensitivity to ethanol (Moore *et al*. 1998), and to our knowledge, has not been tested for defects in learning or behavioral plasticity.

Discussion

Olfactory startle attenuation resembles classically defined habituation

Habituation is defined as 'a response decrement as a result of repeated stimulation' (Harris 1943). It is considered to be a form of nonassociative learning, one of the simplest forms of learning whereby an organism learns to suppress its response to repeated inconsequential stimuli. However, other mechanisms may produce response attenuation to repetitive stimuli. Among these are peripheral mechanisms,
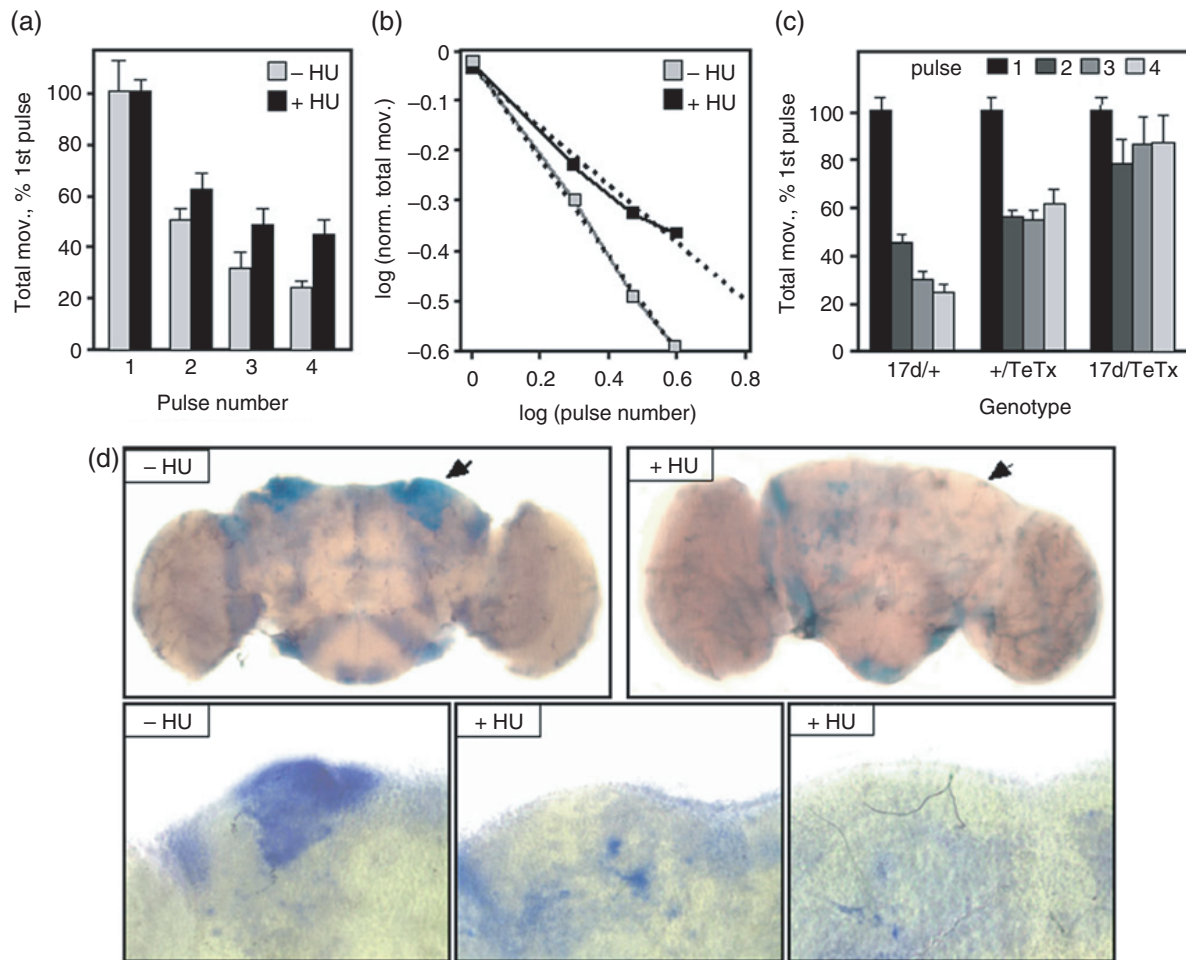
Figure 7: Mushroom bodies are involved in olfactory habituation. (a) Flies were exposed to hydroxyurea (HU) during early larval development. HU-treated flies (+ HU) were compared to mock-treated control flies (– HU) processed in parallel. Ethanol was delivered in 30-second pulses (30/35 relative flow rate of ethanol/air) every six minutes. Habituation of ethanol-induced olfactory startle (normalized to the first peak) is reduced by HU treatment. The habituation index was significantly different ($P < 0.01$) using Student's two-tailed t-tests assuming unequal variances, $n = 6$ for control flies, $n = 8$ for treated flies. The total movement during the first exposure to ethanol was 105 ± 12.5 mm (mean $\pm$ SEM) for the control group and 135 ± 6.0 mm (mean $\pm$ SEM). (b) The data presented in (a) are fit to power functions ($y = Cx^{-k}$) with the resultant equations being $y = 0.96x^{-0.518}$ for the HU-treated group and $y = 1.0x^{-1.006}$ for the control group. These data and their respective power function fits are log-transformed in (b) to demonstrate the differences in k-values (slopes). There is a significant difference in the k-values (slopes) between the HU-treated and the control groups ($P = 0.005$) using two-tailed t-tests. (c) Tetanus toxin inactivation of the MBs impairs habituation. Flies carrying P[GAL4]17d (17d) and UAS-TeTx (TeTx), as well as control flies carrying each transgene alone, were exposed to 30-second pulses of ethanol every six minutes. ANOVA of the habituation index revealed a significant effect of genotype ($P < 0.00001$); *post hoc* analysis demonstrated a significant difference between 17d/TeTx and both controls ($P < 0.05$), $n = 15$. (d) The efficacy of the HU-treatment was ascertained in rut^{769} flies, which show strong β-galactosidase expression in the MBs. Upper panels show whole-mount fly brains stained with X-GAL and lower panels show higher magnification views of the MB areas (indicated by arrows in the upper panels). Strong β-galactosidase expression is seen in the MBs of untreated flies (– HU). Reduced or nearly absent expression was seen in HU-treated flies (+ HU). See text for details.

defined by Thompson and Spencer (1966) to include adaptation of sensory systems and fatigue of effector systems. Habituation is commonly distinguished from these peripheral mechanisms by exhibiting both spontaneous recovery and reversibility through the presentation of a novel or strong stimulus (Thompson & Spencer 1966). While often used to demonstrate that habituation has occurred, these are not absolute criteria; they are operational criteria and do not suggest mechanism. For example, olfactory adaptation in *Drosophila* has been well defined, and shown to spontaneously recover (Stortkuhl *et al.* 1999). In our assay, the attenuated state demonstrated spontaneous recovery after
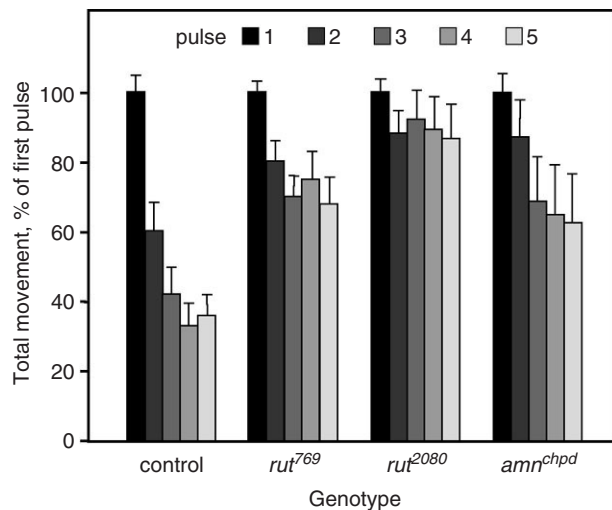
Figure 8: *rutabaga* mutants exhibit reduced habituation of olfactory startle. rut^{769}, rut^{2080}, amn^{chpd} and control flies were tested for habituation to ethanol vapor (30/35 relative flow rates of ethanol/air) delivered in 30-second pulses every six minutes. A one-way ANOVA was performed on the habituation index as a function of genotype ($P < 0.0001$). By this analysis, the mutants rut^{769} and rut^{2080} exhibited reduced habituation compared to control flies. *Post hoc* comparisons by Newman-Keuls revealed a significant difference between control flies and rut^{769} ($P = 0.007$) and between control flies and rut^{2080} ($P = 0.0004$). There was no statistical difference between control flies and amn^{chpd} ($P = 0.9$). Bars represent means ± SEM, $n = 6$–10.

30 min (Fig. 4a) and was readily reversed by a brief mechanical stimulus (Fig. 3). However, the dishabituated response differed from the naive startle response in that the dishabituated response achieved maximal velocity more slowly (Fig. 3c). This latency of the non-naïve startle responses may represent a form of adaptation or fatigue, a component of behavioral attenuation that was only partially reversed by the disruptive mechanical stimulus. Alternatively, the mechanical stimulus may not have been strong enough to produce complete recovery.

It has often been observed that the kinetics of habituation are related to the frequency of stimulus delivery, such that higher frequency stimulation produces faster habituation (Davis 1970; Rankin & Broster 1992; Thompson & Spencer 1966). This characteristic of habituation was recapitulated in our data as well. The degree of startle attenuation was greater at shorter ISIs. At the longer ISIs tested, 12 and 18 min, mechanisms of spontaneous recovery may be competing with mechanisms of habituation.

Olfactory habituation differs from classical habituation in that we fail to observe a correlation between stimulus intensity and the rate of habituation. In classical descriptions of habituation, weaker stimuli are more effective than stronger stimuli in producing response attenuation, and extremely strong, noxious stimuli fail to produce response attenuation at all (Braun & Bicker 1992; Corfas & Dudai 1989; Pinsker *et al.* 1970; Thompson & Spencer 1966; Wood 1970). Though the naïve startle response demonstrated dose dependency through the range of vapor concentrations tested, the kinetics of habituation were similar throughout this range. However, the classical inverse relationship between stimulus strength and habituation efficacy was often but not universally observed. For example, younger *Aplysia*, unlike older *Aplysia*, did not show a relationship between stimulus strength and habituation (Ludowiak & Peretz 1980). Additionally, crickets habituate to cricket-song-like sound pulses; stronger sound pulses produce greater habituation (Givois & Pollack 2000). Thus, the relationship between stimulus strength and habituation appears to vary with sensory modality and preparation, and may not be applicable to the olfactory sensory system of *Drosophila*. It is also possible that the ethanol doses used in our experiments are not noxious to flies.

Neuroanatomical sites of habituation

Because removal of the third antennal segments abolished the startle response to ethanol vapor, we conclude that the increased locomotor activity is initiated by the detection of ethanol through the olfactory organs. Odor detection begins at primary olfactory neurons within the sensilla of the antennae and maxillary palps, such that a particular odor activates a specific subset of primary olfactory neurons (de Bruyne *et al.* 2001). Simple adaptation of specific primary olfactory neurons is not adequate to explain olfactory habituation, since olfactory-independent mechanical stimulation reverses the attenuated state. This cross-modal dishabituation suggests that the central nervous system regulates olfactory startle habituation. While the olfactory neurons responding to ethanol have not been identified, those activated by EA and IA have been defined electrophysiologically and have been shown to be distinct (de Bruyne *et al.* 2001). Therefore, cross-habituation between IA and EA (Fig. 6e) is unlikely to be caused solely by adaptation of common primary olfactory neurons. Of note, Stortkuhl *et al.* (1999) also observed behavioral cross-adaptation between different odorants, including *iso*-amyl acetate and butanol, two odorants which do not share common primary olfactory neurons in their detection (de Bruyne *et al.* 2001). Taken together, these experiments suggest that cross-attenuation between different odorants utilizes central brain regions. As a caveat, odor-evoked activation of antennal lobe glomeruli has recently been visualized by fluorescent calcium-binding compounds (Wang *et al.* 2003). This study revealed that odorants at low concentrations activated specific subsets of glomeruli, whereas higher concentrations activated glomeruli more widely. It is not known whether the broader activity in glomeruli represents a broader activation of primary olfactory neurons. If high concentrations of EA and IA are capable of activating a common set of primary olfactory neurons, it remains a

possibility that these neurons participate in olfactory cross-habituation. Alternatively, mechanisms of cross-habituation may occur in the glomeruli or other parts of the brain.

The MBs receive olfactory input from the antennal lobes and constitute an important site for sensory integration and learning (Roman & Davis 2001; Zars 2000). The MBs are therefore likely candidate sites of olfactory-mediated habituation. We provide several lines of evidence that the MBs are involved in regulating olfactory habituation. Inactivation by tetanus toxin or ablation of the MBs by HU treatment significantly reduced, but did not abolish, the magnitude of habituation in our assay. These results may be explained by incomplete ablation of the MBs by HU or the preferential expression of the line 17d in the α/β lobes, a subset of MB cells (Zars *et al.* 2000). Alternatively, habituation of olfactory mediated startle may be distributed throughout several neuroanatomical sites, including the MBs. Finally, *rut*, whose function is required for habituation, is highly expressed in the MBs where it functions in olfactory conditioning (Zars *et al.* 2000). However, the loss of *rut* function has a more severe effect on habituation than that caused by HU ablation of the MBs, suggesting that *rut* more completely inactivates the MBs or that *rut* may function at multiple sites in olfactory habituation.

A central localization of habituation mechanisms is supported by other studies in *Drosophila*. For example, prolonged application of sucrose to one foot produced habituation of the proboscis extension reflex to sucrose applied to the contralateral foot, indicating that habituation must have occurred within the central nervous system (Duerr & Quinn 1982). Additionally, the *Drosophila* central complex structural mutant *no bridge* exhibited defective habituation of the proboscis extension reflex (Bouhouche *et al.* 1993). Using a different reflex, the giant fiber-mediated jump-and-flight response, Engel and Wu reported that much of the habituation of this escape reflex occurred in the brain (Engel & Wu 1996). In this paradigm, the giant fiber system was stimulated by electrodes placed in the eyes while measuring the electrophysiological responses of the thoracic jump and flight muscles. A long-latency response was produced by stimulation within the brain, at afferents to the giant fibers; a short-latency response was elicited further 'downstream', by directly stimulating the cervical region of the giant fibers. The authors observed that the long latency response habituated, whereas the short latency response did not, suggesting that regions in the brain were important for habituation of this reflex.

In summary, this study demonstrates that *Drosophila* react to ethanol as an olfactory stimulus with a characteristic increase in locomotion. This response attenuates with repeated exposures to ethanol, and the attenuation is readily reversed by a novel, disruptive stimulus and by spontaneous mechanisms. Thus, olfactory startle attenuation resembles classically defined habituation. Habituation to ethanol generalizes to other odorants, namely EA and IA. Cross-habituation between these two odorants, which are known to activate different sets of primary olfactory neurons (de Bruyne *et al.* 2001), suggests that mechanisms of attenuation occur in the central nervous system. Consistent with this idea is our finding that MBs, central brain structures crucial for insect learning, contribute to this olfactory mediated habituation. Additionally, the learning/memory mutant *rut* habituates poorly. Despite the intense study of habituation, relatively little is known about the mechanisms involved. By combining our high throughput assay with the genetic tools available in *Drosophila*, we can now carry out large-scale genetic screens for mutations that alter habituation, allowing an unbiased approach towards identifying the molecular and cellular mechanisms of habituation.

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