Molecular Cloning of *linotte* in Drosophila: A Novel Gene That Functions in Adults during Associative Learning

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Summary

The *linotte* (*lio*) gene was identified in a screen for mutations that disrupted 3 hr memory after olfactory associative learning, without affecting the perception of odors or electroshock. The mutagenesis yielded a transposon-tagged gene disruption, which allowed rapid cloning of genomic DNA. The *lio* transcription unit was identified via rescue of the *lio¹* learning/memory defect by induced expression of a *lio⁺* transgene in adults. The perception of odors or electroshock remained normal when the *lio⁺* transgene was expressed in these *lio¹* flies. Learning/memory remained normal when the *lio⁺* transgene was expressed in wild-type (*lio⁺*) flies. The *lio* gene produces only one transcript, the level of expression of which varies throughout development. Sequence analysis indicates that *lio* encodes a novel protein.

Introduction

Behavioral analyses of associative learning and memory have revealed a general functional homology among invertebrates and vertebrates (reviewed by Hawkins and Kandel, 1984; Kesner and Olton, 1990; DeZazzo and Tully, 1995; Hammer and Menzel, 1995). Acquisition requires the temporal association of a reinforcing stimulus (US), which naturally elicits a behavioral response, with a conditioned stimulus (CS), which comes to elicit a conditioned response (CR) as a result of the CS–US pairing(s) (Mackintosh, 1983; Tully and Quinn, 1985). A newly acquired experience initially is susceptible to various forms of disruption. With time, however, the new experience becomes resistant to disruption (McGaugh and Herz, 1972; Erber, 1976; Tully et al., 1990). This observation has been interpreted to indicate that a labile, short-term memory is "consolidated" into a more stable, long-term memory. This consolidation process depends on protein synthesis (Davis and Squire, 1984; Castellucci et al., 1989; Crow and Forrester, 1990; Tully et al., 1994) and is facilitated by multiple training sessions separated by intervals of rest (Ebbinghaus, 1885; Carew et al., 1972; Hintzman, 1974; Frost et al., 1985; Huang and Kandel, 1994; Tully et al., 1994).

This behavioral homology appears to reflect in part an underlying molecular homology. Genetic dissection of olfactory associative learning in fruit flies (reviewed by Davis, 1993; DeZazzo and Tully, 1995) and cellular analyses of heterosynaptic facilitation in Aplysia (reviewed by Kandel et al., 1987; Byrne et al., 1993) or of synaptic long-term potentiation (LTP) in vertebrates (reviewed by Bliss

and Collingridge, 1993; Eichenbaum and Otto, 1993) have all revealed the involvement of the cAMP second messenger system. Temporally paired stimuli induce an increase in cAMP (Byers et al., 1981; Livingstone et al., 1984; Frey et al., 1993; Weisskopf et al., 1993; Huang and Kandel, 1994; Wu et al., 1995), which activates a cAMP-dependent protein kinase (PKA). PKA then serves two functions. In the cytoplasm, activated PKA phosphorylates targets, such as ion channels, thereby modulating synaptic efficacy for minutes to hours (Cowan and Siegel, 1986; Montarolo et al., 1986; Dale et al., 1988; Drain et al., 1991; Skoulakis et al., 1993). Activated PKA is also translocated to the nucleus; there, it phosphorylates a cAMP-responsive transcription factor, CREB (Dash et al., 1990; Backsai et al., 1993; Yin et al., 1995b). Phosphorylated CREB (activator) then initiates a cascade of immediate-early genes, including cis-regulatory enhancer-binding protein (C/EBP; Alberini et al., 1994; Bourtchuladze et al., 1994; Yin et al., 1994, 1995a), presumably culminating in a protein synthesis–dependent synaptic growth process (Greenough, 1984; Montarolo et al., 1986; Glanzman et al., 1990; Nazif et al., 1991; Bailey et al., 1992; Stewart and Rusakov, 1995). This physical change at the synapse may be responsible (at least in part) for more long-lasting modulations of synaptic efficacy and long-term memory.

All the above observations suggest an evolutionarily conserved molecular mechanism involved with the formation of long-term memory: learning-induced activation of the cAMP second messenger system, which terminates in a CREB-mediated transcription factor cascade involved with synaptic growth and function. Although this process may represent a core mechanism common among many species, many other molecules appear to be involved, especially with short-term plasticity (Malinow et al., 1989; Crow and Forrester, 1990; Choi et al., 1991; Ghirardi et al., 1992; Grant et al., 1992; Silva et al., 1992; Abeliovich et al., 1993; Griffith et al., 1993; Weisskopf et al., 1993; Huang and Kandel, 1994; Mihalek et al., submitted). These observations suggest that the cAMP pathway may be involved only in certain learning tasks and/or that it is more generally necessary but perhaps not sufficient. Indeed, flies homozygous for null mutations of the *dunce* or *rutabaga* genes, which encode a cAMP-specific phosphodiesterase and a calcium/calmodulin-dependent adenylyl cyclase, respectively, nevertheless display significant residual associative learning (Tully and Quinn, 1985; Tully and Gold, 1993). Thus, the molecular and behavioral intricacies of learning and memory suggest that additional genes may participate in these processes, and they remain to be discovered.

To pursue such gene discovery in Drosophila, we have generated and screened about 2200 P element–insertional (transposon-tagged) lines for reduced 3 hr memory retention after Pavlovian olfactory learning. We have reported the behavior-genetic characterization of two novel genes, *latheo* and *linotte* (*lio*), identified from this screen (Boynton and Tully, 1992; Dura et al., 1993). Mutant *latheo*

and *lio* flies both are affected in acquisition of conditioned odor avoidance responses, rather than memory retention thereafter. Moreover, transposon tagging these genes has allowed their expeditious molecular cloning.

Here, we report the molecular identification of the *lio*+ transcription unit. Only one message is detected throughout the development of wild-type flies, and the level of this transcript is reduced in adult *lio*1 mutants. Sequence analysis of a cDNA clone corresponding to this mRNA has revealed one 2.7 kb *lio*+ open reading frame (ORF). Heat-induced expression of a *hslio*+ transgene 3 hr before training fully and specifically rescues the learning and memory defects of *lio*1 mutants. These data constitute definitive proof that the correct (*lio*+) transcription unit has been identified. The deduced amino acid sequence of this transcript bears no homology with any known protein, indicating that the *lio* gene encodes a novel protein involved with associative learning.

Results

Characterization of the Genomic Region Surrounding the *lio*1 P Element Insertion

Tagging the *lio* gene with a *PlacW* transposon allowed its immediate localization in situ to cytological region 37D on the left arm of the second chromosome using P element DNA as a probe (Dura et al., 1993). The *PlacW* transposon also contains an origin of DNA replication and ampicillin resistance gene, which allowed the direct cloning of a 900 bp fragment of genomic DNA flanking the *lio*1 P element insertion as a bacterial plasmid (Figure 1).

This 900 bp genomic DNA fragment was used to probe a Southern blot of wild-type (Canton-S) genomic DNA. The 900 bp probe appeared to be nonrepetitive and was subsequently used to screen a Drosophila genomic DNA bacteriophage λ library (kindly provided by R. L. Davis). A total of eight genomic clones were recovered; restriction mapping indicated that these covered a 24 kb genomic region (Figure 1). Cross-hybridization of the 900 bp "rescue fragment" with restriction fragments of the λ clones indicated that the *lio* P element was inserted in a 1 kb EcoRI–HindIII fragment (Figure 1). The 900 bp genomic fragment also hybridized in situ to chromosomal region 37D (data not shown), thereby verifying that the appropriate flanking DNA was cloned.

Cytological localization of the *lio*1 P element insertion placed it just proximal to the *Dopa decarboxylase* (*Ddc*) gene (37C; Hirsh and Davidson, 1981). Approximately 180 kb of the genomic region surrounding *Ddc* had already been cloned by Stathakis et al. (submitted), so we com-
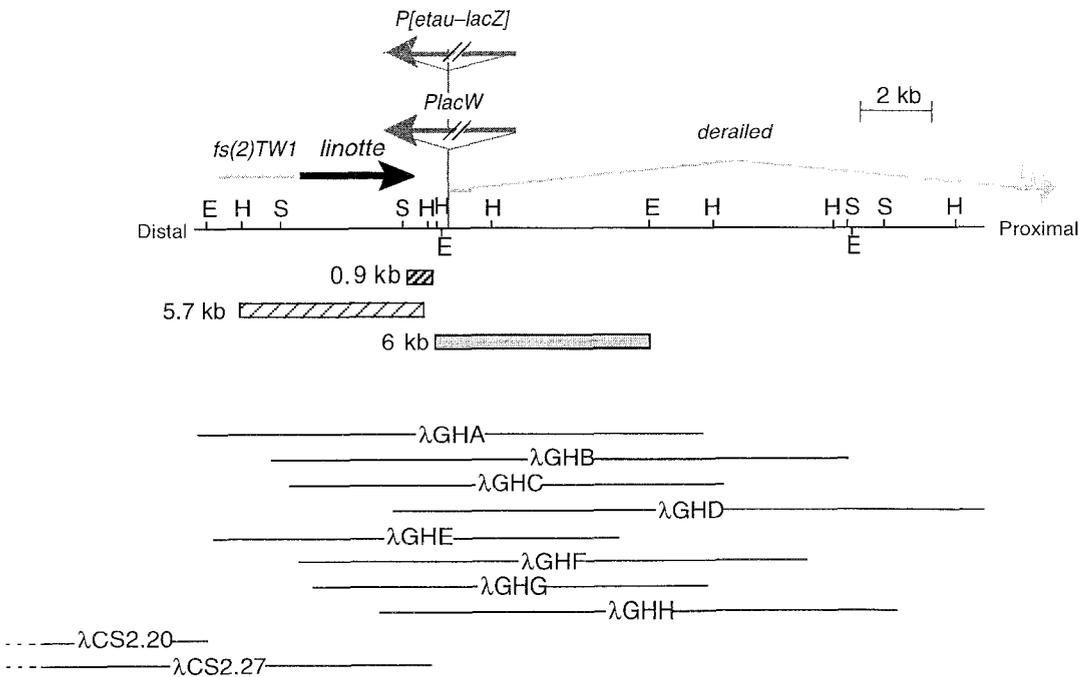

Figure 1. Schematic Map of Genomic DNA in Cytological Region 37D of the Second Chromosome, Which Contains the *lio* Gene

The restriction fragment map shows sites for EcoRI (E), HindIII (H), and Sac II (S). The *PlacW* P element transposon, which causes the *lio*1 mutation, is depicted as a gray arrow indicating the transcriptional orientation of the *lacZ* reporter gene. Its point of insertion in the genome is marked by a descending line. The *lio*+ transcript (black arrow) lies distal (left) of the *PlacW* insertion. The P[etau–lacZ] P element, which causes the *derailed* (*drl*) mutation, is represented by a gray arrow indicating the transcriptional orientation of the *etau–lacZ* reporter gene (Callahan et al., 1995). This transposon is inserted 6 bp proximal (right) of the *lio*1 *PlacW* P insertion. The *drl* transcript lies proximal (right), and the *fs(2)TW1* transcript lies distal (left), to the *lio*1 transcript (see Discussion). The 5.7 kb HindIII–HindIII, the 6 kb EcoRI–EcoRI, and the 0.9 kb SacII–HindIII genomic restriction fragments, which were used to probe cDNA or genomic libraries, are represented by boxes below the restriction map. Genomic phage clones (λ), identified either with the 0.9 kb SacII–HindIII restriction fragment (λGHA–λGHH) or from the *Ddc* project (λCS2.20 and λCS2.27), are indicated as lines below.

pared the *lio¹* and *Ddc* genomic regions. The most proximal *Ddc* genomic clone (λCS2.27) cross-hybridized with all our λ clones on a Southern blot and contained overlapping restriction maps (Figure 1). This placed the *lio¹* P element insertion ~65 kb proximal to the *Ddc* locus.

Identification of RNA Transcripts in the *lio* Genomic Region

To identify RNA transcripts near the *lio¹* P element insertion, subcloned restriction fragments from the genomic λ clones were used initially to probe Northern blots of wild-type (Canton-S) adult whole-fly poly(A)⁺ RNA. A 5.7 kb HindIII fragment distal to the P element insertion (Figure 1) hybridized to a 3.5 kb and a 1.9 kb RNA species (data not shown). This 5.7 kb genomic fragment was then used to screen a Canton-S adult head cDNA library (kindly provided by R. L. Davis). Five cDNA clones were identified; cDNA restriction mapping and Southern and Northern blot analyses revealed that these clones fell into two non-cross-hybridizing classes. One cDNA class hybridized only to the 1.9 kb RNA transcript and to the 1.2 kb HindIII–SacII genomic fragment (Figure 1). By these criteria and via direct sequencing of one of the cDNA clones (data not shown), this cDNA class was determined to correspond to the female sterility gene, *fs(2)TW1* (see Stathakis et al., submitted). Female fertility appeared normal in the original *lio¹* mutants and in over 100 lines homozygous for independent excisions of the *lio¹* P element insertion. Thus, we did not consider *fs(2)TW1* likely to correspond to the *lio* gene.

The second cDNA class hybridized only to the 3.5 kb RNA transcript and to the 0.9 kb SacII–HindIII genomic fragment situated just 800 bp distal to the *lio¹* P element insertion (Figure 1). A 3.1 kb cDNA clone of this class was used as a probe on Northern blots from two independent extractions of poly(A)⁺ RNA from wild-type and *lio¹* adult heads, revealing in *lio¹* mutants 54% ± 2% of normal levels of the 3.5 kb transcript (Figure 2, compare lanes 1 and 3; also see Experimental Procedures). The 3.5 kb transcript was detected with only one strand-specific probe from the 3.1 kb cDNA (data not shown), thereby indicating the direction of transcription. We also probed such a Northern blot with a 6 kb EcoRI genomic restriction fragment (Figure 1) just proximal to the *lio¹* P element insertion. No transcripts were detected (data not shown).

Together, these data suggested that the *lio* gene encodes the 3.5 kb transcript. The *lio¹* P element insertion is ~800 bp proximal to this transcript, and the *fs(2)TW1* transcript lies immediately distal to this putative *lio* transcript (Stathakis et al., submitted). The *lio¹* P element does not appear to be inserted in the transcription unit itself but nevertheless reduces its level of expression, thereby suggesting the *lio¹* mutation to be hypomorphic. Previously published genetic data (Dura et al., 1993), in contrast, have suggested that the *lio¹* mutation is amorphic. The resolution of this apparent discrepancy must await further investigations of the effect of various levels of lio activity on learning and of the spatial distribution of lio in adult heads.
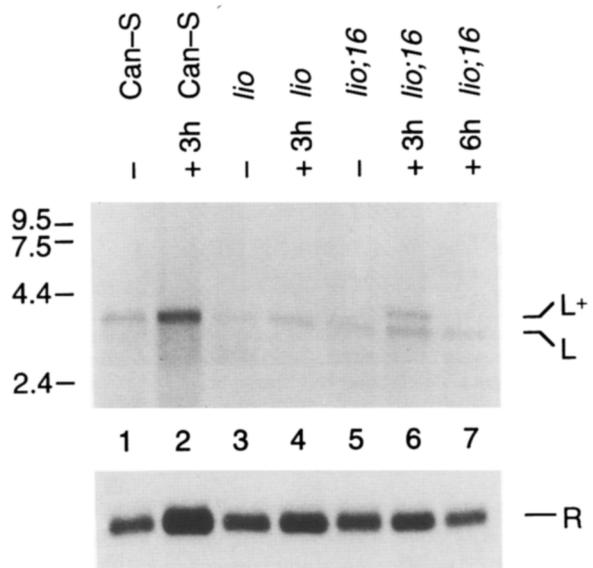

Figure 2. Expression of the *lio⁺* Transcript Is Reduced in *lio¹* Mutants and Is Not Affected by Heat Shock, while Expression of the *hslio⁺* Transgene Is Induced by Heat Shock

In the absence of heat shock, twice as much *lio⁺* transcript (L) is expressed in wild-type (Can-S) adult heads (lane 1) as in mutant *lio¹* heads (lane 3) or in transgenic *lio¹;hslio⁺-16* heads (lane 5), while expression of the *hslio⁺* transgene (L+) was not detected. At 3 hr after heat shock (when flies were subjected to olfactory learning; see Figure 3A), expression of the *lio⁺* transcript was unchanged in wild-type (lane 2), mutant *lio¹* (lane 4), or transgenic *lio¹;hslio⁺-16* (lane 6) flies. In contrast, the *hslio⁺* transgene was expressed at a high level (lane 6). At 6 hr after heat shock (when 3 hr memory after olfactory learning was assayed; see Figure 3B), expression of the *hslio⁺* transgene (lane 7) was greatly diminished. Poly(A)⁺ RNA was extracted from wild-type (Can-S), *lio¹* (*lio*), and *lio¹;hslio⁺-16* (*lio;16*) adult heads, electrophoresed on a 1% denaturing agarose gel, transferred to a charged nylon membrane, and probed with ³²P-radiolabeled *lio⁺* (3.1 kb) cDNA. To quantitate relative amounts of RNA loaded into each lane, this Northern blot was reprobed with a ³²P-radiolabeled DNA fragment from the *ribosomal protein 49* (*rp49*) gene (R). Radiolabeled signals finally were quantified with a phosphorimager, and signal intensities of bands hybridizing with the *lio⁺* probe were normalized using *rp49* signal intensities from each corresponding lane. The relative mobility of single-stranded RNA molecular weight markers (data not shown) is indicated on the left.

Induced Expression of a *hslio⁺* Transgene Rescues the *lio¹* Mutant Learning and Memory Deficits

Since the *lio¹* mutant strain was originally isolated from a transposon-mediated mutagenesis, we realized that this foreign piece of DNA might influence the expression of more than one gene in the region. Thus, no molecular or histological (see below) data were sufficient to identify the transcript associated with the *lio¹* learning defect (see Discussion). Correct identification of the *lio⁺* transcript was obtained, however, via transgenic rescue of the *lio¹* learning deficit. We fused the 3.1 kb cDNA clone to the *heat shock-70* (*hs-70*) promoter sequence to construct an inducible *hslio⁺* minigene (see Experimental Procedures). Several transgenic lines carrying independent genomic insertions of this *hslio⁺* construct were generated first on a *lio⁺* (wild-type) background. Then, the *hslio⁺* insertions
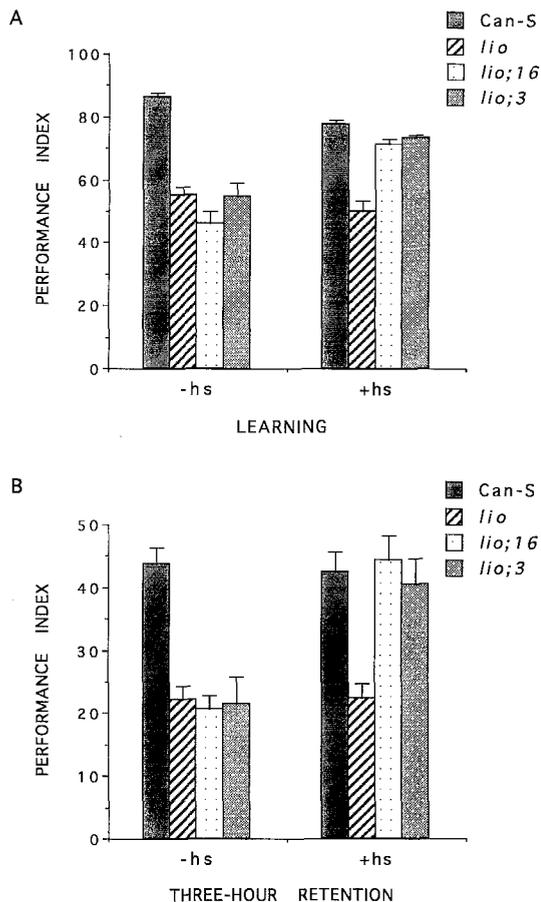
A

LEARNING

B

THREE-HOUR RETENTION

Figure 3. Heat Shock–Induced Expression of the *hslio*[+] Transgene Rescues the Learning and Memory Deficits of *lio*[1] Mutants

Conditioned odor avoidance after olfactory learning was quantified as a mean performance index (PI) ± SEM. If no flies learn to avoid the shock-paired odor, then PI = 0; if all flies learn, then PI = 100 (see Experimental Procedures; n = 6 PIs per group).

(A) Conditioned odor avoidance immediately after olfactory learning (LEARNING) in wild-type (Can-S), mutant *lio*[1] (*lio*) and transgenic *lio*[1]; *hslio*[+]-16 (*lio;16*) and *lio*[1];*hslio*[+]-3 (*lio;3*) flies in the absence of heat shock (−hs) or 3 hr after a 30 min heat shock (+hs). In the absence of heat shock, learning in *lio*[1] mutants was significantly lower than in wild-type flies (p < .001), while learning in transgenic *lio*[1];*hslio*[+]-16 and *lio*[1];*hslio*[+]-3 flies was similar to that in *lio*[1] mutants (p = .015 and .87, respectively). When trained 3 hr after heat shock, learning in *lio*[1] mutants was still significantly lower than in wild-type flies (p < .001), but learning in transgenic *lio*[1];*hslio*[+]-16 and *lio*[1];*hslio*[+]-3 flies was significantly improved (p < .001 for each) and did not differ from that of wild-type flies (p = .071 and .22, respectively). In contrast to this clear effect of heat shock on learning in transgenic flies, heat shock had no effect on learning in wild-type flies (p = .006) or in *lio*[1] mutants (p = .084).

(B) Memory retention 3 hr after olfactory learning (THREE-HOUR RE-TENTION) in wild-type (Can-S), mutant *lio*[1] (*lio*), and transgenic *lio*[1]; *hslio*[+]-16 (*lio;16*) and *lio*[1];*hslio*[+]-3 (*lio;3*) flies trained in the absence of heat shock (−hs) or 3 hr after a 30 min heat shock (+hs). As was true for learning, 3 hr retention in *lio*[1] mutants was significantly lower than in wild-type flies (p < .001) in the absence of heat shock, while learning in transgenic *lio*[1];*hslio*[+]-16 and *lio*[1];*hslio*[+]-3 flies was similar to that in *lio*[1] mutants (p = .76 and .91, respectively). When trained 3 hr after heat shock, 3 hr retention in *lio*[1] mutants still was signficantly lower than in wild-type flies (p < .001), but 3 hr retention in transgenic *lio*[1]; *hslio*[+]-16 and *lio*[1];*hslio*[+]-3 flies was significantly improved (p < .001 for each) and did not differ from that of wild-type flies (p = .67 and .69, respectively). In contrast to this clear effect of heat shock on 3 hr retention in transgenic flies, heat shock had no effect on 3 hr retention in wild-type flies (p = 0.73) or in *lio*[1] mutants (p = .93).

were crossed into *lio*[1] mutants (see Experimental Procedures).

Mutant *lio*[1] flies originally were isolated because of a 3 hr memory retention deficit but were subsequently shown to have impaired learning as well (Dura et al., 1993). Thus, both learning and 3 hr retention were assayed in *hslio*[+] transgenic flies. Our previous experiments have shown robust heat shock induction of *hs-70* promoter–driven transgenes with minimal nonspecific effects on learning and memory formation by exposing adults flies to one 30 min heat shock (37°C) 3 hr before training (Yin et al., 1994, 1995a). This heat shock regimen was used here.

In the absence of heat shock, 2 *hslio*[+] transgenic lines (*lio*[1];*hslio*[+]-3 and *lio*[1];*hslio*[+]-16) displayed learning and 3 hr retention scores (performance indices [PIs]; see Experimental Procedures) similar to those of the *lio*[1] mutant (Figure 3). After heat shock induction of the *hslio*[+] transgenes, however, learning and 3 hr retention scores were similar to those of wild-type flies. In contrast, this heat shock regimen had no effect on the learning or 3 hr retention scores of wild-type flies or *lio*[1] mutants lacking a transgene. Learning was also assayed in 2 other transgenic lines (*lio*[1];*hslio*[+]-1 or *lio*[1];*hslio*[+]-21). Mean (PI ± SEM) scores for *lio*[1];*hslio*[+]-21 transgenic flies were 63 ± 3 (n = 6) in the absence of heat shock and 80 ± 2 (n = 6) 3 hr after heat shock, while those for *lio*[1];*hslio*[+]-1 were 50 ± 6 (n = 2) in the absence of heat shock and 61 ± 2 (n = 2) 3 hr after heat shock. Thus, while results from *lio*[1];*hslio*[+]-21 transgenic flies were similar to those of *lio*[1];*hslio*[+]-3 and *lio*[1];*hslio*[+]-16 transgenic flies, induced expression of the transgene in *lio*[1];*hslio*[+]-1 flies appeared to yield intermediate results.

Consistent with the apparent behavioral rescue of the *lio*[1] mutation by induced expression of the *hslio*[+] transgene, a Northern blot analysis on poly(A)[+] RNA from adult *lio*[1]; *hslio*[+]-16 heads showed an increased level of expression of the *hslio*[+] transcript 3 hr after heat shock induction, while levels of expression of the endogenous *lio*[+] transcript in wild-type, *lio*[1], or *lio*[1];*hslio*[+]-16 flies remained unchanged (see Figure 2). The transgenic transcript was undetectable in the absence of heat shock (see Figure 2, lane 5), indicating little leaky expression of the transgene, and again was undetectable 6 hr after heat shock (see Figure 2, lane 7). This same heat shock regimen induces high levels of expression of the *hs-dCREB2-b* or *hs-dCREB2-a* transgenic transcripts, and they, in contrast, then remain detectable for more than 9 hr (Yin et al., 1994, 1995a). Thus, turnover of the *hslio*[+] transcript appears to be relatively rapid.

Induced Expression of the *hslio*[+] Transgene Does Not Affect Olfactory Acuity or Shock Reactivity

To understand the effects of single gene mutations on learning/memory, we have consistently argued that poor performance in learning/memory assays cannot be interpreted properly without also assessing the task-relevant sensory/motor responses evoked in untrained animals by the stimuli used in the learning/memory procedures (Gailey et al., 1991; Boynton and Tully, 1992; Luo et al., 1992; Dura et al., 1993; Tully et al., 1994; Yin et al., 1994, 1995a; Mihalek et al., submitted). To this end, we have developed assays of olfactory acuity and shock reactivity that quanti-

Table 1. Olfactory Acuity and Shock Reactivity in Wild-Type (Canton-S), Mutant, and Transgenic Flies

Heat Shock	Group	Olfactory Acuity				Shock Reactivity	
		OCT		MCH			
		10^0	10^{-2}	10^0	10^{-2}	60 V	20 V
-hs	Wild-type	74 ± 3	35 ± 7	85 ± 3	42 ± 7	81 ± 5	42 ± 10
	lio^1	82 ± 3	42 ± 5	81 ± 2	35 ± 7	74 ± 4	54 ± 4
-hs	Wild-type	69 ± 5	44 ± 6	72 ± 3	33 ± 4	87 ± 2	62 ± 4
	lio^1; $hslio^+$-16	75 ± 4	43 ± 6	68 ± 4	38 ± 5	84 ± 3	59 ± 6
+hs	Wild-type	52 ± 6	31 ± 3	62 ± 4	21 ± 3	87 ± 3	57 ± 7
	lio^1; $hslio^+$-16	60 ± 6	32 ± 5	52 ± 3	25 ± 5	92 ± 1	51 ± 6

The heat shock regiment was identical to that used for Pavlovian learning/memory assays; flies were assayed 3 hr after heat shock.
Olfactory acuity and shock reactivity were assayed in untrained flies with the methods of Boynton and Tully (1992) and Luo et al. (1992), respectively (see Experimental Procedures; n = 8 PIs per group). Planned comparisons between wild-type and mutant flies failed to detect any significant differences (10^0, undiluted odorants; 10^{-2}, 100-fold dilution in mineral oil).
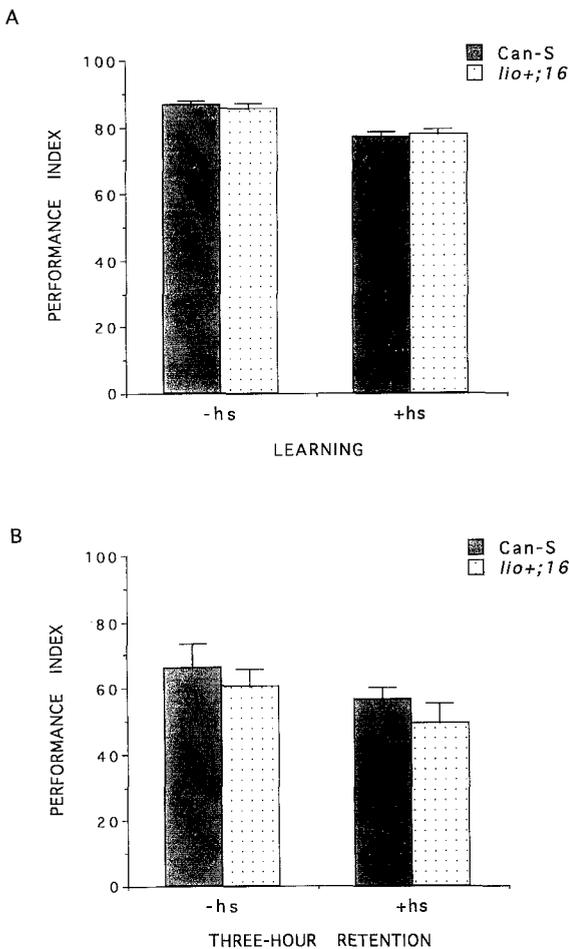
A

B

Figure 4. Heat Shock–Induced Expression of the $hslio^+$ Transgene Does Not Affect Learning or Memory in lio^+ (Wild-Type) Flies

Conditioned odor avoidance after olfactory learning was quantified as in Figure 3 (n = 6 PIs per group).

(A) Conditioned odor avoidance immediately after olfactory learning (LEARNING) in wild-type (Can-S) and transgenic lio^+;$hslio^+$-16 (lio^+;16) flies in the absence of heat shock (−hs) or 3 hr after a 30 min heat shock (+hs). Learning in transgenic flies did not differ signficantly from that in wild-type flies in the absence of heat shock (p = 0.60) or when trained 3 hr after heat shock (p = .73).

(B) Memory retention 3 hr after olfactory learning (THREE-HOUR RE-TENTION) in wild-type (Can-S) and transgenic lio^+;$hslio^+$-16 (lio^+;16) flies trained in the absence of heat shock (−hs) or 3 hr after a 30 min

tate the abilities of the flies to sense the same odors and electroshock in the same T maze apparatus used for assays of Pavlovian learning/memory (see Experimental Procedures). For this study, olfactory acuity and shock reactivity were assayed in untrained flies in the absence of, and 3 hr after, the usual heat shock regimen (see above). This post–heat shock time point was chosen to correspond to the time when flies were trained in the Pavlovian learning experiments.

Table 1 lists the olfactory acuity and shock reactivity scores from wild-type and transgenic (lio^1;$hslio^+$-16) flies with or without the heat shock treatment (± hs) and from mutant lio^1 flies without heat shock. In the absence of heat shock, olfactory acuity and shock reactivity mean scores (PI ± SEM) were similar among wild-type, lio^1;$hslio^+$-16, and lio^1 flies (cf. Dura et al., 1993). This observation indicates that the genetic backgrounds of the 3 strains were similar (see Experimental Procedures). Three hours after heat shock, olfactory acuity and shock reactivity mean scores still did not differ between wild-type and lio^1; $hslio^+$-16 flies. In light of these data, the behavioral rescue of mutant lio^1 flies by induced expression of the $hslio^+$ transgene observed in Pavlovian learning/memory experiments (see above) now can be interpreted as a specific rescue of learning/memory per se.

Induced Expression of the $hslio^+$ Transgene Does Not Affect Learning or Memory of Wild-Type Flies

Does the improved performance of induced lio^1;$hslio^+$-16 transgenic flies reflect a general enhancement of learning/memory or rather a specific rescue of the lio^1 mutation? We addressed this issue by studying the $hslio^+$-16 transgenic insertion in a lio^+ (wild-type) background rather than the lio^1 mutant background. In this manner, the effects on learning and memory of induced (and ectopic) overexpression of the lio^+ transgene were quantified (Figure 4).

In the absence of heat shock, mean learning or 3 hr retention scores (PI ± SEM) did not differ between wild-type and transgenic lio^+;$hslio^+$-16 flies, again indicating

heat shock (+hs). As was true for learning, 3 hr retention in transgenic flies did not differ signficantly from that of wild-type flies in the absence of heat shock (p = .47) or when trained 3 hr after heat shock (p = .40).
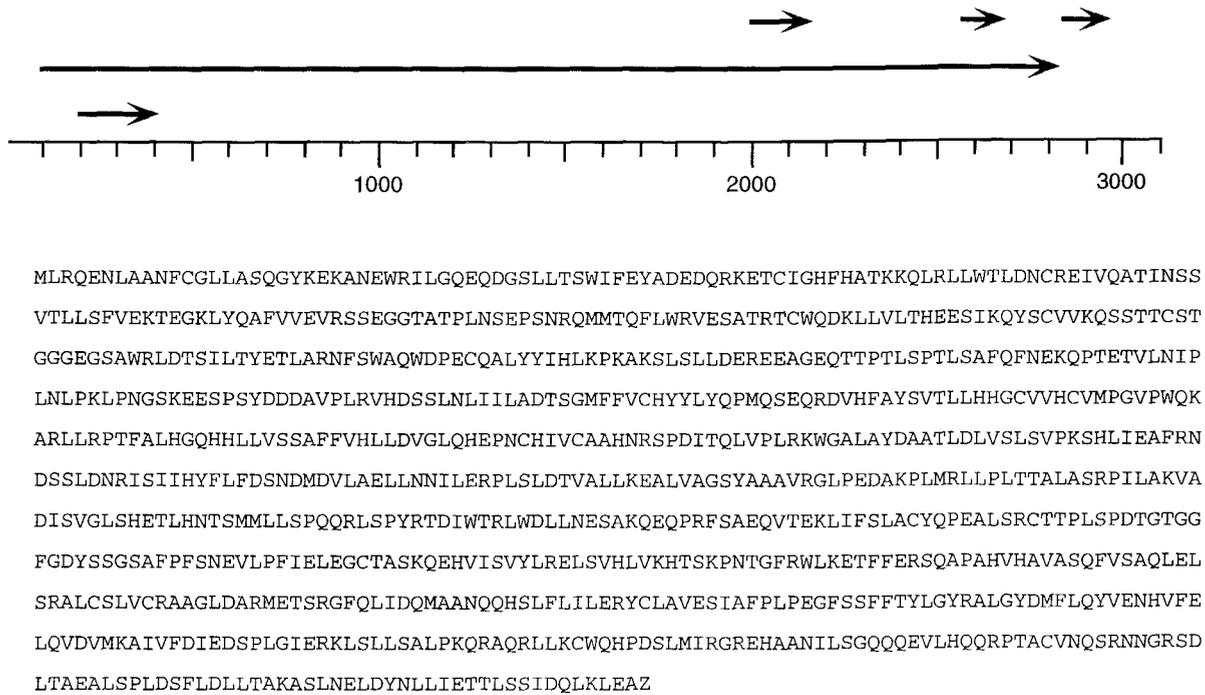
Figure 5. The Open Reading Frame and Deduced Amino Acid Sequence of the *lio*⁺ Gene

MLRQENLAANFCGLLASQGYKEKANEWRILGQEQDGSLLTSWIFEYADEDQRKETCIGHFHATKKQLRLLWTLDNCREIVQATINSS

VTLLSFVEKTEGKLYQAFVVEVRSSEGGTATPLNSEPSNRQMMTQFLWRVESATRTCWQDKLLVLTHEESIKQYSCVVKQSSTTCST

GGGEGSAWRLDTSILTYETLARNFSWAQWDPECQALYYIHLKPKAKSLSLLDEREEAGEQTTPTLSPTLSAFQFNEKQPTETVLNIP

LNLPKLPNGSKEESPSYDDDAVPLRVHDSSLNLIILADTSGMFFVCHYYLYQPMQSEQRDVHFAYSVTLLHHGCVVHCVMPGVPWQK

ARLLRPTFALHGQHHLLVSSAFFVHLLDVGLQHEPNCHIVCAAHNRSPDITQLVPLRKWGALAYDAATLDLVSLSVPKSHLIEAFRN

DSSLDNRISIIHYFLFDSNDMDVLAELLNNILERPLSLDTVALLKEALVAGSYAAAVRGLPEDAKPLMRLLPLTTALASRPILAKVA

DISVGLSHETLHNTSMMLLSPQQRLSPYRTDIWTRLWDLLNESAKQEQPRFSAEQVTEKLIFSLACYQPEALSRCTTPLSPDTGTGG

FGDYSSGSAFPFSNEVLPFIELEGCTASKQEHVISVYLRELSVHLVKHTSKPNTGFRWLKETFFERSQAPAHVHAVASQFVSAQLEL

SRALCSLVCRAAGLDARMETSRGFQLIDQMAANQQHSLFLILERYCLAVESIAFPLPEGFSSFFTYLGYRALGYDMFLQYVENHVFE

LQVDVMKAIVFDIEDSPLGIERKLSLLSALPKQRAQRLLKCWQHPDSLMIRGREHAANILSGQQQEVLHQQRPTACVNQSRNNGRSD

LTAEALSPLDSFLDLLTAKASLNELDYNLLIETTLSSIDQLKLEAZ

(Top) Open reading frame (ORF) maps of each reading frame of the *lio*⁺ sense strand. The transcribed orientation of the *lio*⁺ cDNA was determined by strand-specific probing of a Northern blot of whole-fly Canton-S poly(A)⁺ RNA (see text). All ORFs initiating with an AUG and longer than 100 nucleotides are indicated with arrows above a linear representation of the *lio*⁺ cDNA. Only one ORF yields a deduced amino acid sequence of appreciable length. This ORF initiates 89 nucleotides from the 5' end of the 3.1 kb cDNA, extends 2748 nucleotides, and terminates 262 nucleotides from the 3' end.

(Bottom) The deduced 916 amino acid sequence of the 2748 nucleotide *lio*⁺ ORF. Comparison of this sequence to the those contained in the GenBank, Swiss-Prot, and Pro-Site databases revealed no significant homologies between the putative *lio*⁺ protein and any known protein or functional domain.

that genetic backgrounds of the two strains were similar. When trained 3 hr after heat shock, mean learning and 3 hr retention scores between these two strains still did not differ. These data demonstrate that induced overexpression of the *lio*⁺ product does not enhance learning or memory generally. Thus, we can conclude that induced rescue of the learning/memory deficit in *lio¹;hslio*⁺ transgenic flies (see above) represents a specific rescue of the *lio¹* mutation.

The *lio*⁺ Transcript Encodes a Novel Protein

Full rescue of the *lio¹* learning/memory deficits by induced expression of a *hslio*⁺ transgene constituted definitive proof that we had identified the correct RNA transcript. Thus, a closer (molecular) look at the corresponding 3.1 kb cDNA clone was warranted. Sequence analysis of the 3.1 kb cDNA revealed one prominent 2.7 kb translational ORF in the transcribed orientation (Figure 5). Several stop codons occurred 5' of this putative ORF, and the nucleotides immediately preceding the translational start site conformed with the Drosophila (and general) conservation rules for active translational start sites (Cavener, 1987; Kozak, 1987).

This ORF encodes a 916 amino acid (103 kDa) deduced polypeptide and terminates 262 nucleotides upstream of the 3' end of the cDNA sequence. The deduced amino

acid sequence (Figure 5) bears no significant homology with any previously characterized protein in the BLAST, Swiss-Protein, or Pro-Site databases. The *lio*⁺ gene, therefore, appears to encode a novel protein involved in associative learning.

The *lio*⁺ Transcript Is Detected in Embryos, Pupae, and Adults but Not Larvae

To investigate the developmental expression of the *lio*⁺ transcript, the 3.1 kb cDNA was used in Northern blot analyses to probe poly(A)⁺ RNA from various developmental stages (Figure 6). At all stages, only one 3.5 kb transcript was detected. Levels of expression of this message differed significantly during development. The *lio*⁺ mRNA was expressed at a high level in early embryos (0–4 hr) but not in late embryos (>16 hr), suggesting a maternal origin for the early signal. The *lio*⁺ message was not detected during the larval stages but then reappeared during pupal development and was expressed at high levels in adult head and body.

The Developmental Pattern of Expression of the Enhancer-Trap Reporter Gene in *lio¹* Mutants Does Not Correspond to That of the *lio*⁺ Transcript

The *lio¹* mutation resulted from the insertion of a transposable enhancer-trap P element (*PlacW*) containing a *lacZ*
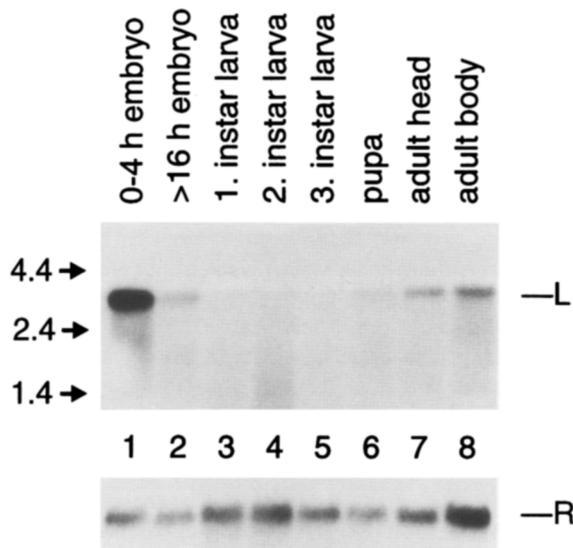
Figure 6. The *lio*[+] Transcript Is Expressed in Embryos, Pupae, and Adults but Not during Larval Development

A high level of *lio*[+] expression in early embryos followed by much lower levels in late embryos suggests that *lio*[+] initially is maternally derived. Appreciable levels of expression are not observed again until the pupal and adult stages. Poly(A)[+] RNA was extracted from wild-type (Canton-S) early (0–4 h embryo) and late (>16 h embryo) embryos; first, second, or third instar larvae (1. instar larva, 2. instar larva, and 3. instar larva, respectively); pupae, adult heads, and adult bodies. Extracted RNA was electrophoresed on a 1% denaturing agarose gel, transferred to a charged nylon membrane, and probed with ^{32}P-radiolabeled *lio*[+] (3.1 kb) cDNA (L) and *rp49* (R) as in Figure 2. The relative mobility of single-stranded RNA molecular weight markers (data not shown) is indicated on the left.

reporter gene, which could be activated transcriptionally by regional enhancer elements (Bier et al., 1989). New mutant alleles of *rutabaga*, for instance, were recovered with *PlacW* insertions in the 5' untranslated region of the gene (Levin et al., 1992). The pattern of *lacZ* reporter gene expression in these mutants overlapped extensively with expression of the *rutabaga* gene product (Han et al., 1992).

To observe *lacZ* reporter gene activity in the CNS of *lio*[1] mutants, we stained embryos, third instar larvae, and adults with the chromogenic *lacZ* substrate, X-Gal (Figure 7). In stage 12 embryos (Figure 7A), *lacZ* reporter gene activity was detected in the CNS and PNS and in several other locations. In third instar larvae (Figure 7B), a high level of *lacZ* reporter gene activity was observed in the lateral hemispheres of the brain lobes, where the adult visual system develops, and to a lesser degree in the dorsal medial region of the brain and in the ventral ganglia. In frontal sections of adult heads (Figure 7C), *lacZ* reporter gene activity also was observed in a few neurons in the dorsal medial region of the protocerebrum and in the optic lobes and subesophogeal ganglion. No prominent *lacZ* activity was detected in the calyces of the mushroom bodies, where the *dunce* and *rutabaga* genes are preferentially expressed (Nighorn et al., 1991; Han et al., 1992). Notably, this developmental pattern of *lacZ* expression does not coincide with the temporal pattern of *lio*[+] expression (see Figure 6 and Discussion).

Figure 7. Enhancer-Trap-Driven Expression of the *lacZ* Reporter Gene Contained within the *lio*[1] P Element Insertion throughout Development

The different preparations were fixed and stained with the chromogenic substrate X-Gal, which appears blue (see Experimental Procedures).
(A) *lacZ* activity in a whole-mount stage 14 embryo (100×) was detected in the brain (br) and in the ventral nerve cord (vn). *lacZ* activity was also detected in the PNS and in the posterior midgut.
(B) In whole-mount, dissected third instar larval CNS (200×), enhancer-trap-driven expression of the *lacZ* reporter gene was appreciable in the dorsal medial region of the brain (dm), in the lateral brain hemisperes (bh; the developing adult visual system), and in the ventral ganglia (vg).
(C) In 10 μm frontal sections of adult heads (200×), enhancer-trap-driven expression of the *lacZ* reporter gene was observed in the dorsal–medial region (dm), in the optic lobes (ol), and in the subesophogeal ganglion (sg). No staining was apparent in cells surrounding the mushroom body calyces (data not shown).

Gross Anatomy of Mushroom Bodies and Central Complex Is Normal in *lio*[1] Mutants

Genetic or chemical lesions two anatomical regions of the adult brain, the mushroom bodies and the central com-

plex, disrupt olfactory learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994). Subtler aspects of mushroom body development also are defective in *dunce* and *rutabaga* mutants (Balling et al., 1987), and defects in the central complex have in fact been reported for *lio* mutants (Dura et al., 1995).

Full rescue of the *lio¹* learning/memory deficit by induced expression of the *hslio⁺* transgene in adults (see above), however, brought the latter claim into question. Thus, we decided to reassess adult brain structure in *lio¹* mutants. We visually inspected the mushroom bodies and central complex and quantified their neuropillar volumes via planimetric analysis in wild-type (*lio⁺*) flies, *lio¹* homozygous mutants, and hemizygous flies carrying either the wild-type (*lio⁺*) or *lio¹* chromosome and a second chromosome deletion (*Df*; see Experimental Procedures) of the *lio* region. (The dosages of *lio⁺* or *lio¹* in these hemizygous flies were only 50% of those in corresponding homozygous flies, thereby potentially yielding more severe phenotypic defects.) In a double-blind experiment, frontal sections of *lio¹/lio¹*, *lio¹/Df*, *lio⁺/Df*, and *lio⁺/lio⁺* heads were serially sectioned in the laboratory of Dr. M. Heisenberg, and then planimetric analyses of the mushroom bodies and central complex were carried out in our laboratory. We failed to detect any qualitative (Figures 8A and 8B) or quantitative (Figure 8C) differences among the four genotypes in these two brain structures.

Discussion

The *lio¹* Learning and Memory Deficits Are Rescued Completely by Induced Expression of a *hslio⁺* Transgene

Two transgenic lines (*lio¹;hslio⁺-16* and *lio¹;hslio⁺-3*) carrying independently isolated genomic insertions of a *hslio⁺* transgene show normal olfactory learning and memory after expression of the transgene is induced by heat shock in adults (see Figure 4). This rescue effect is behaviorally specific, since induced expression of the *hslio⁺* transgene did not affect the flies' task-relevant abilities to sense the odors (olfactory acuity) or electroshock (shock reactivity) used in the Pavlovian experiments (Table 1; cf. Dura et al., 1993).

Complete rescue of the *lio¹* learning/memory defect in induced *hslio⁺* flies indicated that ectopic expression of the *lio⁺* transgene does not produce any deleterious effects on conditioned olfactory behavior. We also considered the other extreme, that (ectopic) expression of the *hslio⁺* transgene might produce a general enhancement, thereby improving learning/memory in *lio¹* mutants nonspecifically. This possibility was tested by inducing (over)expression of the *hslio⁺* transgene in *lio⁺* (wild-type) flies, rather than in *lio¹* mutants. In such transgenic *lio⁺;hslio⁺-16* flies, learning/memory was normal (see Figure 5). Thus, induced expression of the *hslio⁺* transgene did not produce a general enhancement of learning or memory. Consequently, we can conclude that the deleterious effects on learning and memory of the *lio¹* mutation were specifically rescued by induction of the *hslio⁺* transgene.

In the absence of heat shock, transgenic *lio¹;hslio⁺* flies show learning/memory deficits similar to those of *lio¹* mutants (see Figure 4), and expression of the transgene is not detected in adult heads (see Figure 3). These data indicate little leaky expression of the transgene. At 3 hr after heat shock, in contrast, learning and memory are rescued completely, and expression of the transgene in adult heads is high. Combined with data from the developmental Northern blots, which revealed undetectable levels of the *lio⁺* transcript throughout larval development (see Figure 6), and from histological studies of mutant adult brain, which revealed no structural abnormalities in the mushroom bodies or central complex (Figure 8), these results indicate clearly that the learning/memory deficit of *lio¹* mutants does not derive secondarily from developmental abnormalities. Instead, the *lio* gene appears to function more acutely during adult associative learning (cf. Ewer et al., 1988). This inducible, complete, and behaviorally and molecularly specific rescue of the *lio¹* learning/memory deficit constitutes definitive proof that we have correctly cloned and identified the *lio⁺* transcription unit.

The Patterns of Expression for the *lio⁺* Gene and the *lio¹* PlacW Enhancer-Trap Reporter Gene Do Not Coincide

When comparing the temporal patterns of expression between the *lio⁺* transcript and the enhancer-trap reporter gene encoded within the *lio¹* P element insertion, an apparent discrepancy exists. The former cannot be detected in any larval stage (see Figure 6), while the latter is expressed at high levels in the lateral brain hemispheres of third instar larvae (see Figure 7). Recent identification of the *derailed* (*drl*) gene, however, has revealed a more specific resolution to this discrepancy for *lio¹* (Callahan et al., 1995).

By DNA sequence comparison, we have determined that our *lio¹* P element is inserted 6 bp distal to their independently isolated P element insertion in *drl* (see Figure 1). Consistent with this finding, the embryonic CNS patterns of reporter gene expression for both P element insertions correspond to the expression pattern of the *drl* gene itself (C. Cahallan and J. Thomas, personal communication). Moreover, the *drl* transcript is expressed throughout larval development but is undetectable in adult flies. The latter result is consistent with our Northern blot analysis of adult head RNA, in which a genomic DNA fragment proximal to the *lio¹* P element insertion (and including at least some of the *drl* exonic sequence) failed to detect any transcript.

These data indicate that the *lio¹* P element affects the level of expression of the *lio* transcript in adult flies, thereby producing a learning/memory deficit, even though it is inserted in or near the 5' end of the *drl* transcription unit. We do not yet know whether the *lio¹* P element insertion also produces the *drl* mutant phenotype, axonal misguidance in a subset of neurons during development of the nervous system in embryos (Callahan et al., 1995). Nevertheless, these potential pleiotropic defects do not prevent complete rescue of the *lio¹* learning/memory defect after
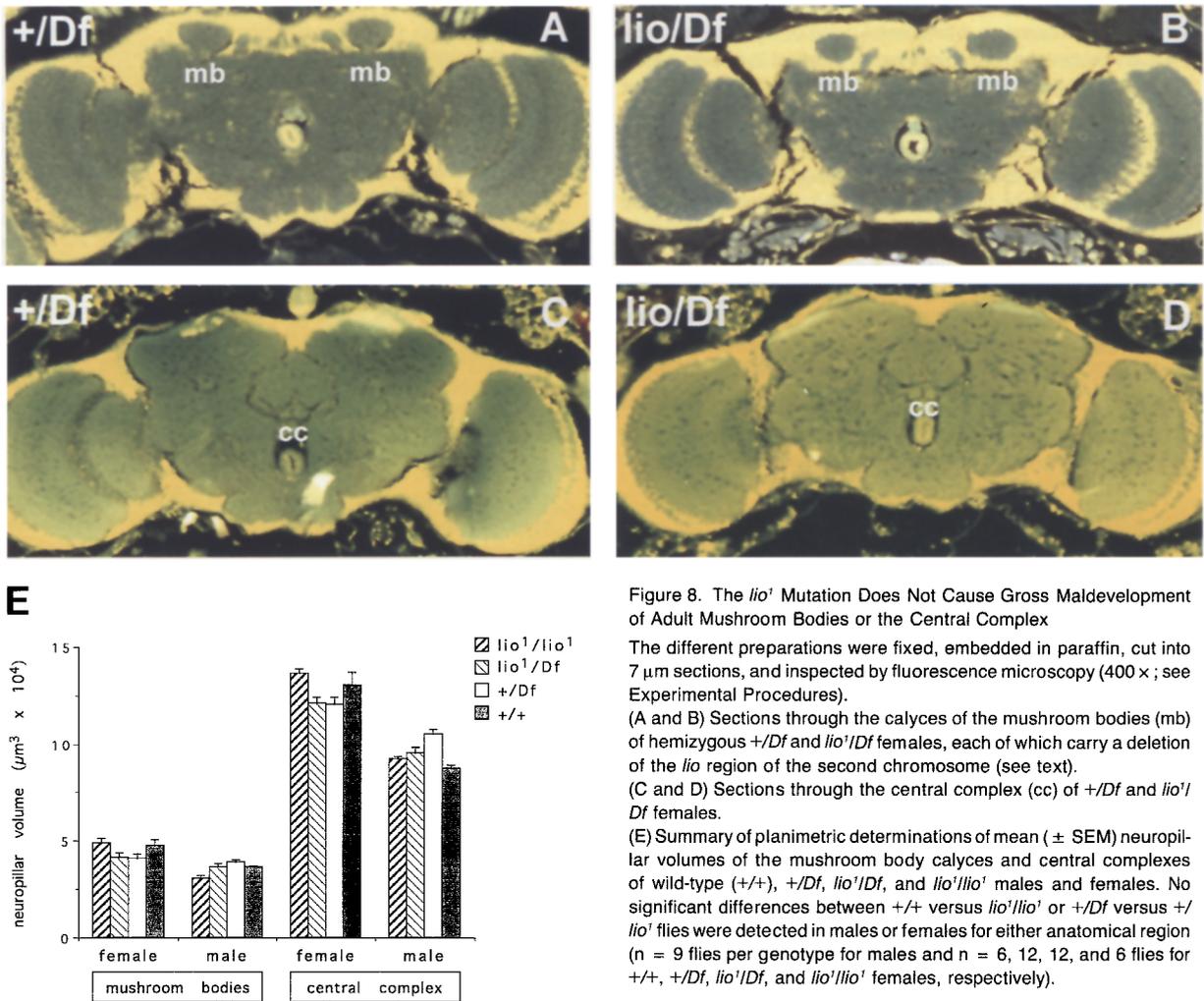
Figure 8. The *lio¹* Mutation Does Not Cause Gross Maldevelopment of Adult Mushroom Bodies or the Central Complex

The different preparations were fixed, embedded in paraffin, cut into 7 μm sections, and inspected by fluorescence microscopy (400 × ; see Experimental Procedures).

(A and B) Sections through the calyces of the mushroom bodies (mb) of hemizygous +/Df and *lio¹/Df* females, each of which carry a deletion of the *lio* region of the second chromosome (see text).

(C and D) Sections through the central complex (cc) of +/Df and *lio¹/Df* females.

(E) Summary of planimetric determinations of mean (± SEM) neuropillar volumes of the mushroom body calyces and central complexes of wild-type (+/+), +/Df, *lio¹/Df*, and *lio¹/lio¹* males and females. No significant differences between +/+ versus *lio¹/lio¹* or +/Df versus +/*lio¹* flies were detected in males or females for either anatomical region (n = 9 flies per genotype for males and n = 6, 12, 12, and 6 flies for +/+, +/Df, *lio¹/Df*, and *lio¹/lio¹* females, respectively).

the *hslio⁺* transgene is induced in adults. When and where, then, is *lio* expressed? To date, RNA in situ hybridization studies using *lio⁺* RNA probes have failed to produce any detectable signal in adult brain sections. Immunocytochemical studies using antibodies raised against the *lio⁺* gene product, however, may reveal its cellular localization.

What Are Sufficient Criteria for Identifying Novel Genes Involved with Learning/Memory?

The *lio* gene was originally isolated in a behavioral screen for P element insertional mutations that disrupted 3 hr retention after Pavlovian olfactory learning. To date, behavioral characterization of 2200 P element lines has yielded four new learning/memory genes: *lio¹*, *latheo^P¹*, *nalyot*, and *golovan* (Boynton and Tully, 1992; Dura et al., 1993; and T. T., unpublished data). The transposon tagging method was chosen for two reasons particularly relevant to behavioral phenotypes. First, the P element mutator contained a selectable eye color marker, which yielded morphologically tagged behavioral mutants. Consequently, subsequent genetic experiments were greatly facilitated. Second, the P element mutator represented a

molecular tag with which to clone genomic DNA flanking the insertion site. This approached gained access to genomic DNA in the region of the P element insertion but was not designed to identify unambiguously the transcription unit specifically responsible for the learning/memory deficit.

To this end, the particular set of molecular–genetic, histological, and behavioral data derived from our *lio* cloning project has yielded important information. First, insertion of the P element into a transcription unit is not sufficient evidence to conclude that that particular transcript is involved with the behavioral defect. Second, correspondence between patterns of expression of an enhancer-trap reporter gene (contained within the transposon) and of a nearby transcript also does not constitute sufficient evidence to conclude that that particular transcript is involved with the behavioral phenotype. Third, the only evidence sufficient to draw such a conclusion is rescue of the mutant phenotype by expression of a (wild-type) transgenic transcript, along with controls for behavioral and molecular specificity. Although the issue of behavioral specificity seems trivial in light of the complete rescue of mutant

lio¹ learning/memory, it becomes quite relevant when only partial rescue of a learning/memory defect is observed, as has recently been reported for the *dunce* gene (Dauwalder and Davis, 1995).

This singular criterion is particularly relevant for P element–derived mutations, since these large, foreign pieces of DNA are likely to disrupt the expression of several nearby genes. Phenotypic rescue in transgenic flies is sufficient, as well, for ethylmethane sulfonate–induced mutations, which tend to produce more restricted (even single nucleotide) molecular lesions. When multiple, independently derived mutant alleles are available, however, a second approach can be used to identify the relevant transcription unit: molecular lesions corresponding to each of several mutations can be shown to reside within the same transcription unit (e.g., *bithorax*; Bender et al., 1983).

lio⁺ Encodes a Novel Protein and Constitutes a Novel Gene Involved with Associative Learning

Of particular interest is the observation that the *lio⁺* gene encodes an unknown protein (see Figure 6). Future studies obviously and eventually will reveal the biological function(s) of this gene. More germaine to this study, however, is the reiteration that discovery of novel genes involved with associative learning/memory was the primary goal of the original P element mutagenesis. Given the frequency with which learning/memory mutants were identified from our screen (one mutant per 550 P element lines) and the speed with which the correct transcript was identified for *lio*, this approach in Drosophila appears particularly expeditious. With substantial molecular and behavioral homology for associative learning and memory processes already documented among fruit flies, bees, mollusks, and vertebrates (see DeZazzo and Tully, 1995, for a review), we anticipate trans-species homologs of the *lio* gene.

Experimental Procedures

Plasmid Rescue and cDNA cloning

Genomic sequences flanking the *lio¹* P element were cloned by plasmid rescue using standard techniques (Sambrook et al., 1989; Wilson et al., 1989). Briefly, *lio¹* genomic DNA was digested with SacI, followed by ligation to form a rescue plasmid, which was propagated in Escherichia coli LE392. The rescue fragment was then radiolabeled with ^{32}P by random priming and used to screen 3×10^7 phage plaques from a Drosophila genomic bacteriophage λ-DASH library (Sambrook et al., 1989). The 5.7 kb HindIII λ genomic fragment (see Figure 1) was subcloned into the plasmid vector pBS–KS+, radiolabeled, and used to probe a Northern blot of adult whole-fly poly(A)⁺ RNA and a λgt11 Drosophila adult head cDNA library. A 6 kb EcoRI fragment (see Figure 1) corresponding to the sequences distal to the *lio¹* P element was subcloned into the plasmid vector pBS–KS+, radiolabeled, and used to probe a Northern blot of adult head poly(A)⁺ RNA. A 3.1 kb EcoRI *lio* cDNA fragment was excised from the λ phage and subcloned into the plasmid vector pBS–KS+. This 3.1 kb insert (containing the 2.7 kb ORF) was cloned into the EcoRI (polylinker) site of the transformation vector CaSpeR-hs, which contains a *white⁺* minigene as a selectable marker and P element sequences to facilitate insertion into the genomic DNA (Pirrotta, 1988). This transgene construct was designated *hslio⁺*.

RNA Isolation and Northern Blotting

Flies were collected and sacrificed immediately by flash freezing in liquid nitrogen. Where applicable, Drosophila adult head and body mRNA was made by vigorously shaking frozen flies and separating the frozen heads and bodies by sifting over dry ice. The frozen parts were pulverized in a mortar on dry ice and then extracted using the acidic guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Poly(A)⁺ RNA subsequently was selected by oligo(dT)⁺ chromatography (Chirgwin et al., 1979). The poly(A)⁺ RNA then was electrophoresed on a 1% formaldehyde agarose gel, transferred to a charged nylon membrane, and probed with the radiolabeled 3.1 kb cDNA fragment or with a fragment from the Drosophila *ribosomal protein 49* (*rp49*) gene (Church and Gilbert, 1984; O'Connell and Rosbash, 1984). For experiments summarized in Figure 2, two Northern blots were generated, each containing poly(A)⁺ RNA from wild-type flies and *lio¹* mutants. Bands intensities were determined by phosphorimage analysis (Fuji Photo Film Co.). Intensity of the *lio⁺* transcript was normalized to that of *rp49* within a given lane (wild-type or mutant). Levels of expression of the *lio⁺* transcript in *lio¹* mutants then were expressed as percentages of the levels of expression of the *lio⁺* transcripts in wild-type flies in each corresponding Northern blot. Finally, these two percentages of expression were averaged (see text).

Fly Stocks

The genetic background of *w;lio¹* mutants was equilibrated with that of the wild-type (Canton-S) strain by repeatedly backcrossing heterozygous *w/w;lio¹/+* females (which carried the *mw⁺* eye color marker) to *w(CS10)* males for more than five generations. The *w(CS10)* strain was derived by backcrossing *w¹¹¹⁸* flies to wild-type (Canton-S) flies for ten generations (Dura et al., 1993); the *w(isoCJ1)* strain was derived from *w(CS10)* and carries isogenic X, second, and third chromosomes (Yin et al., 1994, 1995a). Homozygous *w;lio¹* flies (hereafter referred to as *lio¹*) were bred a few months before behavioral experiments. For histological experiments, *lio¹* homozygotes or flies from another "wild-type" *PlacW* transposant strain, *E₁4(TP)*, which showed normal olfactory acuity, shock reactivity, and olfactory learning, were coded and then crossed to flies carrying the second chromosome deletion *Df(2L)VA12* (hereafter referred to as *Df*) and the *CyO* second chromosome balancer. Straight-winged flies from each cross, *lio¹* mutants, and *E₁4(TP)* flies were then processed together and decoded after the planimetric analysis (see below). To generate transgenic flies, ~3000 *w(isoCJ1)* embryos were dechorionated with 40% aqueous bleach for 60 s, rinsed in water, desiccated at 18°C and 60% relative humidity for ~20 min, aligned on acetate-based double-sided tape (3M type 415, 3M, St. Paul, MN), and coinjected with *hslio⁺* and the transposase-source plasmid pUChspD2-3wc (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Approximately 200 G₀ flies were recovered and mated to *w(isoCJ1)*, from which 18 independent, fertile transformant lines were established. These transgenic lines were designated *lio⁺; hslio⁺-X*. Flies from 4 transgenic lines carrying *hslio⁺* insertions in the third chromosome (*lio⁺;hslio⁺-16*, *lio⁺;hslio⁺-3*, *lio⁺;hslio⁺-21*, and *lio⁺; hslio⁺-1*) were crossed with *lio¹* mutants to recover heterozygous *lio¹/ +;hslio⁺/+* progeny, which were identified by eye color. These heterozygotes then were mated to a "cantonized" *CyO/Sp;TM6B/Sb* double balancer strain to yield *lio¹/CyO;hslio⁺/TM6B* progeny. Intermating of these flies yielded 4 lines homozygous for the *lio¹* mutation on the second chromosome and for the *hslio⁺* transgene on the third chromosome. These transgenic lines were designated *lio¹;hslio⁺-X*.

Histology

To quantitate the neuropillar volumes of the mushroom bodies and central complex, paraffin sections of *lio¹*, *lio⁺* [*E₁4(TP)*], *lio¹/Df*, and *lio⁺/ Df* flies were prepared as in Ashburner (1989) with a few modifications: heads were first incubated in 1:1 methylbenzoate:low melting paraffin, followed by six 30 min incubations with pure paraffin. Frontal sections (7 μm) were inspected visually at 400× magnification. The volumes of mushroom body or central complex neuropil were then quantified via planimetric analysis using an MTI CCD 725 camera connected to a Screen Machine Classic Videoboard (FAST electronic GmbH, Munich, Germany) in an MS-DOS PC with custom software developed by R. Wolf and M. Heisenberg (Heisenberg et al., 1995). The operator traced the outlines of mushroom body calyces and the central complex (including the noduli and fan-shaped body) through serial sections while blind to genotype.

X-Gal Staining of Tissues

Embryos were dechorionated, fixed in 3.7% formaldehyde in PBS,

and stained with X-Gal as described (Ashburner, 1989). Larval central nervous systems were dissected in Ringer's solution, fixed in 30% glutaraldehyde, and stained with X-Gal as described (Ashburner, 1989) for 1 hr at 37°C. Adult heads were imbedded in 3-octanol (OCT), sectioned in a cryostat, fixed in 1% glutaraldehyde/PBS, stained with X-Gal, and mounted as described by Han et al. (1992).

Behavioral Analysis of Transgenic Flies
Preparation of Flies
Before behavioral assays, ~600 1- to 2-day-old flies were placed in a foam-plugged half-pint glass bottle with standard food and a wad of paper towel. These flies were stored overnight at 25°C and 50% relative humidity. The next morning, groups that were destined for the heat shock treatment (37°C for 30 min) were transferred to foam-plugged, 15 × 85 mm glass vials with a 10 × 20 mm strip of Whatman 3M filter paper. The vials were placed in a water bath, ensuring that the fly chamber was completely submerged. After this heat shock regimen, the flies were transferred to a standard food vial, where they recovered for 3 hr at 25°C and 50% relative humidity, at which time behavioral assays commenced.

Pavlovian Learning/Memory
To analyze associative learning, we used the Pavlovian conditioning procedure of Tully and Quinn (1985). Briefly, groups of about 100 flies were trained in a tube with an internal electrifiable grid. The tube was sequentially ventilated with two odorants, OCT (ICN Biochemical, Aurora, OH) and 4-methylcyclohexanol (MCH; Fluka Chemie AG, Buchs CH) at concentrations equally aversive to untrained flies. The flies were exposed for 60 s to OCT (CS+) while being given twelve 1.5 s pulses of 60 V (DC) electroshock every 5 s, followed by a 45 s rest period. The flies then were exposed for 60 s to MCH (CS−) without any electroshock, which again was followed with a 45 s rest interval. To test for learning, the trained flies were tapped into a T maze immediately after this discriminative conditioning procedure. Air laced with the CS+ or CS− was drawn through each of the two respective arms of the T maze, and the flies were allowed 120 s to migrate into either T maze arm. At the end of this test trial, the flies were trapped in the T maze arms, anesthetized, and counted. For one complete experiment, this training/testing procedure was repeated with a second group of flies using the reciprocal odor combination (MCH as CS+ and OCT as CS−). The total numbers of flies in the T maze arms were then used to calculate the proportions "correctly avoiding" the CS+ (they were in the CS− T maze arm), and the two values from reciprocal experiments were averaged. Finally, a PI for one complete experiment was calculated by normalizing the average proportion correctly avoiding. PIs could range from 0 (a 50:50 distribution in the T maze; no learning) to 100 (all flies avoid the CS+). To measure 3 hr retention, trained flies were transferred to a food vial, where they were stored at 25°C during the retention interval. At 75 s before the test trial, flies were transferred to the choice point of the T maze and tested as described above.

Olfactory Acuity
The flies' ability to smell the odorants used during Pavlovian conditioning experiments was quantified by exposing groups of untrained flies for 120 s to odor versus air in the T maze (see Boynton and Tully, 1992). Typically, two odor concentrations were used: undiluted, as in the Pavlovian experiments, and a 100-fold dilution. PIs were calculated as above but for each group separately. To control for side bias, equal numbers of groups were assayed with odor in the right or left arm of the T maze.

Shock Reactivity
The flies' ability to sense the electroshock used during Pavlovian conditioning experiments was quantified by introducing groups of untrained flies into a testing T maze where both arms contain electrifiable grids. One of the two arms was electrified as above, and the flies chose between shock and no shock for 120 s (see Luo et al., 1992; the primary reference to this method was incorrectly stated as Dura et al., 1993, in Tully et al., 1994; Yin et al., 1994, 1995a). Typically, two shock voltages were used: 60 V, as in the Pavlovian experiments, and 20 V. PIs were calculated as in olfactory acuity experiments.

Statistical Analysis of Behavioral Data
PIs are distributed normally (Tully and Gold, 1993), so untransformed data were analyzed parametrically with the Macintosh software package JMP 3.1 (SAS Institute, Inc.). All pairwise comparisons were planned. To maintain an experiment-wise error rate of $\alpha = 0.05$, the critical P values were adjusted accordingly (Sokal and Rohlf, 1981; Audesirk and Audesirik, 1989) and are listed below for each experiment. All behavioral experiments were performed in a balanced fashion, with n = 2 PIs collected per day per group (genotype ± hs). In these experiments, the experimenter was blind to genotype.

Learning in Wild-Type, Mutant, and Transgenic Flies with or without Heat Shock
PIs from four GENOtypes (wild type, lio^1, $lio^1;hslio^+$-16, or $lio^1;hslio^+$-3) and two HEAT shock regimens (−hs or +hs) were subjected to a two-way ANOVA with GENO ($F_{(3,72)} = 71.30$, $p < .001$) and HEAT ($F_{(1,72)} = 17.74$, $p < 0.001$) as main effects and GENO × HEAT ($F_{(3,72)} = 21.68$, $p < .001$) as an interaction term. The 10 planned comparisons were deemed significant if $p \leqslant .005$ and are summarized in Figure 3A.

Three Hour Retention in Wild-Type, Mutant, and Transgenic Flies with or without Heat Shock
PIs from four GENOtypes (wild type, lio^1, $lio^1;hslio^+$-16, or $lio^1;hslio^+$-3) and two HEAT shock regimens (−hs or +hs) were subjected to a two-way ANOVA with GENO ($F_{(3,72)} = 20.54$, $p < .001$) and HEAT ($F_{(1,72)} = 20.49$, $p < .001$) as main effects and GENO × HEAT ($F_{(3,72)} = 7.67$, $p < 0.001$) as an interaction term. The 10 planned comparisons were deemed significant if $p \leqslant .005$ and are summarized in Figure 3B.

Olfactory Acuity in Wild-Type and Transgenic Flies
PIs from two GENOtypes (wild-type or $lio^1;hslio^+$-16), four ODOR/concentration groups (OCT 10^{-2}, OCT 10^0, MCH 10^{-2}, or MCH 10^0), and two HEAT shock regimens (−hs or +hs) were subjected to a three-way ANOVA with GENO ($F_{(1,128)} = 0.29$, $p = .59$), ODOR ($F_{(3,128)} = 61.76$, $p < .001$), and HEAT ($F_{(1,128)} = 33.33$, $p < .001$) as main effects; with GENO × ODOR ($F_{(3,128)} = 1.79$, $p = .15$), GENO × HEAT ($F_{(1,128)} = 0.04$, $p = .84$), and ODOR × HEAT ($F_{(3,128)} = 0.15$, $p = .93$) as two-way interaction terms; and with GENO × ODOR × HEAT ($F_{(3,128)} = 0.15$, $P = 0.93$) as the three-way interaction term. The 12 planned comparisons were judged significant if $p \leqslant .004$ and are summarized in Table 1.

Shock Reactivity in Wild-Type and Transgenic Flies
PIs from two GENOtypes (wild-type or $lio^1;hslio^+$-16), two VOLTages (20 or 60 V), and two HEAT shock regimens (−hs or +hs) were subjected to a three-way ANOVA with GENO ($F_{(1,64)} = 0.57$, $p = .45$), VOLT ($F_{(1,64)} = 97.47$, $p < .001$), and HEAT ($F_{(1,64)} = 0.14$, $p = .71$) as main effects; with GENO × VOLT ($F_{(1,64)} = 0.63$, $p = .43$), GENO × HEAT ($F_{(1,64)} = 0.21$, $p = .65$), and VOLT × HEAT ($F_{(1,64)} = 2.81$, $p = .10$) as two-way interaction terms; and with GENO × VOLT × HEAT ($F_{(1,64)} = 0.63$, $p = .43$) as the three-way interaction term. The 6 planned comparisons were judged significant if $p \leqslant .01$ and are summarized in Table 1.

Learning in Wild-Type and Transgenic Flies with or without Heat Shock
PIs from two GENOtypes (wild-type or $lio^+;hslio^+$-16) and two HEAT shock regimens (−hs or +hs) were subjected to a two-way ANOVA with GENO ($F_{(1,20)} = 0.02$, $p = .89$) and HEAT ($F_{(1,20)} = 36.04$, $p < .001$) as main effects and GENO × HEAT ($F_{(1,20)} = 0.39$, $p = .54$) as an interaction term. The 2 planned comparisons were deemed significant if $p \leqslant .05$ and are summarized in Figure 4A.

Three Hour Retention in Wild-Type and Transgenic Flies with or without Heat Shock
PIs from two GENOtypes (wild-type or $lio^+;hslio^+$-16) and two HEAT shock regimens (−hs or +hs) were subjected to a two-way ANOVA with GENO ($F_{(1,20)} = 1.27$, $p = .27$) and HEAT ($F_{(1,20)} = 3.37$, $p = .08$) as main effects and GENO × HEAT ($F_{(1,20)} = 0.01$, $p = .93$) as an interaction term. The 2 planned comparisons were deemed significant if $p \leqslant .05$ and are summarized in Figure 4B.

Statistical Analysis of Histological Data
Planimetric estimates of neuropillar volume of mushroom bodies (sum of both hemispheres) and central complex were distributed normally, so raw data were analyzed parametrically with the Macintosh software package JMP 3.1 (SAS Institute, Inc.). Data from each anatomical region were subjected separately to a two-way ANOVA with GENO ($F_{(3,64)} = 1.49$, $p = .23$ for central complex; $F_{(3,64)} = 0.77$, $p = .52$ for mushroom bodies) and SEX ($F_{(1,64)} = 172.13$, $p < .001$ for central complex; $F_{(1,64)} = 38.68$, $p < .001$ for mushroom bodies) as main effects and GENO × SEX ($F_{(3,64)} = 8.47$, $p < .001$ for central complex; $F_{(3,64)}$

$= 6.21$, $p < .001$ for mushroom bodies) as an interaction term. To maintain an experiment-wise error rate of $\alpha = 0.05$, critical p values for the 4 planned comparisons (see Figure 8E) were adjusted to $p \leqslant .01$. Experimenters were blind to genotype during histological preparations of tissue sections and during planimetric analyses.

References

Abeliovich, A., Paylor, R., Chen, C. K. J. J., Wehner, J. M., and Tonegawa, S. (1993). PKCγ mutant mice exhibit moderate deficits in contextual learning. Cell 75, 1263–1271.

Alberini, C. M., Ghirardi, M., Metz, R., and Kandel, E. R. (1994). C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in Aplysia. Cell 76, 1099–1114.

Ashburner, M. (1989). Drosophila: A Laboratory Manual (Plainview, New York: Cold Spring Harbor Laboratory Press).

Audesirik, G., and Audesirik, T. (1989). Effects of in vitro lead exposure on voltage-sensitive calcium channels differ among cell types in central neurons of Lymnaea stagnalis. NeuroToxicology 10, 659–670.

Backsai, B. J., Hochner, B., Mahaut-Smith, M., Adams, S. R., Kaang, B.-K., Kandel, E. R., and Tsien, R. Y. (1993). Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. Science 260, 222–226.

Bailey, C. H., Chen, M., Keller, F., and Kandel, E. R. (1992). Serotonin-mediated endocytosis of apCAM: an early step of learning-related synaptic growth in Aplysia. Science 256, 645–649.

Balling, A., Technau, G. M., and Heisenberg, M. (1987). Are the structural changes in adult Drosophila mushroom bodies memory traces? Studies on biochemical learning mutants. J. Neurogenet. 4, 65–73.

Bender, W., Akam, M., Karch, F., Beachy, P. A., Peifer, M., Spierer, P., Lewis, E. B., and Hogness, D. S. (1983). Molecular genetics of the bithorax complex in Drosophila melanogaster. Science 221, 23–29.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Caretto, R., Uemura, T., Grell, E., Jan, L. Y., and Jan, Y. N. (1989). Searching for pattern and mutations in the Drosophila genome with a P-lacZ vector. Genes Dev. 3, 1273–1287.

Bliss, T. V. P., and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation. Nature 361, 31–39.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A. J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 79, 59–68.

Boynton, S., and Tully, T. (1992). latheo, a new gene involved in associative learning and memory in Drosophila melanogaster identified from P element mutagenesis. Genetics 131, 655–672.

Byers, D., Davis, R. L., and Kiger, J. A., Jr. (1981). Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in Drosophila melanogaster. Nature 289, 79–81.

Byrne, J. H., Zwartjes, R., Homayouni, R., Critz, S. D., and Eskin, A. (1993). Roles of second messenger pathways in neuronal plasticity and in learning and memory. In Advances in Second Messenger and Phosphoprotein Research, S. Shenolikar and A. C. Nairn, eds. (New York: Raven Press), pp. 47–107.

Callahan, C. A., Muralidhar, M. G., Lundgren, S. C., Scully, A. L., and Thomas, J. B. (1995). Control of neuronal pathway selection by a Drosophila receptor protein-tyrosine kinase family member. Nature 376, 171–174.

Carew, T. J., Pinsker, H. M., and Kandel, E. R. (1972). Long-term habituation of a defensive withdrawal reflex in Aplysia. Science 175, 451–454.

Castellucci, V. F., Blumenfeld, H., Goelet, P., and Kandel, E. R. (1989). Inhibitor of protein synthesis blocks long-term behavioral sensitization in the isolated gill-withdrawal reflex of Aplysia. J. Neurobiol. 20, 1–9.

Cavener, D. R. (1987). Comparison of the consensus sequence flanking translational start sites in Drosophila and vertebrates. Nucl. Acids Res. 15, 1353–1361.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acids from sources enriched in ribonuclease. Biochemistry 18, 5294.

Choi, K.-W., Smith, R. F., Buratowski, R. M., and Quinn, W. G. (1991). Deficient protein kinase C activity in turnip, a Drosophila learning mutant. J. Biol. Chem. 266, 15999–16006.

Chomczynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA 81, 1991.

Cowan, T., and Siegel, R. W. (1986). Drosophila mutations that alter ionic conduction disrupt acquisition and retention of a conditioned odor avoidance response. J. Neurogenet. 3, 187–201.

Crow, T., and Forrester, J. (1990). Inhibition of protein synthesis blocks long-term enhancement of generator potentials produced by one-trial in vivo conditioning in Hermissenda. Proc. Natl. Acad. Sci. USA 87, 4490–4494.

Dale, N., Schacher, S., and Kandel, E. R. (1988). Long-term facilitation in Aplysia involves increase in transmitter release. Science 239, 282–285.

Dash, P., Hochner, B., and Kandel, E. R. (1990). Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. Nature 345, 718–721.

Dauwalder, B., and Davis, R. L. (1995). Conditional rescue of the dunce learning/memory and female fertility defects with Drosophila or rat transgenes. J. Neurosci. 15, 3490–3499.

Davis, H. P., and Squire, L. R. (1984). Protein synthesis and memory: a review. Psychol. Bull. 96, 518–559.

Davis, R. L. (1993). Mushroom bodies and Drosophila learning. Neuron 11, 1–14.

de Belle, J. S., and Heisenberg, M. (1994). Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. Science 263, 692–695.

DeZazzo, J., and Tully, T. (1995). Dissection of memory formation: from behavioral pharmacology to molecular genetics. Trends Neurosci. 18, 212–217.

Drain, P., Folkers, E., and Quinn, W. G. (1991). cAMP-dependent protein kinase and the disruption of learning in transgenic flies. Neuron 6, 71–82.

Dura, J. M., Preat, T., and Tully, T. (1993). Identification of linotte, a new gene affecting learning and memory in Drosophila melanogaster. J. Neurogenet. 9, 1–14.

Dura, J. M., Preat, T., and Taillebourg, E. (1995). The linotte learning and memory gene encodes a putative tyrosine kinase required for normal developement of the Drosophila central brain. J. Neurogenet. 10, 25.

Ebbinghaus, H. (1885). Über das Gedächtnis (New York: Dover).

Eichenbaum, H., and Otto, T. (1993). Odor-guided learning and memory in rats: is it 'special'? Trends Neurosci. 16, 22–24.

Erber, J. (1976). Retrograde amnesia in honeybees (Apis mellifera carnica). J. Comp. Physiol. Psychol. 89, 41–46.

Ewer, J., Rosbash, M., and Hall, J. C. (1988). An inducible promoter fused to the period gene in Drosophila conditionally rescues adult per⁻ mutant arrhythmicity. Nature 333, 82–84.

Frey, U., Huang, Y., and Kandel, E. R. (1993). Effects of cAMP stimulate a late stage of LTP in hippocampal CA1 neurons. Science 260, 1661–1664.

Frost, W. N., Castellucci, V. F., Hawkins, R. D., and Kandel, E. R. (1985). Monosynaptic connections made by the sensory neurons of the gill- and siphon-withdrawal reflex in Aplysia participate in the storage of long-term memory for sensitization. Proc. Natl. Acad. Sci. USA 82, 8266–8269.

Gailey, D. A., Villella, A., and Tully, T. (1991). Reassessment of the effect of biological rhythm mutations on learning in Drosophila melanogaster. J. Comp. Physiol. 169, 685–697.

Ghirardi, M., Braha, O., Hochner, B., Montarolo, P. G., Kandel, E. R., and Dale, N. (1992). Roles of PKA and PKC in facilitation of evoked and spontaneous transmitter release at depressed and nondepressed synapses in Aplysia sensory neurons. Neuron 9, 479–489.

Glanzman, D. L., Kandel, E. R., and Schacher, S. (1990). Target-dependent structural changes accompanying long-term synaptic facilitation in Aplysia neurons. Science 249, 799–802.

Grant, S. G. N., O'Dell, J., Karl, K. A., Stein, P. L., Soriano, P., and Kandel, E. R. (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. Science 258, 1903–1910.

Greenough, W. T. (1984). Structural correlates of information storage in the mammalian brain: a review and hypothesis. Trends Neurosci. 7, 229–283.

Griffith, L. C., Verselis, L. M., Aitken, K. M., Kyriacou, C. P., Danho, W., and Greenspan, R. J. (1993). Inhibition of calcium/calmodulin-dependent protein kinase in Drosophila disrupts plasticity. Neuron 10, 501–509.

Hammer, M., and Menzel, R. (1995). Learning and memory in the honeybee. J. Neurosci. 15, 1617–1630.

Han, P.-L., Levin, L. R., Reed, R. R., and Davis, R. L. (1992). Preferred expression of the Drosophila rutabaga gene in mushroom bodies, neural centers for learning in insects. Neuron 9, 619–627.

Hawkins, R. D., and Kandel, E. R. (1984). Is there a cell biological alphabet for learning? Psychol. Rev. 91, 375–391.

Heisenberg, M., Borst, A., Wagner, S., and Byers, D. (1985). Drosophila mushroom body mutants are deficient in olfactory learning. J. Neurogenet. 2, 1–30.

Heisenberg, M., Heusipp, M., and Wanke, C. (1995). Structural plasticity in the Drosophila brain. J. Neurosci. 15, 1951–1960.

Hintzman, D. L. (1974). Theoretical implications of the spacing effect. In Theories in Cognitive Psychology: The Loyola Symposium, R. L. Solso, ed. (Hillsdale, New Jersey: Lawrence Erlbaum Association), pp. 77–99.

Hirsh, J., and Davidson, N. (1981). Isolation and characterization of the dopa decarboxylase gene of Drosophila melanogaster. Mol. Cell. Biol. 1, 475–485.

Huang, Y. Y., and Kandel, E. R. (1994). Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. Learning Mem. 1, 74–82.

Kandel, E. R., Klein, M., Hochner, B., Shuster, M., Siegelbaum, S. A., Hawkins, R. D., Glanzman, D. L., Castellucci, V. F., and Abrams, T. W. (1987). Synaptic Modulation and Learning: New Insights into Synaptic Transmission from the Study of Behavior (New York: John Wiley & Sons).

Kesner, R. P., and Olton, D. S. (1990). Neurobiology of Comparative Cognition (Hillsdale, New Jersey: Lawrence Erlbaum Association).

Kozak, M. (1987). An analysis of 5′-noncoding sequence from 699 vertebrate messenger RNAs. Nucl. Acids Res. 15, 8125–8148.

Levin, L. R., Han, P.-L., Hwang, P. M., Feinstein, P. G., Davis, R. L., and Reed, R. R. (1992). The Drosophila learning and memory gene

rutabaga encodes a Ca^{2+}/calmodulin-responsive adenylyl cyclase. Cell 68, 479–489.

Livingstone, M. S., Sziber, P. P., and Quinn, W. G. (1984). Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a Drosophila learning mutant. Cell 37, 205–215.

Luo, L., Tully, T., and White, K. (1992). Human amyloid precursor protein ameliorates behavioral deficit of flies deleted for Appl gene. Neuron 9, 595–605.

Mackintosh, N. J. (1983). Conditioning and Associative Learning (New York: Oxford University Press).

Malinow, R., Schulman, H., and Tsien, R. W. (1989). Inhibition of post-synaptic PKC or CaMKII blocks induction but not expression of LTP. Science 245, 862–866.

McGaugh, J. L., and Herz, M. L. (1972). Memory Consolidation (San Fransisco, California: Albion).

Montarolo, P. G., Goelet, P., Castelucci, V. F., Morgan, J., Kandel, E. R., and Schacher, S. (1986). A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. Science 234, 1249–1254.

Nazif, F. A., Byrne, J. H., and Cleary, L. J. (1991). cAMP induces long-term morphological changes in sensory neurons of Aplysia. Brain Res. 539, 324–327.

Nighorn, A., Healy, M. J., and Davis, R. L. (1991). The cyclic AMP phosphodiesterase encoded by the Drosophila dunce gene is concentrated in the mushroom body neuropil. Neuron 6, 455–467.

O'Connell, P., and Rosbash, M. (1984). Sequence, structure, and codon preference of the Drosophila ribosomal protein 49 gene. Nucl. Acids Res. 12, 5495–5513.

Pirrotta, V. (1988). Vectors for P-mediated transformation in Drosophila. Biotechnology 10, 437–456.

Rubin, G. M., and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable vectors. Science 218, 348–353.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Silva, A. J., Paylor, R., Wehner, J. M., and Tonegawa, S. (1992). Impaired spatial learning in α-calcium-calmodulin kinase II mutant mice. Science 257, 206–211.

Skoulakis, E. M. C., Kalderon, D., and Davis, R. L. (1993). Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. Neuron 11, 197–208.

Sokal, R. R., and Rohlf, F. J. (1981). Biometry (New York: Freeman).

Spradling, A. C., and Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. Science 218, 341–347.

Stewart, M. G., and Rusakov, D. A. (1995). Morphological changes associated with stages of memory formation in the chick following passive avoidance training. Behav. Brain Res. 66, 21–28.

Tully, T., and Gold, D. (1993). Differential effects of dunce mutations on associative learning and memory in Drosophila. J. Neurogenet. 9, 55–71.

Tully, T., and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant Drosophila melanogaster. J. Comp. Physiol. 157, 263–277.

Tully, T., Boynton, S., Brandes, C., Dura, J.-M., Mihalek, R., Preat, T., and Villella, A. (1990). Genetic dissection of memory formaton in Drosophila melanogaster. Cold Spring Harbor Symp. Quant. Biol. 55, 203–211.

Tully, T., Preat, T., Boynton, S. C., and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in Drosophila. Cell 79, 35–47.

Weisskopf, M. G., Zalutsky, R. A., and Nicoll, R. A. (1993). The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. Nature 362, 423–427.

Wilson, C., Pearson, R. K., Bellen, H. J., O'Kane, C. J., Grossniklaus, U., and Gehring, W. J. (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmen-

tally regulated genes in *Drosophila*. Genes Dev. *3*, 1301–1313.

Wu, Z. L., Thomas, S. A., Villacres, E. C., Xia, Z., Simmons, M. L., Chavkin, C., Palmiter, R. D., and Storm, D. R. (1995). Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. Proc. Natl. Acad. Sci. USA *92*, 220–224.

Yin, J. C. P., Wallach, J. S., Del Vecchio, M., Wilder, E. L., Zhou, H., Quinn, W. G., and Tully, T. (1994). Induction of a dominant-negative CREB transgene blocks long-term memory in Drosophila. Cell *79*, 49–58.

Yin, J. C. P., Del Vecchio, M., Zhou, H., and Tully, T. (1995a). CREB as a memory modulator: induced expression of a cCREB2 isoform enhances long-term memory in Drosophila. Cell *81*, 107–115.

Yin, J. C. P., Wallach, J. S., Wilder, E. L., Klingensmith, J., Dang, D., Perrimon, N., Zhou, H., Tully, T., and Quinn, W. G. (1995b). Drosophila CREB/CREM homolog encodes multiple isoforms including a PKA-responsive transcriptional activator and antagonist. Mol. Cell. Biol. *15*, 5123–5130.

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