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Drosophila fasciclinII Is Required for the Formation of Odor Memories and for Normal Sensitivity to Alcohol

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Summary

Drosophila fasciclinII **(*fasII*) mutants perform poorly after olfactory conditioning due to a defect in encoding, stabilizing, or retrieving short-term memories. Performance was rescued by inducing the expression of a normal transgene just before training and immediate testing. Induction after training but before testing failed to rescue performance, showing that Fas II does not have an exclusive role in memory retrieval processes. The stability of odor memories in *fasII* mutants are indistinguishable from control animals when initial performance is normalized. Like several other mutants deficient in odor learning, *fasII* mutants exhibit a heightened sensitivity to ethanol vapors. A combination of behavioral and genetic strategies have therefore revealed a role for Fas II in the molecular operations of encoding short-term odor memories and conferring alcohol sensitivity. The preferential expression of Fas II in the axons of mushroom body neurons furthermore suggests that short-term odor memories are formed in these neurites.**

Introduction

Significant efforts have been made in recent years to assign the function of an assortment of molecules to distinct temporal phases of memory, including short-term, intermediate-term, and long-term memory. Some molecules appear to function rather specifically in distinct temporal phases, which is due to the fact that different cellular mechanisms underlie different phases. For instance, the transcription factor CREB functions in long-term memory, evidenced by experiments which show that its inactivation impairs long-term but not short-term processes (Dash et al., 1990; Yin et al., 1994; Bourtchuladze et al., 1994). The calcium-dependent phosphatase, calcineurin, appears to participate in intermediate-phase processes since intermediate-phase LTP is blocked by a transgene overexpressing the enzyme but early-phase LTP is preserved (Winder et al., 1998). These effects on LTP correlate with the behavior of the transgenic animals; short-term behavioral memories are spared while those of longer duration are impaired (Mansuy et al., 1998).

Although these and other genetic studies have shown a requirement for certain molecules in specific temporal phases of behavioral memory, the specific operations effected by these molecules within each phase have remained unsolved. In other words, these molecules could, in principle, be involved in the molecular operations needed to encode memories of a given temporal phase, provide for the stability of those memories, or facilitate their retrieval. A genetic disruption of any of these operations would impair animal performance in the temporal phase during which the animal is tested.

Related issues have been approached pharmacologically for LTP. Pharmacological inhibitors applied before stimulation block LTP if the inhibited molecule is involved in LTP induction (Malinow et al., 1988, 1989; Otmakhov et al., 1997). If LTP is attenuated in an irreversible way by application of the pharmacological agent after induction, then the inhibited molecule is argued to be involved in LTP maintenance. A few pharmacological experiments have also been carried out in behaving animals before and after training to determine the behavioral impact of such disruptions. One informative study utilized reversible inactivation of the entire hippocampus with local infusion of an inhibitor of fast glutaminergic synaptic transmission (Riedel et al., 1999). Infusion of the inhibitor during training, chronically after training, or just before testing impaired performance of a spatial task two weeks later, leading to the conclusion that hippocampal activity is required for all three operations: encoding, storage, and retrieval of spatial memories. In principle, an analogous strategy could be used in genetic studies of memory formation by removing or supplying gene activity before or after training to dissect the role of specific genes in the operational aspects of memory formation.

By using a combination of behavioral and genetic manipulations, we provide evidence here that the transmembrane form of Fas II is involved in encoding short-term odor memories but not in memory stability or retrieval. The *fasII* gene encodes three isoforms of a homophilic cell adhesion receptor similar to the isoforms of vertebrate NCAM and it has been well studied for its roles in cellular migration (Grenningloh et al., 1990, 1991; Holmes and Heilig, 1999; Wright et al., 1999), axon bundling (Lin and Goodman, 1994; Lin et al., 1994), and synapse stabilization and growth (Schuster et al., 1996). All three forms share the same extracellular portion with five immunoglobulin-like repeats and two fibronectin type III domains, but they differ in sequences at their carboxy termini (Grenningloh et al., 1991; Lin and Goodman, 1994). Two forms have a transmembrane domain with short but divergent intracellular tails; one contains a PEST sequence for rapid protein turnover and the other is without this sequence. A third form is missing the transmembrane domain and is attached to the cellular membrane via a GPI linkage. In addition, we show that *fasII* mutation confers a heightened sensitivity to etha-

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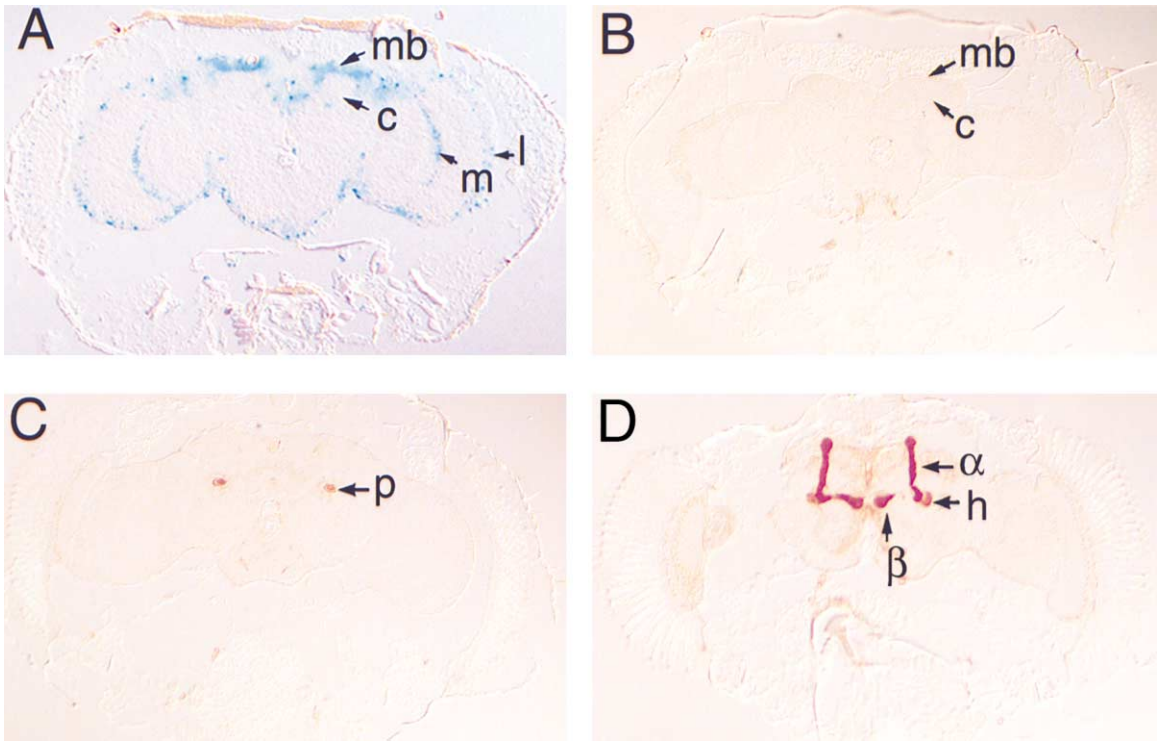


Figure 1. *fasII* Expression in the Brain

Frontal sections of the adult brain stained for the reporter βGAL (A) or with an anti-Fas II antibody (B-D).

(A) Expression of the nuclear localized βGAL reporter in the enhancer detection line, *fasII*^{MB2225}. Expression of the *fasII* gene is observed primarily in the mushroom bodies (mb), lamina (l), and medulla (m).

(B–D) Sections at different levels from posterior (B) to anterior (D) showing that Fas II protein is not expressed in the cell bodies of the mushroom body neurons (mb) nor in the calyx (c), the neuropil housing mushroom body cell dendrites. The antigen is found in the peduncle (p), the heel (h), and the α and β lobes of the mushroom bodies. Weak staining is also observed in the γ lobes (not shown) but is undetectable in the α′/β′ lobes.

nol. Key to the dissection of the operational functions of Fas II in memory were the use of an inducible transgene to provide gene product either before or after training, and a behavioral strategy to normalize the initial performance of control and *fasII* mutants so that memory stability could be compared.

Results

The *fasII* Gene Is Preferentially Expressed in the Mushroom Bodies

We have previously utilized enhancer detection as a method to screen for genes preferentially expressed in the mushroom bodies and potentially involved in their physiology and odor learning. This screen yielded insertions at several loci involved in odor learning including *dunce* (*dnc*; unpublished data), *rutabaga* (*rut*; Levin et al., 1992; Han et al., 1992), protein kinase A (*DC0*; Skoulakis et al., 1993), *leonardo* (*leo*; Skoulakis and Davis, 1996), and *Volado* (*Vol*; Grotewiel et al., 1998). Another line selected from the same screen (Han et al., 1996) that shows preferential expression of the *lacZ* reporter in the mushroom bodies is MB2225, but this line also has reporter expression in the lamina, medulla, and portions of the central brain (Figure 1A). From plasmid rescue of MB2225 and DNA sequencing, we determined that the enhancer detector element in this line resides in the first exon of the *fasII* gene (Figure 2A).

We utilized immunohistochemistry to assay for the expression of Fas II within sections of the adult brain. No Fas II expression was detected in the cell bodies of the mushroom body neurons or in the calyces, the neuropil region housing the dendrites of these neurons (Figure 1B). However, highly preferential expression was detected in the peduncle, the nerve consisting of mushroom body axons that project from posterior to anterior in the brain (Figure 1C) and in the lobes, the neuropil regions that contain mushroom body axons and their termini (Figure 1D). Nevertheless, the expression was not uniform within different types of mushroom body neurons (Crittenden et al., 1998; Lee et al., 1999). No expression was detected in the α′/β′ mushroom body neurons, neurons that have axon collaterals projecting dorsally into the α′ lobe and medially into the β′ lobe. And a modest level of expression was detected in the γ mushroom body neurons, neurons that send a single projection medially from the heel toward the midline and comprise the γ lobe neuropil. Marked expression was observed in mushroom body neurons that project into the α and β lobes and are referred to as α/β mushroom body neurons. These observations confirm and extend a prior report (Crittenden et al., 1998) of Fas II expression in the mushroom bodies and show that the transmem-

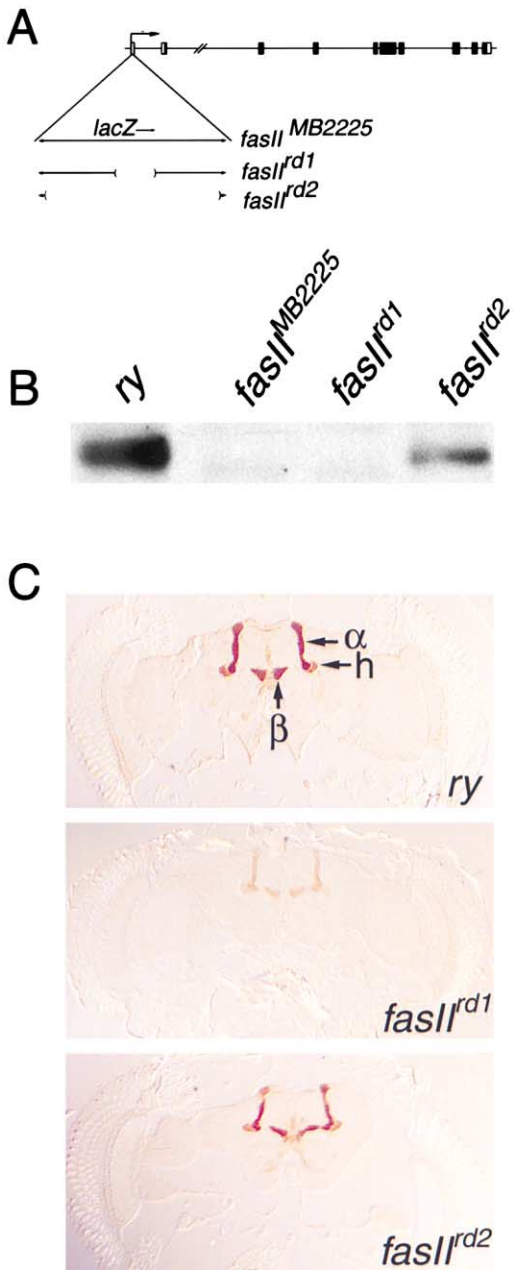


Figure 2. The *fasII* Gene, Mutants, and Expression

(A) The intron/exon structure of *fasII* was deduced from genomic sequence (Adams et al., 2000) and cDNA sequences (Grenningloh et al., 1991). Filled boxes indicate protein coding sequences and open boxes the untranslated sequences. The direction of transcription is indicated by the arrow above exon 1. The *fasII*^{MB2225} mutant is an insertion of a pIArB element at residue 123 in the 1st exon of *fasII*. Two excision derivatives of this insertion are shown in an expanded view below exon 1.

(B) Western blotting of head protein showing that Fas II expression in *fasII*^{MB2225} and *fasII*^{rd1} is undetectable. The Fas II signal in *fasII*^{rd2} was measured at 47 ± 4% of the *ry* control (n = 6).

(C) Anti-Fas II immunohistochemistry showing a severe reduction of Fas II expression in *fasII*^{rd1} and a modest reduction in *fasII*^{rd2} relative to *ry* control animals.

brane form of Fas II, the form detected by MAb 1D4, is rather specifically expressed in the α/β type of mushroom body neurons.

Characterization of *fasII* Mutants

The *fasII* gene is large (Figure 2A) with 10 exons distributed over 74 kb of genomic DNA (Grenningloh et al., 1991; Adams et al., 2000). The three known isoforms of Fas II are produced from alternative splicing of transcripts that originate from the same transcriptional start site. Thus, the *fasII*^{MB2225} insertion in the 5′ untranslated region and any derivatives should disrupt the expression of all three isoforms equally.

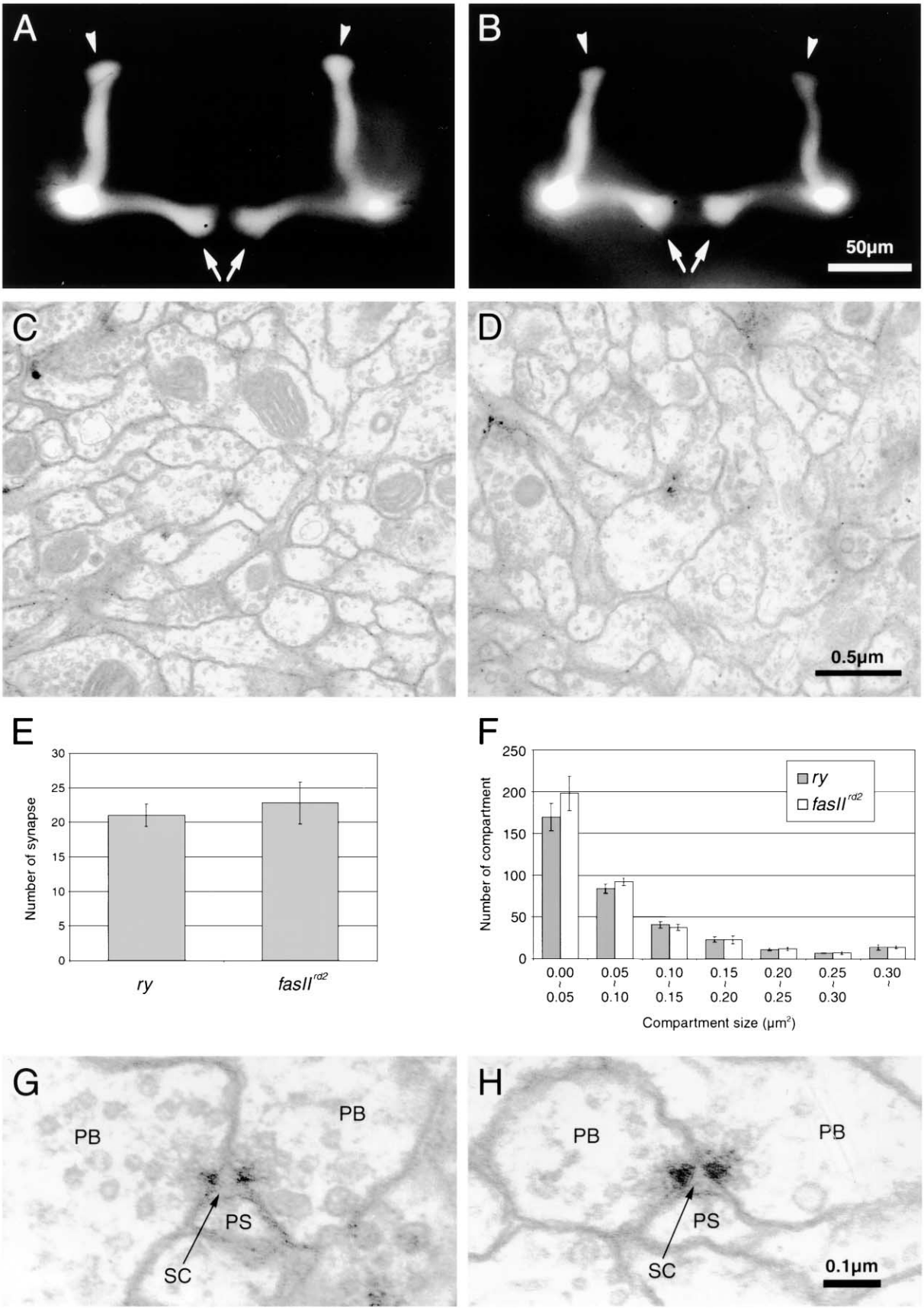
The *fasII*^{MB2225} stock is viable but quite weak with low fecundity. To identify additional alleles of *fasII* that could potentially be used for adult behavioral assays, we performed genetic crosses to obtain imprecise excisions. Several excision derivatives that affect *fasII* function were obtained. Two of these are characterized in detail here. The alleles *fasII*^{rd1} and *fasII*^{rd2} contain deletions internal to the enhancer detector element in *fasII*^{MB2225}. The *fasII*^{rd1} allele has a remnant of the original enhancer detector element larger than 4 kb and it retains both 5′ and 3′ inverted repeats of the P factor. The allele *fasII*^{rd2} is similar but retains only a 66 bp insertion (Figure 2A).

These mutants were characterized for Fas II expression level by Western blotting and by immunohistochemistry of the adult head. The levels of Fas II expression in *fasII*^{MB2225} and *fasII*^{rd1} were undetectable by Western blotting experiments (Figure 2B). Fas II protein in the *fasII*^{rd2} mutant was reduced to 47 ± 4% of the control (n = 6). Immunohistochemical assays were broadly consistent with these results (Figure 2C). Thus, the alleles with insertions of DNA at the same position in exon 1 have differing effects upon Fas II expression.

Mushroom Body Morphology in *fasII* Mutants

We studied the structure of the mushroom body neuropil in *fasII* mutants using light and electron microscopy. Since the gene is preferentially expressed in α and β lobes of the mushroom bodies, we focused on the α/β type of mushroom body neuron. GFP was expressed in the α/β lobes of control animals as well as *fasII* ^{rd1} and *fasII*^{rd2} males using the GAL4 driver c739 (Figures 3A and 3B; not shown). No difference was observed in the intensity of fluorescence or in the size or shape of the dorsally projecting α lobe or medially projecting β lobe. No fluorescence suggestive of misrouted axons was observed, and the swellings at the tips of the α and β lobes that are characteristic of wild-type lobes were readily apparent and unchanged in the *fasII* mutants. Identical results were obtained using the mushroom body preferential-GAL4 drivers 201Y and c772 (not shown).

Electron micrographs of the α lobe neuropil from *fasII* mutants also failed to reveal any differences (Figures 3C and 3D). The frequency distribution of the size of membranous compartments measured in the α lobe neuropil was indistinguishable in the mutant compared to control animals (Figure 3F). Structures typical of synapses were observed that consisted of an electron-dense presynaptic ribbon surrounded by synaptic vesicles. We found that the density of these synapses within


A
B
50μm
C
D
0.5μm
E
Number of synapse
30
25
20
15
10
5
0
ry
fasII rd2
F
Number of compartment
250
200
150
100
50
0
ry
fasII rd2
0.00 / 0.05
0.05 / 0.10
0.10 / 0.15
0.15 / 0.20
0.20 / 0.25
0.25 / 0.30
0.30 /
Compartment size (μm2)
G
PB
PB
PS
SC
H
PB
PB
PS
SC
0.1μm

the neuropil was identical between mutant and control (Figure 3E; $p = 0.632$). In addition, we failed to find any difference in the overall ultrastructure of the synapses between control and mutant (Figures 3G and 3H) with respect to morphology, size, or the presence of synaptic vesicles.

We conclude that the overall mushroom body structure, its neuropil, and the contained synapses have no detectable physical alteration due to the loss of *fasII* function. This is in contrast to the marked effect that the loss of *fasII* function has upon synapses at the larval neuromuscular junction (Schuster et al., 1996). This suggests that Fas II must have functions at the NMJ that are unimportant or of little consequence for adult mushroom body neurons.

fasII Mutants, Odor, and Shock Perception

Some reports have suggested that mushroom bodies are involved in olfactory processing (Ito et al., 1998). To determine whether *fasII* functions within mushroom bodies to mediate odor perception, we tested whether the mutants avoid two odors used frequently in odor learning experiments.

The mutant $fasII^{rd1}$ and $fasII^{rd2}$ both avoided the aversive odors of octanol and benzaldehyde to the same degree as the *ry* control animals at two different odor concentrations (Figure 4A), indicating that the perception of these odors and the activation of motor circuits is normal in the mutants. Preat (1998) demonstrated that electric shock administered prior to an odor avoidance task decreases the odor avoidance compared to nonshocked controls, presumably due to the generation of stress. Furthermore, some mutants exhibit an exaggerated odor response after preshock relative to control animals. We tested *fasII* mutants for this behavior (Figure 4B). Octanol avoidance of the control and the two *fasII* mutants was depressed after preshock (compare 4B with 4A), but the magnitude of this depression was identical in all three genotypes. Benzaldehyde responses were unaffected by preshock. Therefore, if the behavioral responses elicited by the perception of these two aversive odorants require mushroom body activity, this activity must be independent of *fasII* function.

In addition, we examined whether the mutants perceive and avoid electrified grids used for delivering negative reinforcement for learning (Figure 4C). No difference in avoidance was observed between the genotypes at two different applied voltages. Therefore, both $fasII^{rd1}$ and $fasII^{rd2}$ appear to have normal sensory functions required for olfactory learning tasks.

Normal Odor Learning Requires *fasII* Function in Adults

The highly preferred expression of Fas II in the mushroom bodies prompted us to search for a role in odor learning. Control animals and the two *fasII* mutants were given olfactory classical conditioning using the odors described above as conditioned stimuli and electric shock as the unconditioned stimulus. We used this training to examine the memory of the conditioned stimulus at 3 min, 20 min, 60 min, and 180 min after training. The *fasII* mutants exhibit defective memory of the conditioned stimulus compared with *ry* control flies at all times tested (Figure 5A). The $fasII^{rd1}$ and $fasII^{rd2}$ mutants show similar but relatively poor performance compared to *ry* despite a difference in Fas II expression level. Moreover, three other *fasII* alleles that we isolated showed a similar reduction in 3 min memory (not shown). Given that the learning defect was apparent at the earliest time after training (3 min), these data suggest that *fasII* is required for the molecular operations underlying short-term memory.

To prove that the memory deficit is due to mutation at the *fasII* locus, we constructed a transgene in which the heat shock protein 70 (hs) promoter drives the expression of the cDNA for the transmembrane form of Fas II. This transgene, carried in two independent transgenic lines, *hs-fasII-1* and *hs-fasII-2*, was crossed into the *fasII* mutant background and animals homozygous for the transgene were tested for 3 min memory after olfactory classical conditioning. Some groups were given heat shock 3 hr prior to training and others were given no heat shock (Figure 5B). The *hs-fasII-2* transgene in either the $fasII^{rd1}$ or $fasII^{rd2}$ mutant backgrounds partially rescued the poor performance of the mutants. This rescue was independent of heat shock, indicating that constitutive expression of the transgene produced the elevated memory scores. The *hs-fasII-1* transgene fully rescued the 3 min memory deficit of the mutants with or without heat shock. The improved rescue with *hs-fasII-1* correlates with the expression level of the transgene. Western blotting experiments have shown that both *hs-fasII* transgenes are leaky at 25°C, with *hs-fasII-1* exhibiting higher Fas II expression levels than *hs-fasII-2* with or without heat shock (data not shown).

These behavioral rescue experiments establish that Fas II function is required for normal odor learning. Furthermore, they also establish that the expression of the

Figure 3. Structure of the Mushroom Bodies in *fasII* Mutants

Analysis of the general organization of the mushroom bodies (A and B), the mushroom body neuropil by electron microscopy (C, D, and F), and neuropil synapses (E, G, and H) in $fasII^{rd2}$ (B, D, and H) and the *ry* control (A, C, and G).

(A and B) The general organization of mushroom bodies as revealed by GFP fluorescence, in which UAS:GFP was driven by the GAL4 line, c739. No significant difference was observed in the overall organization of the α lobes (arrowheads) or the β lobes (arrows).

(C and D) Electron micrographs of the α lobe neuropil in *ry* (C) and $fasII^{rd2}$ (D) mutants. No differences were detected.

(E) Mean number of synapses counted in the α lobe of *ry* and $fasII^{rd2}$. An electron-dense, presynaptic ribbon with surrounding vesicles defined each synapse. The mean number was about 21 synapses per 38.5 μm^2 of neuropil area. $p = 0.63$; Student's t test with $n = 4$.

(F) Frequency distribution of the membrane compartment size in the α lobe of *ry* and $fasII^{rd2}$. $n = 4$.

(G and H) High-powered micrographs of synapse structure in the α lobe of *ry* (G) and $fasII^{rd2}$ (H). No differences were detected. Note the two presynaptic boutons (PB) in each panel synapsing on one postsynaptic compartment (PS) through the triangle-shaped synaptic cleft (SC).

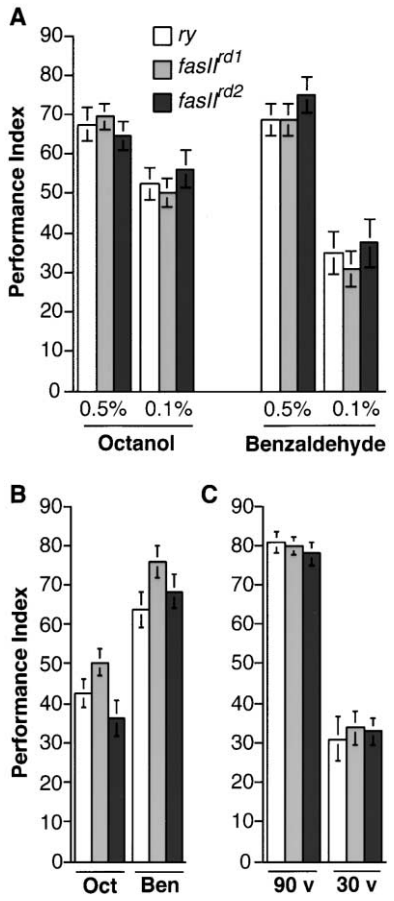


Figure 4. Sensory Functions in *fasII* Mutants

(A) Odor avoidance of *fasII* mutants and *ry* control animals to 0.5% and 0.1% octanol and benzaldehyde. There was no effect of genotype after one factor ANOVA for 0.5% octanol ($F_{(2,42)} = 0.51$; NS), 0.1% octanol ($F_{(2,42)} = 0.33$; NS), 0.5% of benzaldehyde ($F_{(2,42)} = 0.70$; NS), or 0.1% benzaldehyde ($F_{(2,42)} = 0.44$; NS). n = 15 for each group.

(B) Odor avoidance after electric shock. One group of flies was exposed to 12 electric shocks in the presence of 0.5% benzaldehyde and subsequently tested for avoidance to 0.5% octanol (Oct). The other group was exposed to 12 electric shocks in the presence of 0.5% octanol and subsequently tested for avoidance to 0.5% benzaldehyde (Ben). One factor ANOVA for 0.5% octanol avoidance after shock and benzaldehyde exposure revealed a significant effect of genotype ($F_{(2,42)} = 3.63$; $p = 0.035$). n = 15 for each group. Post hoc comparisons using Bonferroni correction revealed no significant difference between *ry* and *fasII*^{rd1} ($p = 0.14$) or *ry* and *fasII*^{rd2} ($p = 0.24$), but a significant difference between *fasII*^{rd1} and *fasII*^{rd2} ($p = 0.01$). This is irrelevant since each mutant is not significantly different from the control. One factor ANOVA for 0.5% benzaldehyde avoidance after shock and octanol exposure revealed no significant effect of genotype ($F_{(2,42)} = 2.26$; NS). n = 15 for each group. Octanol avoidance after shock (B), however, is decreased relative to octanol avoidance with no pre-shock (A).

(C) Electric shock avoidance of *fasII* mutants. There was no effect of genotype after one factor ANOVA for shock at 90V ($F_{(2,21)} = 0.56$, NS, n = 8) or at 30V ($F_{(2,24)} = 0.44$, NS, n = 9).

transmembrane form of Fas II by itself is sufficient to correct the odor learning defect. Nevertheless, the constitutive rescue by the transgenes in the absence of heat shock fails to discriminate whether the expression that rescues the phenotype occurs during development of the animals or just prior to training of adult flies.

To address this issue, we first asked whether the rescue observed by raising *fasII*^{rd2}*;hs-fasII-1* flies at 25°C would be reversed with an overnight incubation at 18°C prior to training. If so, this would indicate that the leakiness of the *hs-fasII* transgene is reduced at 18°C and that the behavioral rescue was due to transgene expression in the adult. Such treatment did indeed reverse the rescue observed with *fasII*^{rd2}*; hs-fasII-1* flies raised at 25°C (Figure 5C, bars 3 and 4). Incubation of the *ry* control animals at 18°C for 20 hr prior to training, however, had no effect upon performance (Figure 5C, bars 1 and 2). This established the required temperature shift conditions to modulate transgene expression and also strongly suggested that the transgene must be expressed during adulthood for the rescue of the memory deficit.

To further define the temporal requirements for rescue, we incubated *fasII*^{rd2}*; hs-fasII-1* flies at 18°C for 20 hr to produce mutant learning and then gave them a brief incubation at 25°C just prior to training. Incubation of these animals at 25°C for either 1 hr (Figure 5C, bar 5) or 2 hr (Figure 5C, bar 6) produced memory performance indistinguishable from *ry* controls. This clearly establishes that Fas II expression just prior to training corrects the memory deficit of the mutants. Furthermore, this correction is reversible. After a 2 hr incubation at 25°C, one group of *fasII*^{rd2}*;hs-fasII-1* flies were transferred back to 18°C for 24 hr and then trained and tested. These animals performed at mutant levels similar to *fasII*^{rd2} mutants treated in an identical fashion (Figure 5C, bars 8 and 9). The rapid behavioral rescue with a temperature shift and the reversibility of this rescue make improbable the possibility that the mushroom body synapses have developed improperly in the *fasII* mutants and produced an odor learning defect. Rather, the results collectively indicate that Fas II performs a physiological function for odor learning.

fasII Mutants Are Defective in Memory Formation but Not Memory Stability nor Retrieval

There are three explanations for the deficit in odor memory of *fasII* mutants at 3 min after training (Figure 5A). First, Fas II may have a role in the formation of odor memories (encoding) such that in the absence of Fas II, the mutants exhibit poor performance immediately after training. Second, *fasII* mutants may form normal odor memories but the stability of these is compromised early after training, leading to early performance deficits. The parallel decay of memory in *fasII* mutants compared to the *ry* control (Figure 5A) could be interpreted as favoring the former possibility, but this is weak evidence. A strict comparison of memory decay rates between mutant and control cannot be made since their initial performance differs and the kinetics of memory decay may vary as a function of the initial level of performance. A third possibility is that *fasII* mutants are defective in memory retrieval. That is, they may form normal memories with normal stability, but the retrieval of these memories at testing is defective, producing poor performance at all time points after training.

We trained *fasII* mutants along with controls with a SHORT PROGRAM schedule (Beck et al., 2000) to help distinguish these possibilities. The SHORT PROGRAM

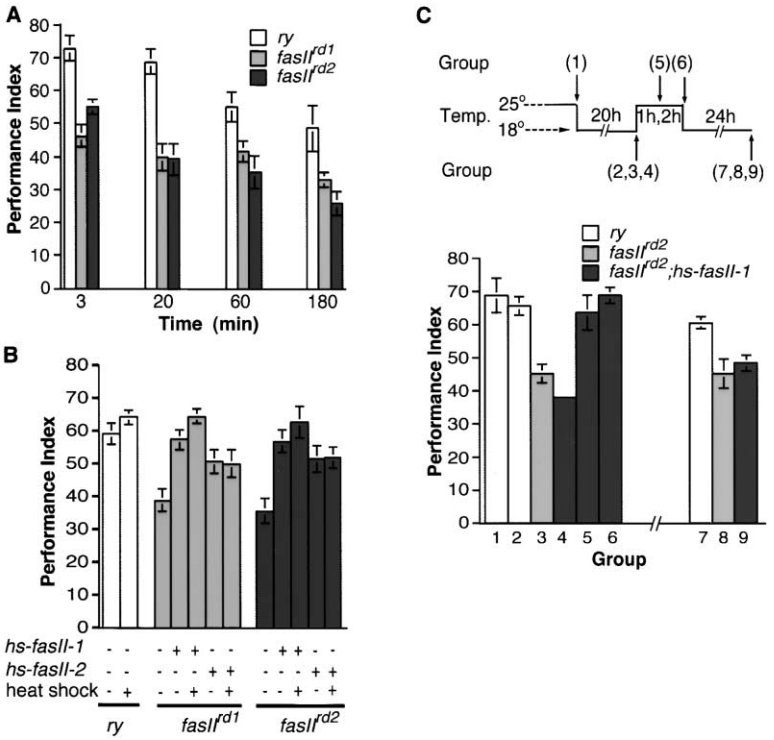


Figure 5. *fasII* Mutant Performance after Odor Conditioning

(A) Memory time course for the *fasII* mutants, *fasII*^{rd1} and *fasII*^{rd2}. Mutants and controls were given LONG PROGRAM training and performance was assayed at various times up to 3 hr after training. Two factor ANOVA revealed a significant effect of time and genotype (time, $F_{(3,60)} = 16.1$, $p < 0.0001$; genotype, $F_{(2,60)} = 38.1$, $p < 0.0001$; $n = 6$ per group) but no interaction between time and genotype ($F_{(6,60)} = 1.25$, NS). Post hoc comparisons with Bonferroni correction revealed a significant difference between *ry* and *fasII*^{rd1} at 3 min ($p < 0.0001$) and 20 min ($p = 0.0002$) but no significant difference at 60 min ($p = 0.0447$) or 180 min ($p = 0.0181$). Similar comparisons revealed a significant difference between *ry* and *fasII*^{rd2} at 3 min ($p < 0.0015$), 20 min ($p = 0.0001$), 60 min ($p = 0.0055$), and 180 min ($p = 0.0038$). No significant differences were found between *fasII*^{rd1} and *fasII*^{rd2} at any time point.

(B) The deficit in 3 min memory of *fasII* mutants is rescued by *hs-fasII* transgenes. Two transgenic lines, *hs-fasII-1* and *hs-fall-2*, which contained an identical *hs-fasII* transgene, were combined with the two *fasII* mutant alleles and 3 min memory tested as in (A) with (+) or without (−) heat shock. One factor ANOVA revealed a significant effect of group ($F_{(11,96)} = 4.50$, $p < 0.0001$, $n = 10$ for all groups except *fasII*^{rd1} and *fasII*^{rd2} where $n = 4$). Planned comparisons using Dunnett's method with *ry* as a control revealed a significant difference with *fasII*^{rd1} and *fasII*^{rd2}.

(C) Three min memory performance measured after temperature shifts. The control, *ry*, *fasII*^{rd2}, and *fasII*^{rd2}*;hs-fasII-1* animals were subjected to temperature shifts between 18°C and 25°C. A timeline representing the temperature shifts is shown with the groups trained and tested at the times indicated. Two other independent experiments have shown that shifts between 18°C and 25°C have no effect upon the performance index of *ry* or *fasII*^{rd2} animals.

(1) *ry* flies raised and trained at 25°C.
(2) *ry* flies raised at 25°C, incubated at 18°C for 20 hr and immediately trained and tested.
(3) *fasII*^{rd2} mutant flies; same conditions as (2).
(4) *fasII*^{rd2}*; hs-fasII-1* flies; same conditions as (2).
(5) *fasII*^{rd2}*; hs-fasII-1* flies raised at 25°C, incubated at 18°C for 20 hr, and then incubated for 1 hr at 25°C before training and testing.
(6) *fasII*^{rd2}*; hs-fasII-1* flies raised at 25°C, incubated at 18°C for 20 hr, and then incubated for 2 hr at 25°C before training and testing.
(7) *ry* flies raised at 25°C, incubated at 18°C for 20 hr, then at 25°C for 2 hr, and subsequently at 18°C for 24 hr before training and testing.
(8) *fasII*^{rd2} flies; same conditions as (7).
(9) *fasII*^{rd2}*; hs-fasII-1* flies; same conditions as (7).

One factor ANOVA for groups 1–6 revealed a significant effect of genotype ($F_{(5,30)} = 14.43$, $p < 0.0001$, $n = 6$ for all groups). Post hoc comparisons for groups 1–6 with Bonferroni correction revealed a significant difference between bars 2 and 3 ($p < 0.0003$), 2 and 4 ($p < 0.0001$), 1 and 3 ($p < 0.0001$), 3 and 5 ($p = 0.0009$), 3 and 6 ($p < 0.0001$), 1 and 4 ($p < 0.0001$), 4 and 5 ($p < 0.0001$), and 4 and 6 ($p < 0.0001$). This indicates that *hs-fasII-1* rescues the 3 min memory deficit of *fasII*^{rd2}only when flies are incubated at 25°C for at least 1 hr. One factor ANOVA for groups 7–9 revealed a significant effect of genotype ($F_{(2,15)} = 6.99$, $p = 0.007$, $n = 6$ for all groups). Post hoc comparisons for groups 7–9 with Bonferroni correction revealed a significant difference between bars 7 and 8 ($p = 0.0029$) and 7 and 9 ($p = 0.0138$) but not between 8 and 9 ($p = 0.45$). This indicates that the rescue of the 3 min memory deficit of *fasII*^{rd2} with 25°C incubation (bar 6) can be reversed by reincubation at 18°C.

utilizes only a 10 s odor presentation as conditioned stimulus and one shock pulse as the unconditioned stimulus. It produces modest performance after only one training trial and allows the adjustment of initial performance by varying the number of odor:shock pairings. Flies were trained with 1, 2, 3, 5, 7, 10, and 15 trials with a 30 s intertrial interval and the accumulated memory measured immediately after the last training trial. The results show that *fasII*^{rd1} and *fasII*^{rd2} perform poorly relative to *ry*, but equivalently to each other after each training trial, even after the first (Figure 6A). These results establish conditions necessary to normalize immediate performance of the mutants relative to the control so that the decay of behavioral memory can be compared. For example, the performance of *ry* given 3 training trials is equivalent to *fasII* mutants given 10 training trials.

The *fasII*^{rd2} mutants and *ry* controls were trained for 10 and 3 trials, respectively, and their memory measured at 3 min, 60 min, and 180 min after training (Figure 6B). The performance of *ry* and *fasII*^{rd2} under these training conditions was indistinguishable at 3 min and at two subsequent time points. Similarly, when *ry* was trained with 2 trials and *fasII*^{rd2} with 5 trials, 3 min performance was identical (Figure 6B). Moreover, their performance at two subsequent time points after training was indistinguishable. These experiments establish, therefore, that when the initial performance of *ry* and *fasII* mutants are

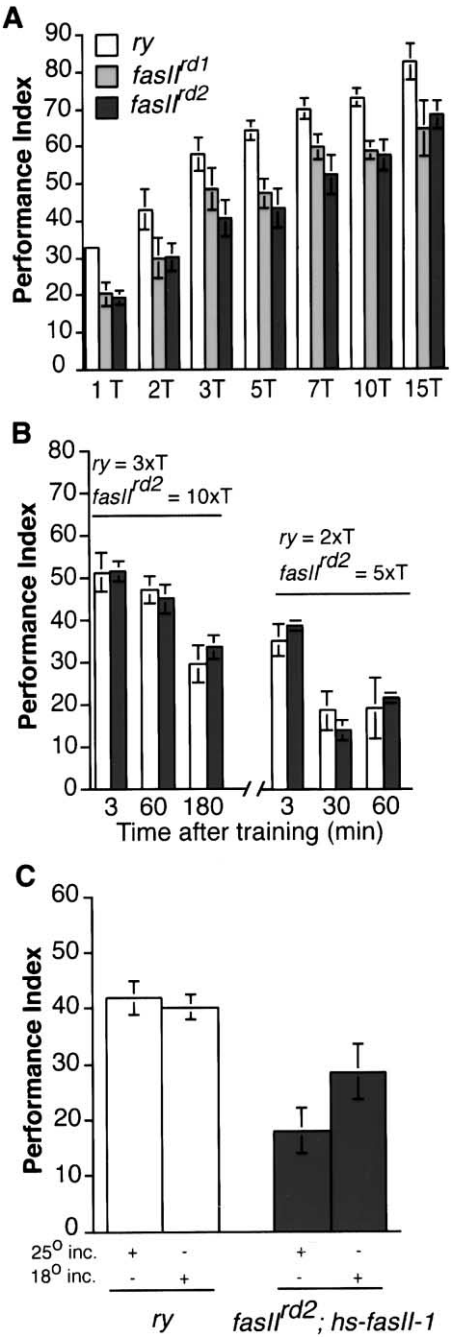


Figure 6. *fasII* Mutants Are Defective in Memory Formation

(A) The *fasII* mutants show a defect in memory acquisition in multiple trial, SHORT PROGRAM training. The two *fasII* mutants were trained with 1 to15 SHORT PROGRAM training trials and tested immediately after the final trial. Performance of the mutants parallels the *ry* control but is delayed. Planned comparisons using Dunnett's post hoc analysis was applied to the groups given the same number of training trials. At trials 1, 5, and 10, the performance of both *fasII*^{rd1} and *fasII*^{rd2} was significantly lower than the *ry* control. At 7 trials, only *fasII*^{rd2} was significantly lower than *ry*. n = 6 for all groups.

(B) Comparison of memory decay after SHORT PROGRAM training with *ry* flies receiving 3 trials and *fasII*^{rd2} flies receiving 10 trials (left half). Comparison of memory decay after SHORT PROGRAM training with *ry* flies receiving 2 trials and *fasII*^{rd2} flies receiving 5 trials (right half). Two factor ANOVA for the *ry*, 3 trial/*fasII*^{rd2}, 10 trial experiment revealed a significant effect of time ($F_{(2,32)} = 17.4$, $p < 0.0001$, n = 6 for 3 and 60 min, n = 7 for 180 min) but no effect of genotype ($F_{(1,32)} = 0.147$, NS, n = 6 for 3 and 60 min, n = 7 for 180 min). There was no significant interaction ($F_{(2,32)} = 0.50$, NS). Two factor ANOVA for the *ry*, 2 trial/*fasII*^{rd2}, 5 trial experiment revealed a significant effect of time ($F_{(2,30)} = 14.9$, $p < 0.0001$, n = 6) but no effect of genotype ($F_{(1,30)} = 0.14$, NS, n = 6). There was no significant interaction ($F_{(2,30)} = 0.57$, NS). These data indicate that behavioral memory decays at the same rate, independent of genotype.

(C) Induction of *hs-fasII-1* after training fails to rescue 2 hr memory. The *ry* and *fasII*^{rd2}; *hs-fasII-1* flies were incubated at 18°C for 24 hr and subsequently trained at 18°C. One group of each genotype was maintained at 18°C and memory tested 2 hr later; a second group of each genotype was shifted to 25°C and memory tested at the same time. Incubation of *fasII*^{rd2}; *hs-fasII-1* flies at 25°C after training failed to rescue 2 hr memory. Planned comparisons using Dunnett's post hoc analysis showed no significant effect of temperature on *fasII*^{rd2}; *hs-fasII-1* flies but a significant difference between the two genotypes. n =11 for all groups.

normalized to each other at two different initial performance levels (52 for Figure 6B, left; and 35 for Figure 6B, right), memory decays at an identical rate irrespective of genotype. Thus, Fas II is not required for the stability of behavioral memory.

We utilized the *fasII*^{rd2}*; hs-fasII-1* transgenic animals to address the issue of whether the behavioral deficit in *fasII* mutants is due to defective retrieval of normally formed and stable memories. As illustrated above (Figure 5C), induction of *hs-fasII-1* with a 2 hr temperature shift before training from 18°C to 25°C rescues the 3 min performance deficit of *fasII*^{rd2}. If this rescue is due to supplying Fas II for a function in retrieval, then supplying Fas II after training and just before testing should also produce behavioral rescue.

To test this, we incubated *fasII*^{rd2}; *hs-fasII-1* flies at 18°C for 24 hr and trained them at 18°C. After training, one group was maintained at 18°C and the other shifted to 25°C. Memory was tested in both groups 2 hr later. Continued 18°C incubation after training or shifting flies to 25°C had no effect upon 2 hr memory of *ry* control animals (Figure 6C), nor was there any difference in 2 hr memory when *fasII*^{rd2}*; hs-fasII-1* flies were shifted to 25°C. These results argue that the behavioral rescue with a 25°C shift just prior to training is not due to a rescue of defective retrieval mechanisms.

Thus, these data collectively argue that the role of Fas II in odor learning is not due to stabilizing memories or providing for retrieval. Rather, these data argue that Fas II functions in the processes allowing for memories to be encoded.

fasII Mutants Are Unusually Sensitive to Alcohol

Previous work has revealed an overlap between genes required for normal odor learning and those that regulate the sensitivity to ethanol in flies. Mutations in *amnesiac* and *rutabaga*, for example, show increased sensitivity to the intoxicating effects of ethanol (Moore et al., 1998). We therefore tested *fasII*^{rd1} and *fasII*^{rd2} mutant flies for ethanol sensitivity in the inebriometer, an assay that quantifies the effect of ethanol on postural control. Flies carrying these *fasII* mutant alleles showed increased ethanol sensitivity, manifested as a reduced mean elu-

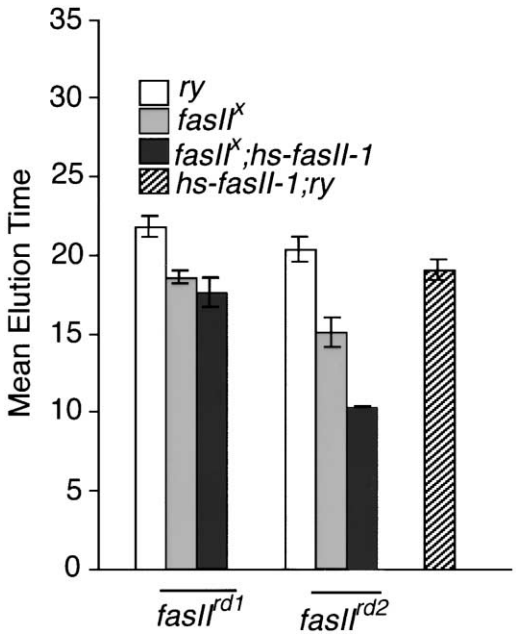


Figure 7. *fasII* Mutants Exhibit Increased Sensitivity to Alcohol

The sensitivity to ethanol vapor of a population of approximately 100 flies was measured in the inebriometer, a column that separates flies based on their ability to maintain postural control. The mean elution time for each *fasII* allele is shown with the *ry* control flies assayed on the same days. Both $fasII^{rd1}$ and $fasII^{rd2}$ mutants, along with two others (not shown), showed a significant reduction in mean elution time ($p < 0.01$, Student's t test). The presence of the *hs-fasII-1* transgene did not alter the ethanol sensitivity of $fasII^{rd1}$ flies ($p = 0.64$, Student's t test), but significantly increased the sensitivity of $fasII^{rd2}$ flies ($p = 0.008$, Student's t test). $n = 3$ for all groups.

tion time in the inebriometer (Figure 7). In addition, two other *fasII* alleles that we isolated and tested blind exhibited sensitivity quantitatively similar to $fasII^{rd2}$ (not shown). This sensitivity was not caused by an alteration in ethanol absorption (data not shown). To determine if the enhanced ethanol sensitivity could be rescued by the *hs-fasII* transgene, we tested $fasII^{rd1}$ and $fasII^{rd2}$ flies carrying the *hs-fasII-1* transgene and raised at 25°C in the inebriometer. Expression from the transgene did not restore normal ethanol sensitivity to either *fasII* mutant (Figure 7). In fact, the presence of the *hs-fasII-1* transgene further increased ethanol sensitivity of the $fasII^{rd2}$ mutant. These data suggest that ethanol sensitivity is related to the levels or function of Fas II protein but that the relationship is complex.

Discussion

Biological Roles of Fas II

The results presented here establish a role for Fas II that is significantly different from those known previously. Antibody perturbation experiments (Harrelson and Goodman, 1988) and genetic analyses (Lin and Goodman, 1994; Lin et al., 1994) have revealed that the molecule functions in the fasciculation and defasciculation of axon bundles. A second function was discovered through studies of the larval neuromuscular junction, where Fas II is required for the stabilization and growth of the synapse (Schuster et al., 1996). The molecule is also required for the expression of the proneural genes, *atonal* and *achaete*, in the eye-antennal imaginal disc (Garcia-Alonso et al., 1995), and for the adhesion or migration of neurons or their precursors in other parts of the nervous system (Holmes and Heilig, 1999; Wright et al., 1999). Most of these biological functions have been ascribed to the molecular function of Fas II as a homophilic cell adhesion molecule.

It is surprising given the known developmental roles for Fas II in axon bundling and synapse growth that no morphological defects of the mushroom bodies were observed in the mutants. We cannot eliminate the possibility that very subtle morphological defects exist in the mushroom bodies of *fasII* mutants. However, a short (1 hr) temperature shift from 18°C to 25°C prior to training is sufficient to rescue the odor learning phenotype and this is reversible with reincubation at 18°C. It is highly unlikely that this temperature shift and subsequent increase in Fas II expression corrects a major morphological defect in the *fasII* mutants. A more plausible conclusion is that rescue is observed due to a function of Fas II in intercellular and/or intracellular signaling processes that underlie memory formation.

How might Fas II be involved in signaling for memory formation? For the role of Fas II in proneural gene expression, there is a dominant interaction of *fasII* with mutants in the Abelson tyrosine kinase gene, suggestive of a role in signaling through nonreceptor tyrosine kinases (Garcia-Alonso et al., 1995). NCAM also signals through nonreceptor tyrosine kinases. Antibody induced clustering of one transmembrane form of NCAM, NCAM-140, induces the association and transient phosphorylation of the nonreceptor kinases, $p59^{fyn}$ and $p125^{fak}$ (Beggs et al., 1997). Furthermore, NCAM-140 clustering activates the mitogen-activated protein kinases, ERK1 and ERK2, and the transcription factor, CREB (Schmid et al., 1999). However, NCAM is also linked to other signaling pathways that modulate intracellular inositol phosphates, calcium, and pH (Schuch et al., 1989). These observations inspire signaling-based models for how Fas II may mediate the formation of memories.

Fas II, Mushroom Bodies, and Information Processing

We have previously proposed that mushroom body neurons serve as the integrators of the conditioned (CS) and unconditioned stimuli (US) during odor learning (Davis, 1993; Roman and Davis, 2001). The CS pathway, which conveys odor information, extends from odor receptors on the antennae to the antennal lobe to the calyces of the mushroom bodies. The calyces, however, do not exhibit high levels of Fas II expression (Figure 1), suggesting that the function of Fas II in odor learning is not for the processing of CS information. Fas II is concentrated along the axons of the mushroom bodies (peduncle) and in the neuropil areas that house the mushroom body axon terminals (lobes, Figure 1). The mushroom body lobes, in particular, are the targets for projections from modulatory inputs that may convey US information, including dopaminergic inputs (Nassel and Elekes, 1992) and peptidergic inputs from *amnesiac*-expressing neurons (Waddell et al., 2000). These observations are consistent with a role for Fas II in the proper presentation

of US information to the mushroom body neurons, or perhaps in events subsequent to the integration of information, such as the strengthening of mushroom body neuron synapses upon follower neurons.

Fas II, NCAM, apCAM, and Memory Processes

Molecules related to Fas II such as NCAM in vertebrates and apCAM in *Aplysia* have been suggested to be involved in learning, memory, and the cellular mechanisms that are thought to underlie these behaviors (see recent reviews by Murase and Schuman [1999] and Crossin and Krushel [2000]). Some studies have suggested a role for these cell adhesion receptors in memory consolidation. For example, amnesia of one-trial passive avoidance training can be produced by injecting anti-NCAM antibodies into the chick brain at 6 hr after training but not at earlier times (Rose, 1995). Similar results have been obtained with the rat, with the sensitive period for effects on passive avoidance memory occurring at 6–8 hr post-training (Doyle et al., 1992). Other studies, however, have suggested a role for NCAM in the early processes of memory formation. For instance, injection of NCAM antisense oligonucleotides before one-trial passive avoidance training blocks memory as early as 3 hr after training (Mileusnic et al., 1999). Moreover, application of anti-NCAM antibodies to hippocampal slice preparations reduces the magnitude of long-term potentiation measured in the CA1 region, with no effect upon basal synaptic transmission (Luthl et al., 1994). Consistent with this observation, a mouse knockout of NCAM (Muller et al., 1996) exhibits a reduction in the magnitude of hippocampal LTP and spatial learning (Cremer et al., 1994), although the mutants have marked defects in brain development which prohibit the discrimination of developmental versus physiological roles for NCAM (see however, Holst et al., 1998). Thus, the specific role for NCAM and similar molecules in memory processes has remained obscure.

Our results demonstrate, through the use of powerful gene knockout and replacement strategies, combined with behavioral assays, that the transmembrane form of Fas II is involved early in the encoding of odor memories and not in memory stability or retrieval. Nevertheless, our results do not eliminate the possibility that Fas II and similar molecules participate in later phases of memory. A parsimonious model is that Fas II and related molecules participate in encoding short-term memory through a cell-signaling function and in forming long-term memory through a cell-adhesion function.

Operational Phases of Memory Formation

Within each temporal phase of memory, we expect the existence of at least three broad operations, including memory formation (encoding), memory stability, and memory retrieval. In other words, each distinct temporal phase must bracket the molecular operations underlying its formation, stability, and retrieval. The results presented here establish that Fas II is involved in the formation of short-term odor memories. Other events that are probably involved in this operation include the activation of the *rutabaga*-encoded adenylyl cyclase, a transient elevation of cAMP, and the activation of protein kinase A, the product of the *DCO* gene (Levin et al., 1992; Han et al., 1992; Drain et al., 1991; Skoulakis et al., 1993; Byrne and Kandel, 1996). In contrast, the formation of long-term memories likely occurs through alterations in the expression of certain gene products (Yin et al., 1994; Bailey et al., 1996). Stability operations for short- versus long-term memory are also likely to be different. The stability of short-term memory may reside in mechanisms to keep protein kinases active while the stability of long-term memory may reside in feedback systems that maintain gene expression states. Retrieval mechanisms could be distinct for different temporal phases of memory but the simplest idea is that retrieval mechanisms are shared among the temporal phases.

Odor Learning and Alcohol Sensitivity

Our observation that *fasII* mutants have heightened ethanol sensitivity extends the overlap between genes involved in odor learning and those involved in alcohol sensitivity (Moore et al., 1998; Harris et al., 1995). However, several observations distinguish the two behaviors and their underlying genetics and neuroanatomy. Whereas raising *fasII* mutants carrying the *hs-fasII-1* transgene at 25°C is sufficient to rescue the odor learning phenotype (Figure 5B), this treatment fails to rescue the ethanol sensitivity (Figure 7). One possible explanation for this is that the level of expression during development or in the adult produced by *hs-fasII-1* in the neural structures mediating ethanol sensitivity is incompatible for normal behavior in the inebriometer. Although the neuroanatomical structures that mediate ethanol sensitivity are not yet defined, they are likely to be distinct from the mushroom bodies, since mushroom body ablation has no effect upon ethanol sensitivity (Moore et al., 1998) but it abolishes odor learning (de Belle and Heisenberg, 1994). Alternatively, it is possible that ethanol sensitivity is caused by a lack of Fas II isoforms other than the transmembrane form expressed from the hs-fasII-1 transgene. Therefore, although the overlap in molecular functions required for normal odor learning and ethanol sensitivity is striking, the two behaviors appear to be mediated by separable neural structures and gene expression requirements.

Experimental Procedures

Fly Genetics, Histology, and Light Microscopy

A Cantonized ry^{506} was used as a control in all the experiments. The *fasII* excision mutants were outcrossed to our Cantonized-ry^{506} control for 6 generations to normalize the genetic background. The two independent transgenic lines, *hs-fasII-1* and *hs-fasII-2*, were obtained by transformation with a vector containing the cDNA coding for the transmembrane form of Fas II. We detected *lacZ* activity histochemically and Fas II by immunohistochemistry as previously described (Han et al., 1996; Crittenden et al., 1998). We visualized overall mushroom body architecture in the brains of male flies carrying $fasII^{+}$, $fasII^{rd1}$ or $fasII^{rd2}$; *P[UAS-GFP]*, and the GAL4 drivers, P[GAL4]c739, P[GAL4]c772, or P[GAL4]201Y. These drivers were generously supplied by K. Kaiser and D. Armstrong.

Electron Microscopy

The heads of adult males without their probosci were fixed for 1 hr at room temperature and then 3 hr at 4°C in 25 mM phosphate buffer, 0.15 mM potassium chloride, 1% glutaraldehyde, and 4% paraformadlehyde. The heads were washed at 4°C several times over 1.5 hr with 25 mM phosphate buffer, 0.15 mM potassium chloride, then post-fixed for 1.5 hr with a 1% osmium tetroxide made

in wash buffer. After the post-fixation, the heads were rinsed three times for 30 min at room temperature with wash buffer, dehydrated in a graded ethanol series (50%–100%), substituted with propylene oxide, and embedding in Epon. Blocks were cured in a 60°C oven for 48 hr. Thick sections of 1 μm were cut and stained with 1% toluidine blue in 1% sodium borate to locate the tip of the α lobe of mushroom body. Thin sections of 80 nm were cut and placed on single slot, Formvar-coated grids. The thin sections were stained with 2% uranyl acetate and Reynolds' lead citrate. A single thin section of the tip of the α lobe was randomly selected from each hemisphere of two individual flies each for *ry* and *fasII*^{rd2}. Electron micrographs of the central area (5.5 × 7.0 μm) of the tip of α lobe were obtained with a transmission electron microscope. The number of synapses were counted on the electron micrographs at a final magnification of 34,800×. Membrane compartment area was measured on scanned images of the electron micrographs using NIH image.

Molecular Biology

PCR and DNA sequencing were used to molecularly characterize the *fasII* mutants that were generated by imprecise excision. For Western blotting, fly head protein was extracted with 0.137 M NaCl, 20 mM Tris (pH 8.0), 10% glyceol, 1% NP40 and 0.1% sodium deoxycholate. An equal mass of protein was loaded per lane on a 6% SDS polyacrylamide gel and the protein blot probed with monoclonal antibody 1D4. Blot signals were quantified after scanning by using NIH software.

Behavioral Analyses

Odor conditioning was performed under dim red light in an environmental room at 65% relative humidity and at 23–25°C. LONG PROGRAM training was performed as described by Beck et al. (2000) and consisted of 60 s of odor A along with 90V shock pulses delivered every 5 s (CS+) followed by 60 s of odor B with no shock (CS−). Testing was performed in a plexiglass T maze in which the flies chose over a 2 min period to avoid the CS+ or CS−. For all tests, flies of 3–7 days of age were used with 0.3% benzaldehyde and 0.6% octanol diluted in light mineral oil as the odorants. SHORT PROGRAM training also followed procedures of Beck et al. (2000). Briefly, flies were exposed to the CS+ odor for 10 s along with a single 1.25 s electric shock delivered at 8 s following the onset of the CS+. This was followed by a 10 s presentation of the CS− odor in the absence of electric shock and a subsequent test. For multiple training trials, a 30 s intertrial interval (ITI) of fresh air was placed between the last CS− and next CS+ presentation.

Odor avoidance, electric shock avoidance, and odor avoidance after shock were performed essentially as reported by Beck et al. (2000) and Preat (1998). For the ethanol sensitivity test, adult male flies, 2–4 days old, were exposed to ethanol vapor in the inebriometer as described (Moore et al., 1998). Ethanol absorption was quantified by measuring the amount of ethanol absorbed after a 30 min exposure to ethanol vapor (Moore et al., 1998).

Data were analyzed with the program Statview. All data presented represent the mean ± the standard error of the mean. In cases where multiple comparisons have been made, the probability values for acceptance of significance is sometimes lower than 0.05 because of Bonferroni adjustments.

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