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Olfactory Learning Deficits in Mutants for *leonardo*, a Drosophila Gene Encoding a 14-3-3 Protein

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Summary

Studies of Drosophila and other insects have indicated an essential role for the mushroom bodies in learning and memory. The *leonardo* gene encodes a Drosophila protein highly homologous to the vertebrate 14-3-3ζ isoform, a protein well studied for biochemical roles but without a well established biological function. The gene is expressed abundantly and preferentially in mushroom body neurons. Mutant alleles that reduce LEONARDO protein levels in the mushroom bodies significantly decrease the capacity for olfactory learning, but do not affect sensory modalities or brain neuroanatomy that are requisite for conditioning. These results establish a biological role for 14-3-3 proteins in mushroom body–mediated learning and memory processes, and suggest that proteins known to interact with them, such as RAF-1 or other protein kinases, may also have this biological function.

Introduction

The ability to acquire and process information about the environment (learning) and to store and retrieve this information over time (memory) is fundamental for many organisms. Learning and memory are expressed as modifications of animal behavior (conditioning) that emerge from the function of molecules within neurons, the integrated action of many neurons comprising neural circuits, and from the engagement of multiple circuits. Drosophila exhibit behavioral conditioning based upon experience to a wide range of olfactory, visual, and tactile stimuli (Davis, 1996). The molecular analysis of Drosophila mutants that are defective in behavioral conditioning has identified several molecules and signal transduction cascades that underlie learning and memory processes (Davis, 1996). One well characterized class of conditioning mutants includes *dunce*, the structural gene for cAMP-dependent phosphodiesterase (Chen et al., 1986; Qui et al., 1991), *rutabaga*, the gene for an adenylyl cyclase (Livingstone et al., 1984; Levin et al., 1992), and *DC0*, the gene encoding the catalytic subunit of cAMP-dependent protein kinase (Foster et al., 1988; Kalderon and Rubin, 1988; Skoulakis et al., 1993a). The identification of these genes and others possibly involved in learning and in the cAMP signaling system (Yin et al., 1994; Feany and Quinn, 1995) has produced the generalization that this cascade is critically involved in the physiology of neurons mediating learning in Drosophila. This concept is in accord with studies of synaptic facilitation in Aplysia, long-term potentiation in the hippocampus, and behavioral conditioning in the mouse, indicating that the cAMP cascade is paramount for these types of neuroplasticity (Sweatt and Kandel, 1989; Schwartz, 1993; Huang et al., 1995).

It is revealing that the products of *dunce*, *rutabaga*, *DCO*, and other genes important for olfactory learning are expressed preferentially in mushroom body neurons (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993a; Wu et al., submitted). Mushroom bodies are bilateral clusters of about 2500 neurons situated in the dorsal and posterior cortex of each brain lobe (Davis and Han, 1996). These cells extend dendrites into a neuropil (calyces) just ventral to the cell bodies, where inputs arrive from the antennal lobes and other centers conveying sensory information. The axons of mushroom body cells fasciculate to form the peduncle that projects to the anterior of the brain. There it bifurcates, with some processes extending medially to comprise the neuropil region known as the β and γ lobes, and others extending dorsally to comprise the α lobe (Strausfeld, 1976). Although the mushroom bodies receive inputs from many sensory modalities through the calyces and lobes and are required for olfactory learning, they are not required for olfaction per se (Heisenberg et al., 1985; Menzel et al., 1991; DeBelle and Heisenberg, 1994). These observations have been combined in a model that envisions mushroom bodies as an integration center for sensory information, utilizing the cAMP cascade as a principle means of physiological modulation (Davis, 1993, 1996)

In this paper, we report on one gene identified in an enhancer detector screen for genes preferentially expressed in mushroom bodies (Han et al., 1996). This gene encodes the Drosophila homolog of the ζ isoform of the 14-3-3 family of proteins. The 14-3-3 proteins are small, highly conserved acidic molecules first discovered as abundant proteins in the brain (Moore and Perez, 1968). Members of the family have since been found in all animals and plants examined (reviewed by Aitken et al., 1992; Aitken, 1995). In vertebrates, the family consists of seven closely related proteins (typical 14-3-3 isoforms) and two more distantly related species (atypical isoforms). Most 14-3-3 proteins are expressed ubiquitously at low levels, but are abundant in the CNS (Boston et al., 1982a, 1982b; Ichimura et al., 1991; Watanabe et al., 1991, 1993), consistent with their having important functions in neurons.

Numerous physiological functions have been ascribed to 14-3-3 proteins. In vitro, they activate tyrosine and tryptophan hydroxylases, the rate-limiting enzymes, in the biosynthesis of catecholamines and serotonin, respectively (Ichimura et al., 1987; Makita et al., 1990; Isobe et al., 1991). They function in Ca^{2+}-regulated exocytosis (Morgan and Burgoyne, 1992a, 1992b; Roth et al., 1994; Chamberlain et al., 1995; Roth and Burgoyne, 1995), cell cycle control (Ford et al., 1994), and in protein kinase C (PKC) regulation (Toker et al., 1990, 1992; Isobe et al., 1992; Tanji et al., 1994). They associate physically with proteins of signal transduction cascades such as Polyoma virus middle T antigen, BCR, PI-3 kinase, CDC25 phosphatase, and RAF-1 kinase (Fantl et al., 1994; Freed et al., 1994; Fu et al., 1994; Irie et al., 1994; Pallas et al., 1994; Reuther et al., 1994; Bonnefoy-Berand

et al., 1995; Conklin et al., 1995; Yamamori et al., 1995). The association with this apparently diverse group of proteins may be due to the intriguing ability of 14-3-3 to bind to phosphoserine residues within the consensus Arg-Ser-X-Ser-X-Pro (Muslin et al., 1996). Their interaction with diverse signaling proteins suggests that the proteins function as modulators of activity or specificity of various kinases (Morrison, 1994), or as coordinators for the assembly of signaling complexes for different cascades (Brasselman and McCormick, 1995; Muslin et al., 1996).

Although some of the molecular roles and interactions of 14-3-3 with other proteins are known, an essential, yet unanswered question concerns the biological processes in which these proteins operate. We report here that a Drosophila 14-3-3 protein encoded by the gene *leonardo*, named so because of the diversity of purported molecular functions attributed to these proteins, is essential for learning and memory. Furthermore, the expression of *leonardo* products in mushroom body neurons and their processes is striking, consistent with the observed behavioral role. Thus, these observations extend our understanding of 14-3-3 proteins from the molecular to the organismal level.

Results

Enhancer Detector Insertions in *leonardo*

The leo^{P1188} and leo^{P1375} alleles were identified in an enhancer detector screen for genes preferentially expressed in the mushroom bodies (Han et al., 1996). In situ hybridization to polytene chromosomes localized the enhancer detector element in both lines to cytogenetic locus 46E. Both insertions result in recessive lethality and are noncomplementary.

The β-galactosidase (β-gal) staining pattern in the brain of a leo^{P1188} heterozygote is shown in Figure 3A and is identical to that obtained with $leo^{P1375}/+$ animals. Intense staining in the perikarya of mushroom bodies suggests that the *lacZ* reporter is driven by an enhancer of a gene expressed preferentially in the mushroom bodies. Additional staining is detected in the cortex of the antennal lobe (data not shown), the thoracic ganglia (data not shown), and apparent glial cells of the medulla. The preferential expression in adult mushroom bodies suggests that the identified gene may play a role in the physiology or development (or both) of these brain structures.

To identify this gene, 26 kb of genomic DNA from the locus were recovered by plasmid rescue and from genomic libraries (Figure 1A). A gene close to the enhancer detector elements, encoding RNAs of 2.9, 1.9, and 1.0 kb (Figure 1B), with the 2.9 kb being specific to head, was identified by probing RNA blots with genomic fragments. Three classes of cDNA clones representing these transcripts (Figure 1A) were isolated and upon sequencing, revealed an open reading frame encoding a protein of 248 amino acids with a predicted molecular weight of 28.3 kD and 88% amino acid identity to the mammalian 14-3-3ζ isoform, a typical member of the family.

To define the insertion sites of the enhancer detector elements relative to this transcriptional unit, portions of the genomic region were sequenced and compared to the cDNAs. One cDNA, c2, contained sequences 5′ to the enhancer detector elements, indicating that the elements reside within an intron. This genomic sequence is not specific to this cDNA, however, as it hybridizes to all three transcript classes (data not shown). Thus, the c10 and c4B cDNAs are partial clones at their 5′ ends. The cDNAs differed primarily in the length of their 3′ untranslated sequence (Figure 1A).

leonardo Encodes One 14-3-3 Isoform

Given the multiplicity of 14-3-3 genes in vertebrates, we wondered whether *leonardo* might be one of a small but highly related gene family. We probed Drosophila genomic blots at low stringency, but surprisingly, these experiments failed to identify any related sequences. This suggests that Drosophila has only one gene encoding 14-3-3 isoform(s) highly homologous to the majority of typical vertebrate 14-3-3 proteins.

To determine whether multiple protein isoforms could be encoded by the *leonardo* gene, we characterized 15 representative cDNA clones by restriction analysis and partial sequencing. This failed to reveal any heterogeneity within the open reading frame, although the differential length of 3′ untranslated sequences in the cDNAs is notable (Figure 1A). Furthermore, the sequence available from an independent clone isolated because of preferential expression in the head and embryos (Swanson and Ganguly, 1992) is identical to that of c10. Thus, any sequence variation of protein coding regions that might exist must be minor within the head RNA population.

Additional evidence for the existence of a single 14-3-3 protein highly related to the typical isoforms was obtained from the generation of two polyclonal antisera against an expressed fusion protein. Both anti-LEO antibodies recognize one protein of 29 KD on Western blots, which is reduced in extracts of leo^{P1188} and leo^{P1375} heterozygotes (Figure 2A). To determine if this band is comprised of multiple species, the anti-LEO antisera were used to probe total protein extracts from Drosophila heads and bodies displayed on blots after two-dimensional gel electrophoresis (Figure 2B). A single spot of the predicted size and pI of about 4.5 was recognized in both heads and bodies, although more protein was detected in heads. Identical results were obtained with the second antisera, whereas preimmune sera from both animals failed to detect immunoreactivity (data not shown).

Collectively, these results suggest that unlike vertebrates, Drosophila lacks other typical 14-3-3 isoforms highly similar to LEONARDO. In support of this conclusion, two independently derived polyclonal sera against mixed mammalian 14-3-3 isoforms (Martin et al., 1993; Tanji et al., 1994) react only with one Drosophila protein species of identical size to that recognized by the anti-LEO antisera (data not shown). Conversely, the LEONARDO antibodies recognize more than one isoform in extracts of vertebrate tissues (data not shown).

Preferential Expression of *leonardo* in the Mushroom Bodies

To establish that the β-gal staining pattern observed in the enhancer detector lines reflects expression of the

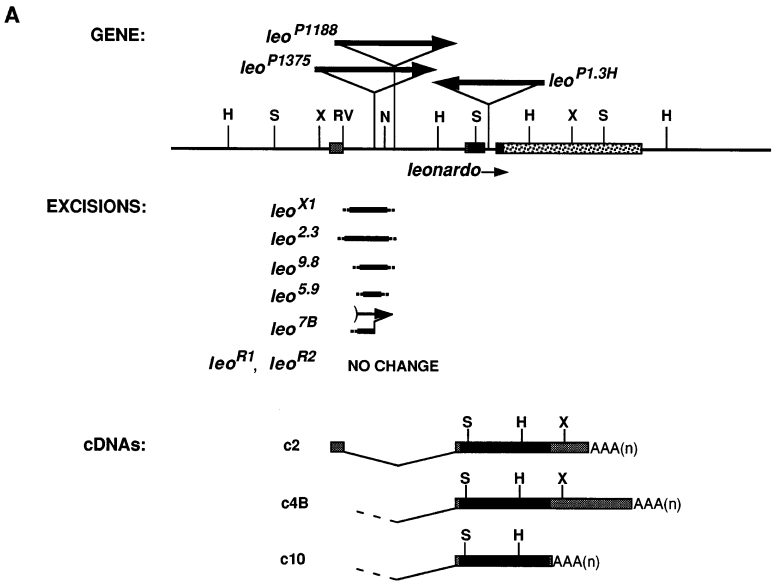
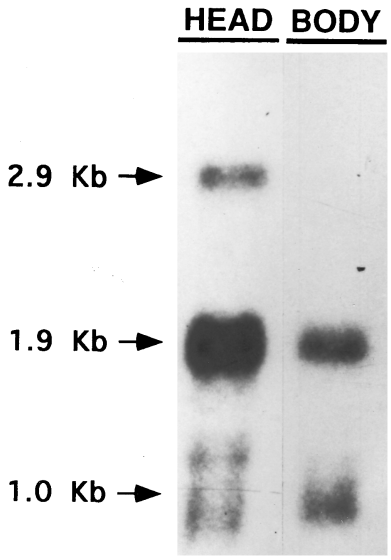


Figure 1. The *leonardo* Gene and Mutants

(A) The genomic region of *leonardo.* The direction of transcription of *leonardo* and of the *lacZ* reporter in the enhancer detector elements are indicated with arrows. Gray boxes represent untranslated sequences, black boxes represent protein coding sequences, and line segments indicate introns and flanking genomic sequences. The intron/exon organization after the second protein coding exon is unknown and indicated by the stippled bar. Molecular alterations in *leonardo* alleles are shown below the map of the gene. The extent of deletions in the *leonardo* alleles shown is indicated by the black bars. For allele leo^{7B}, the remaining P-element sequence is denoted by the partial arrow and parenthesis. The $leo^{P1.3H}$ allele contains a P-element in the second intron of the gene. The organization of three classes of *leonardo* cDNA clones indicates that clone c2 contains untranslated exon sequences upstream of the two insertions, which are missing in the partial clones c4B and c10.

(B) *leonardo* expression. Blots of Poly(A)+ RNA isolated from adult heads and bodies probed with the SalI–HindIII fragment of a *leonardo* cDNA clone. Note the three classes of transcripts, the largest of which is specifically expressed in heads. The band observed around 1.3 kb in the head lane was not detected in all experiments and likely represents an artifact. H: HindIII; N: NotI; RV: EcoRV; S: SalI; X: XbaI.



gene, the distribution of *leonardo* RNA was examined by in situ hybridization. Antisense probes produced remarkably intense signal in mushroom body cells (Figures 3B and 3C). Lower levels of hybridization were observed in cells of the subesophageal ganglion, optic lobes, antennal lobes (data not shown), and the central brain (Figure 3B). The gene is also expressed in cells of the thoracic ganglia (Figure 3C), nurse cells, and maturing oocytes of female animals (data not shown). Notably, there was no hybridization to thoracic muscle, abdominal muscle, fat, or connective tissue cells. Therefore, in adult animals, *leonardo* is expressed predominantly in neurons, especially those that constitute the mushroom bodies.

To examine the spatial distribution of the LEO protein within the mushroom bodies and other brain neuropils, sections were challenged with the anti-LEO polyclonal antibody. The antibody preferentially decorates the neuropil of the mushroom bodies (Figures 3D–3F). The antigen was detected in the dendritic projections (calyces), the cytoplasm of the perikarya (Figure 3D), and the axonal projections that form the peduncle and lobes of mushroom body neurons (Figures 3E and 3F). In addition, the antibody decorates the ellipsoid body, a neuropil structure of the central complex and a group of cell bodies residing just anterior to the "heel" of the mushroom bodies (data not shown; Figure 3F). These cells appear to be the ring neurons that project to the ellipsoid body (Figure 3E). The antibody also decorated neuropil and cell bodies in the antennal lobes, albeit with lower intensity (data not shown). In sagittal sections, modest staining was observed in thoracic ganglia and throughout the cytoplasm of nurse cells and oocytes (data not shown). These patterns were not observed in head and sagittal sections challenged with preimmune sera.

Thus, these results demonstrate that the Drosophila 14-3-3 homolog, *leonardo,* is preferentially expressed in the mushroom body neurons. To test the hypothesis that *leonardo* performs an important role in the physiology and function of mushroom bodies, we tested whether *leonardo* mutants disrupt olfactory learning.

Effect of leo^{P1188} and leo^{P1375} Insertions on *leonardo* Expression

Homozygotes for either leo^{P1188} or leo^{P1375} die before hatching as morphologically normal embryos. The effect

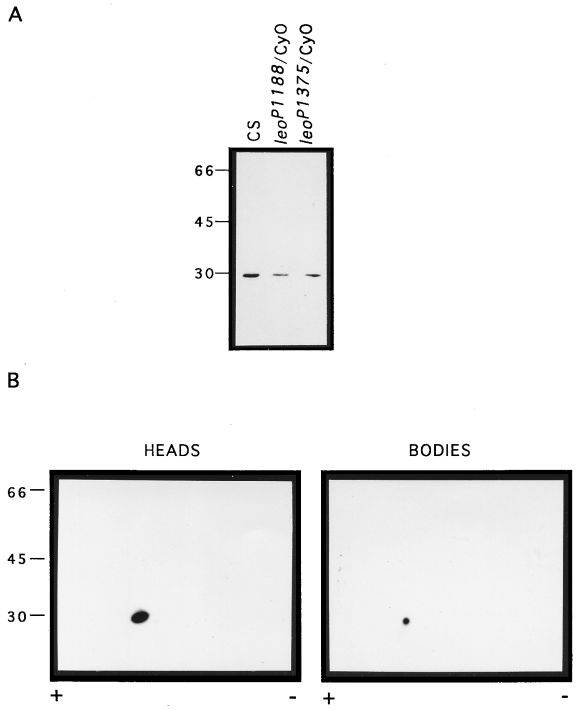


Figure 2. 14-3-3 Isoform Composition in Adult Drosophila

(A) Total protein extracted from one female animal of the indicated genotypes after Western blotting and detection with the anti-LEO antibody. A single protein species of 30 kD was detected, which is reduced in the insertion heterozygotes.

(B) Two-dimensional Western blots of total protein from isolated heads or bodies. One protein of identical electrophoretic mobility and pI was detected in both heads and bodies.

of the insertions on *leonardo* expression was assessed, therefore, by RNA in situ hybridization to prelethal phase embryos. Abundant *leonardo* RNA was present throughout the CNS and lesser amounts were found in the posterior gut and ectoderm of late leo^{P1188} and leo^{P1375} heterozygous (Figure 4A) or wild-type (data not shown) embryos. In contrast, embryos homozygous for either insertion showed a large reduction in *leonardo* RNA (Figure 4B). These results coincide with the observed reduction of *leonardo* protein in extracts from heterozygous adults (see Figure 2A).

Isolation of Viable *leonardo* Alleles

Preliminary behavioral experiments with leo^{P1188} and leo^{P1375} heterozygotes failed to reveal any dominant effects upon olfactory learning and memory. Therefore, we sought novel *leonardo* alleles, which, though viable, compromise the preferential mushroom body expression of the gene. We reasoned that imprecise excisions of the leo^{P1188} and leo^{P1375} insertions might yield such hypomorphic alleles. By mobilization of the leo^{P1375} element, 27 independent viable excision lines and one viable insertion (hop) in the gene were isolated.

We employed the anti-LEO antiserum to determine whether the excision derivatives affected *leonardo* expression. The level of immunoreactivity in the mushroom bodies of the excisions relative to controls was subjectively ranked as normal, high, medium, and low. Of the 26 derivatives screened, 5 appeared indistinguishable from controls and 4 others were classed in the high group with a small, but consistent decrement in staining. The leo^{P1375} and leo^{P1188} heterozygotes, although predicted to have 50% of the normal LEONARDO product, were classed in the high group. Staining was reduced in 17 derivatives, with 11 showing a modest decrease (medium) and 6 showing a strong reduction (low) (Figure 4D).

Molecular characterization of these excision derivatives revealed three classes of alleles. First, some excisions were produced by precise excision (i.e., leo^{R1} and leo^{R2}, see Figure 1A). Second, one allele, $leo^{P1.3H}$, harbored a P-element in the second intron of the gene (see Figure 1A). Third, some imprecise excision derivatives lack both transposon and portions of genomic sequence (i.e. , leo^{X1}, $leo^{2.3}$, $leo^{9.8}$, and $leo^{5.9}$) or portions of both (i.e., leo^{7B}). All derivatives ranked as normal by immunohistochemical staining showed no detectable molecular lesions. Conversely, all alleles that exhibited reduced staining in the mushroom bodies carried genomic aberrations. The genomic excisions were confined to the first intron, suggesting that they disrupt regulatory elements necessary for mushroom body expression or RNA splicing.

Behavioral Effects of *leonardo* Mutations

The effect of reduced LEO in the mushroom bodies on behavioral plasticity was investigated by examining the performance of mutant animals in an associative learning/memory task. The animals were trained and tested in a negatively reinforced olfactory classical conditioning paradigm using electric shock as the unconditioned stimulus, coupled with aversive odors as conditioned stimuli. Because 3 min elapse from the time of association between the stimuli and testing the animals, the earliest measure of their performance represents 3 min memory (immediate memory) of the association. Three control lines were used as follows: first, ry^{506} animals, which are parental to all other lines and carry Cantonized chromosomes, with the exception of an isogenic third with a ry^{506} mutation (Han et al., 1996); second, line P267, which is homozygous for an enhancer detector transposon that does not express β-gal in the brain, thus controlling for possible nonspecific behavioral effects of the P-element; and third, line MB2487, which is homozygous for the enhancer detector transposon at another site and expresses β-gal in the mushroom bodies, thus controlling the potential interference of reporter gene expression on behavioral plasticity.

The MB2487 and P267 animals display immediate performance equivalent to ry^{506} (Figure 5A and Table 1); thus, neither the presence of a P-element in the genome nor expression of the *lacZ* reporter in the mushroom bodies produces behavioral deficits. This lack of effect has been observed independently by Wu et al. (submitted). Furthermore, two precise excisions of leo^{P1375} (leo^{R1} and leo^{R2}) showed normal performance.

The performance of leo^{P1375} and leo^{P1188} heterozygotes, though reduced, was not significantly different from controls (Figure 5A and Table 1). Thus, no dominant effect upon 3 min memory was detected. However, lesions in the gene that reduced LEO in the mushroom

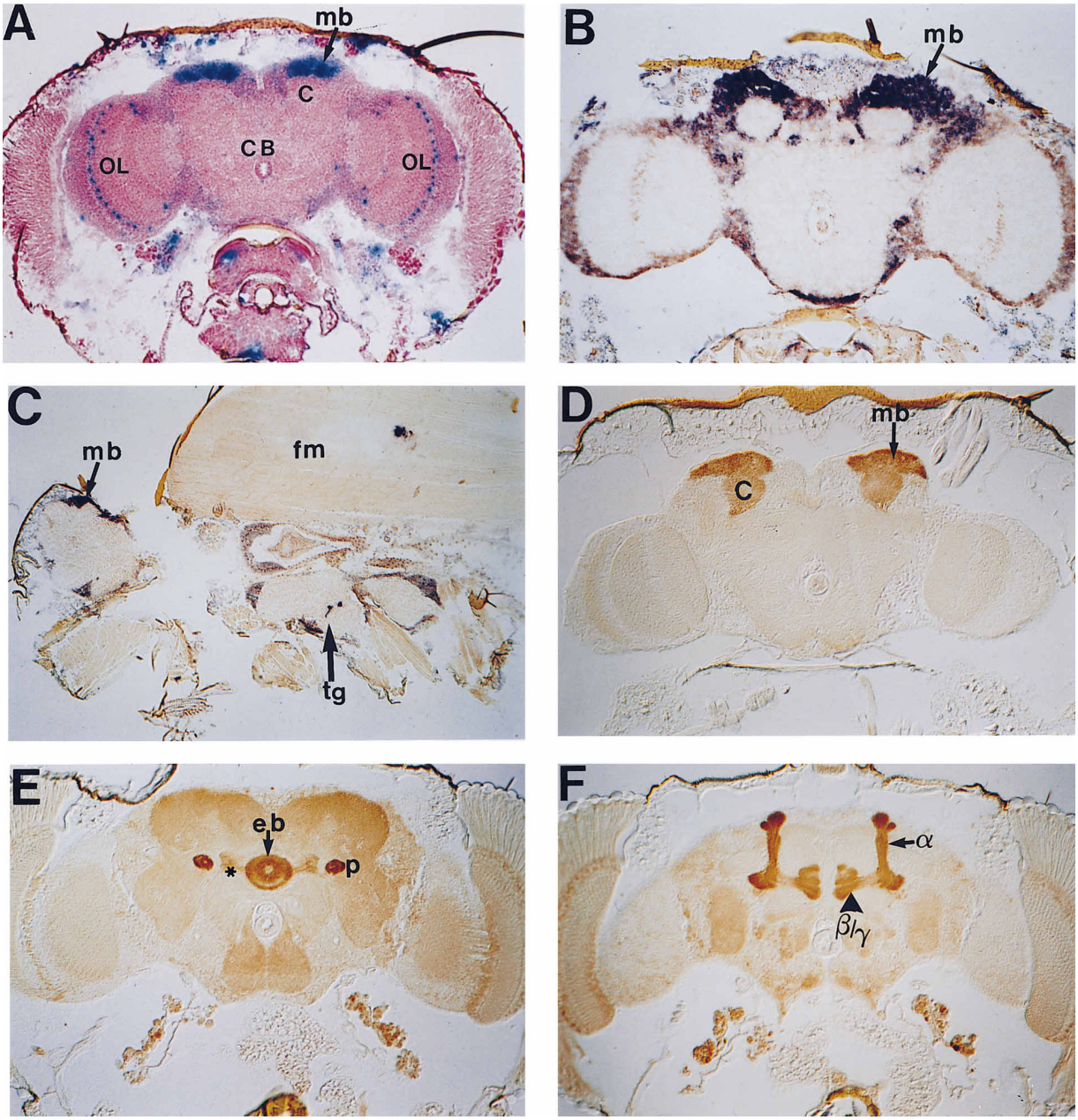


Figure 3. *leonardo* Expression in Adult Heads

Dorsal is up in all photographs.

(A)–(C) Frontal section through the posterior brain of a *leo*^{P1375} adult stained for β-gal activity and showing elevated expression in mushroom body perikarya (arrow). C, calyx; CB, central brain; OL, optic lobes. RNA in situ hybridization to a frontal head section (B) and a sagittal section ([C], anterior to the left) of adult ry^{506} flies with an antisense *leonardo* probe. *leonardo* RNA is preferentially expressed in mushroom body perikarya. Both sense and antisense probes were generated and used in parallel. mb, mushroom body perikarya; tg, thoracic ganglia; fm, flight muscle.

(D)–(F) Immunohistochemical detection of LEONARDO protein in sections of the posterior (D), middle (E), and anterior (F) adult brain. p, peduncle; eb, ellipsoid body; small arrow, α-lobe; arrowhead, β,γ-lobes; asterisk, axons of ring neurons.

bodies resulted in highly significant impairments that were proportional to this reduction. Alleles classed as modest by immunohistochemical criteria exhibited a 20%–25% decrement in 3 min memory, whereas alleles nearly devoid of LEO in the mushroom bodies exhibited a decrement of 30%–35% relative to the controls (Figure 5A). Similar behavioral results were obtained with three other high- and six medium-staining alleles, and one low-staining allele (data not shown). Thus, reductions in LEO staining in the mushroom bodies are paralleled by similar decrements in 3 min memory.

We assessed longer-term memory of the conditioned association for a selected set of mutants at 45, 90, and 240 min after training (Figure 5B). The performance of

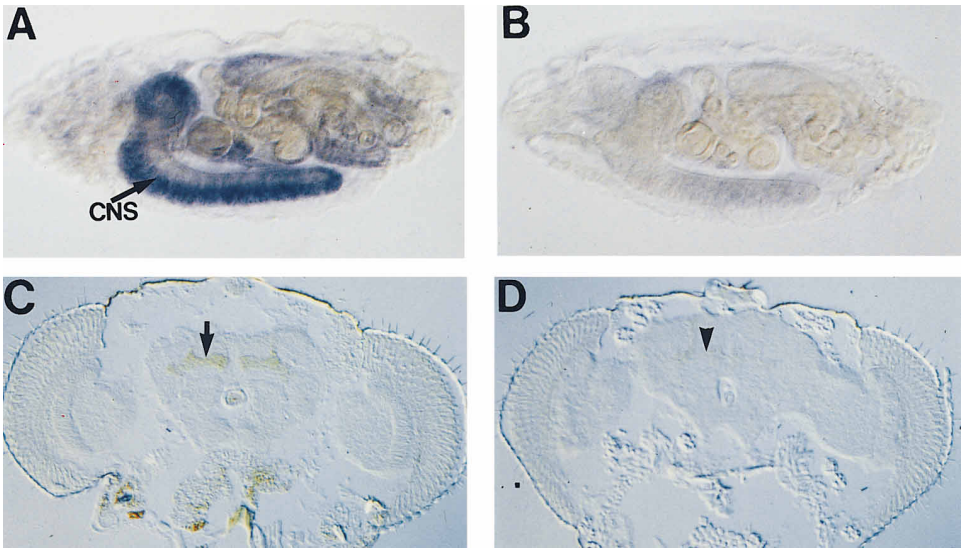


Figure 4. *leonardo* Expression in Mutants

(A) and (B) RNA in situ hybridization with an antisense probe to 16–18 hr heterozygous (A) and homozygous leo^{P1375} (B) embryos. Dorsal is toward the top; anterior to the left. The gene is abundantly expressed in the embryonic CNS (arrow), posterior gut, and epidermis (not apparent in this focal plane), and this expression is greatly reduced in leo^{P1375} homozygous embryos. (C) and (D) Immunohistochemical detection of LEONARDO in frontal head sections of ry^{506} and $leo^{2.3}$ homozygotes. The two animals shown were mounted side-by-side in the same fly collar (see Experimental Procedures). The amount of immunoreactivity in $leo^{2.3}$ homozygotes is significantly reduced (arrowhead) compared with the control (arrow).

the leo^{R1} precise excision derivative was identical to ry^{506}. In contrast, the $leo^{2.3}$ animals exhibited a 30% reduction in 3 min memory and retained this highly significant difference over time through 4 hr. Animals carrying the $leo^{P1.3H}$ insertion exhibited significant differences for initial memory and at 45 min. Though lower, their performance at 90 and 240 min was not significantly different from that of controls. This is probably a reflection of the weakness of this mutation. Similar results were obtained with two additional alleles of the medium- and one of the low-staining groups (data not shown). In addition, memory decay for leo^{P1375} heterozygotes and MB2487 and P267 controls was not appreciably different from that of ry^{506} animals at all time intervals tested (data not shown). Collectively, the results of the behavioral experiments demonstrate that reduction in the *leonardo* protein in the mushroom bodies produces a marked decrease in associative memory.

To confirm that the behavioral deficit is caused by lesions in *leonardo*, complementation tests were performed. Two severe alleles, leo^{X1} and $leo^{2.3}$, were tested in *trans* with the leo^{P1375} allele (Figure 5C). Animals heteroallelic for leo^{P1375} and either leo^{X1} or $leo^{2.3}$ showed a highly significant reduction in 3 min memory. Thus, the lethal leo^{P1375} insertion is allelic to viable *leonardo* alleles for the learning deficit. An independent confirmation that the behavioral deficit mapped to the *leonardo* locus was obtained by backcrossing $leo^{P1.3H}$ to ry^{506} for seven generations. The backcrossed line exhibited a learning deficit similar to the parental animals (data not shown).

The *leonardo* mutants exhibit normal avoidance of the aversive odors used for training. Odor avoidance of all lines was evaluated at two odorant concentrations given a choice of odor or air. No significant difference in the avoidance of octanol (OCT) at either concentration (Figure 6A) or of benzaldehyde (BNZ) at the concentration used for training (Figure 6B) was uncovered between any of the mutants and the controls. The alleles $leo^{9.8}$ and $leo^{2.3}$ exhibited greater BNZ avoidance than controls at the lower concentration. Increased avoidance at one concentration cannot account for the behavioral defects exhibited by these two or the remaining several alleles, since the mutant strains exhibited enhanced avoidance over ry^{506} animals, yet the latter exhibited far better learning than any of the former. Similarly, the avoidance of electrified grids given a choice with a nonelectrified one was quantified. The avoidance of grids kept at two different voltages (Figure 6C) was not different between mutant and control strains. Taken together, the results clearly demonstrate that differential perception of the conditioned or unconditioned stimuli cannot account for the observed behavioral deficits of the mutant animals.

The reduction in LEONARDO does not precipitate developmental abnormalities in the brain anatomy of the mutants. Neuroanatomical differences were not observed between ry^{506} and mutant strains in the immunological screen. Furthermore, histological examination of the gross anatomy of the most extreme behavioral mutants revealed the presence and apparent integrity of all brain structures (Figure 7). For example, specific immunological markers (anti-D-MEF2 and anti-FASII antibodies) were used to examine the structure of the mushroom bodies at the level of the cell bodies and axonal projections (lobes). These analyses demonstrated that in the mutants, the mushroom bodies are organized like those of control animals (Figure 7). Furthermore, since both D-MEF2 and FASII are expressed at the same level as controls, *leonardo* mutations do not appear to affect overall gene expression in the mushroom bodies. Therefore, decreased levels of LEO do not result in abnormalities of the brain that could account for the observed behavioral deficits.

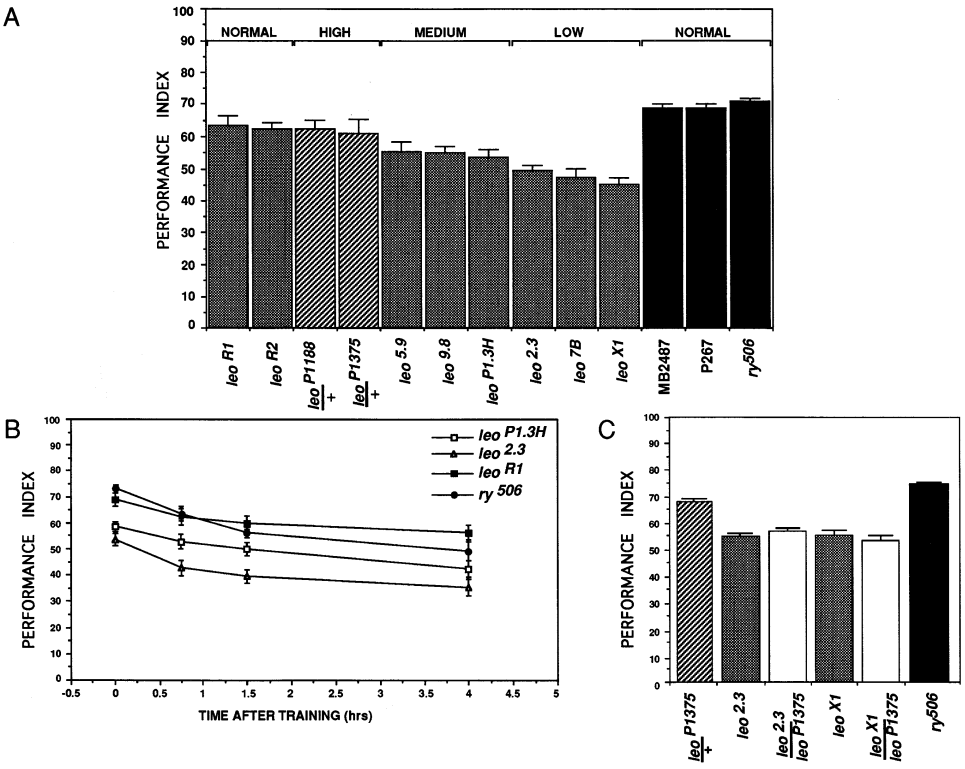


Figure 5. Olfactory Learning/Memory of Viable *leonardo* Alleles

(A) Performance after conditioned odor avoidance. The mean PI ± SEM is shown for each stock. One way ANOVA of the data indicated significant effects of genotype ($F_{[12,123]} = 14.736$, $P < 0.001$). Subsequent planned comparisons among the genotypes were performed to identify differences (see Table 1 in Experimental Procedures). The performance index of control strains is represented by black bars, lethal allele heterozygotes by hatched bars, and the viable alleles by gray bars. n > 9 for all stocks. The brackets above the bars labeled high, medium, low, or normal indicate the relative amount of LEONARDO immunoreactivity in these genotypes (see Figures 4C and 4D for an example).

(B) Decay curve of conditioned odor avoidance for selected *leonardo* alleles. The mean PI ± SEM for the precise excision (leo^{R1}), a medium and low allele, and the parental ry^{506} control strain are shown. A two-way ANOVA indicated significant effects of genotype ($F_{[4,196]} = 38.394$, $P < 0.001$) and retention time ($F_{[3,196]} = 28.342$, $P < 0.001$). Subsequent planned comparisons among the genotypes at each time point were performed to identify differences (see Table 1 in Experimental Procedures). Time 0 indicates 3 min memory. n > 9 for all points.

(C) Noncomplementation of the 3 min memory deficit of *leonardo* alleles. The control strain is represented by black bars, lethal allele heterozygotes by hatched bars, viable alleles by gray bars, and the heteroallelics with open bars. The PI ± SEM for an n 9 is shown. One-way ANOVA revealed significant differences among the genotypes ($F_{[5, 53]} = 22.107$, $P < 0.001$). Both $leo^{2.3}$ and leo^{X1} fail to complement with leo^{P1375}, thus mapping the behavioral deficits to the *leonardo* gene.

These behavioral and anatomical analyses demonstrate that in Drosophila, mutations that reduce the amount of the *leonardo* gene product in the mushroom bodies of adult animals disrupt olfactory learning and memory. The degree of reduction in learning and memory correlates well with the degree of reduction in LEO. Therefore, LEONARDO, the Drosophila 14-3-3ζ isoform, is required in neuroplasticity and behavior.

Discussion

The products of the learning and memory genes *dunce*, *rutabaga,* and *DC0*, i.e., the enzymes cAMP phosphodiesterase, adenylyl cyclase, and protein kinase A, respectively, and their expression pattern underscore the importance of the cAMP signal transduction cascade in mushroom body–mediated olfactory learning and memory. However, it is unlikely that the cAMP cascade operates in isolation in the mushroom bodies. Additional pathways and processes must be required for olfactory learning and memory, as suggested by the variety of additional molecules relevant to these processes that do not appear to participate in the cAMP cascade (reviewed by Davis, 1996). We investigated the role of 14-3-3 proteins in learning and memory because *leonardo*, the gene encoding a Drosophila 14-3-3ζ homolog, is preferentially expressed in mushroom bodies. Disruption of *leonardo* expression in these brain structures results in significant deficits in olfactory memory even within 3 min after training. Since 14-3-3 proteins are not known to participate in the cAMP cascade, the results suggest that the LEONARDO protein is a member of an additional signaling cascade that mediates learning and memory.

leonardo Encodes a 14-3-3ζ Homolog

A common feature of all 14-3-3 proteins that have been isolated to date is the abundance of acidic residues concentrated in highly conserved domains. The seven typical 14-3-3 isoforms from vertebrates are similar in size and share 75%–92% sequence identity. The major differences include variable amino termini and individual residues that are distributed outside of the conserved acidic domains. The atypical isoforms are divergent from

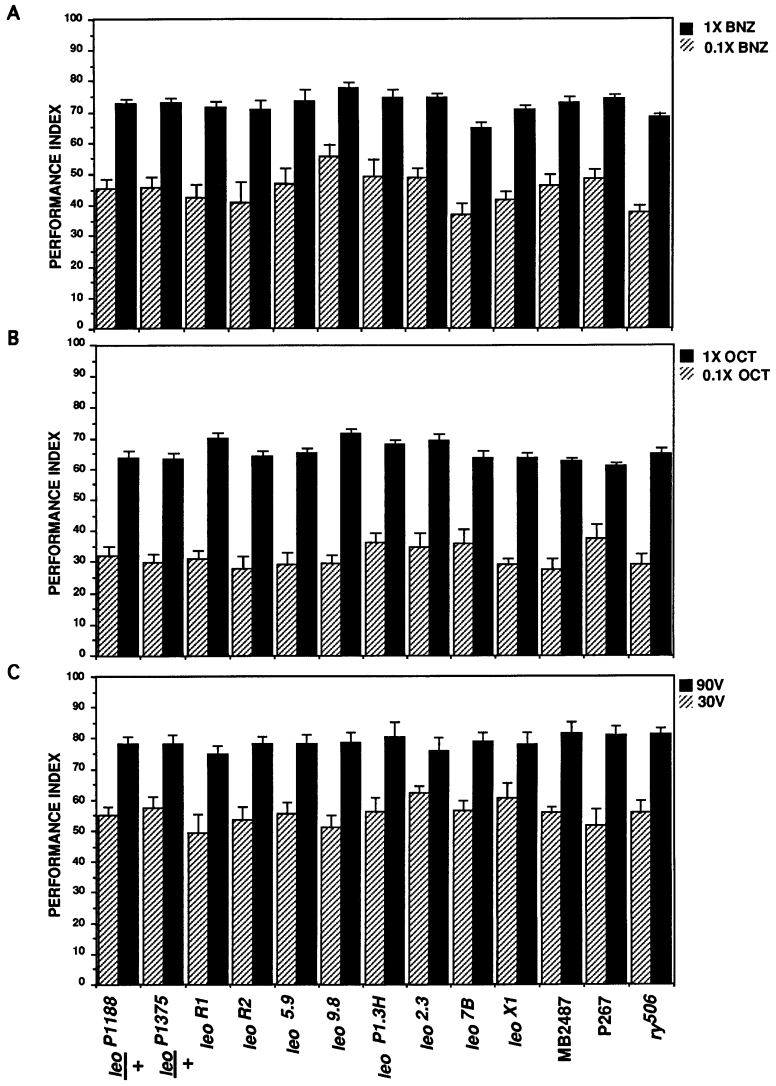


Figure 6. Odor and Shock Avoidance Behavior

(A) Benzaldehyde avoidance (BNZ). The mean PI ± SEM of animals avoiding BNZ at the amount used for conditioning (solid bars) and 10-fold less (hatched bars) is shown. n > 10 for all stocks. One-way ANOVA did not reveal any effects of genotype ($F_{[12,143]} = 1.810$, $P = 0.0517$) for avoidance of 1× BNZ but did for 0.1× BNZ ($F_{[12,131]} = 3.205$, $P = 0.0005$). Subsequent planned comparisons revealed increased avoidance of *leo*$^{9.8}$ and *leo*$^{2.3}$ relative to controls.

(B) Octanol avoidance (OCT). The mean PI ± SEM of animals avoiding OCT at the amount used for conditioning (solid bars) and 10-fold less (hatched bars) is shown. n > 10 for all stocks at the 1× OCT and n > 7 at 0.1× OCT. One-way ANOVA for avoidance of 1× OCT or 0.1× OCT did not reveal any effects of genotype, ($F_{[12,175]} = 2.632$, $P = 0.0029$) and ($F_{[12,106]} = 2.450$, $P = 0.0074$), respectively.

(C) Shock avoidance. The mean PI ± SEM avoidance of an electrified grid at 90 V normally used for training (solid bars) and 30 V (hatched bars) is shown. One-way ANOVA did not reveal any effects of genotype at either level of stimulus strength ($F_{[12, 84]} = 1.390$, $P = 0.1666$) for 90 V, and ($F_{[12, 72]} = 2.704$, $P = 0.0025$) for 30 V, respectively. n > 7 for all stocks.

the rest except for stretches within the conserved acidic domain (Aitken et al., 1992; Martin et al., 1993; Aitken, 1995). *leonardo* encodes the Drosophila homolog of the mammalian ζ isoform, which belongs to the typical group of 14-3-3 proteins (Toker et al., 1990). Although polyclonal antibodies raised against mammalian purified or recombinant proteins react with all 14-3-3 proteins (Patel et al., 1994; Rosenboom et al., 1994), the anti-LEO anti-serum surprisingly reacts with only one protein in Drosophila. This suggests the absence of additional typical group proteins.

The crystal structure of the mammalian ζ isoform (Liu et al., 1995) predicts a dimer, each subunit consisting of nine antiparallel helixes that form a large negatively charged channel. The residues in the acidic channel are highly conserved among the species examined and LEO is 92% identical with the vertebrate ζ in this domain. In addition to homodimers, the vertebrate ζ protein can form heterodimers with atypical and possibly other typical isoforms (Jones et al., 1995). The high degree of identity between the Drosophila and the vertebrate ζ isoform suggests a similar tertiary structure and predicts that LEO could form dimers. The apparent lack of additional typical 14-3-3 proteins in Drosophila predicts that LEO must form homodimers or heterodimers with any coexpressed atypical isoforms.

The carboxy terminus of the vertebrate ζ isoform is phosphorylated in vivo on Ser 185, in the motif Ser-Pro-Glu-Lys. This sequence is unique to this isoform and required for phosphorylation. In the brain, the ratio of phosphorylated forms to dephosphorylated forms approaches 1:1 (Aitken et al., 1995). The corresponding LEO sequence bears the conservative change Ser-Pro-Asp-Lys. Detection of one species in two-dimensional Western analysis suggests that either the protein is phosphorylated with high efficiency, or is not modified at all.

Striking Expression of *leonardo* in the Mushroom Bodies

The *leonardo* RNA is abundant in the perikarya of the mushroom bodies. All three classes of *leonardo* transcripts are abundantly expressed in adult heads. Because the probes used in these experiments encompass the conserved open reading frame and hybridize to all transcripts, it is not possible to determine whether the largest of the RNAs, which is head specific, accumulates

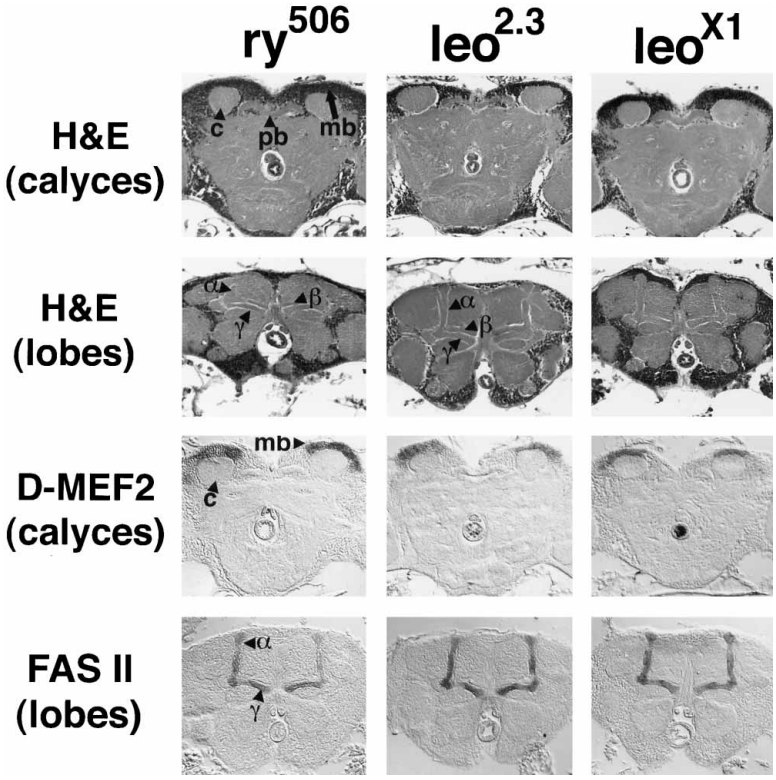


Figure 7. Neuroanatomy of *leonardo* Mutants

Hematoxylin and eosin stained sections of ry^{506}, leo^{X1}, and $leo^{2.3}$ at the level of the calyces and lobes. Sections of the posterior head were challenged with the D-MEF2 antibody to visualize mushroom body perikarya (mb) and calyces (c). The structure of the mushroom body axons in the anterior of the head was examined with the anti-FAS II antibody. No gross anatomical or mushroom body–specific differences between controls and mutant animals were revealed. pb, protocerebral bridge; α, β, γ: respective mushroom body lobes.

in the mushroom bodies differentially. The LEO protein is found throughout the mushroom body neurons, where it appears enriched in the lobes. Furthermore, LEO is found in the perikarya, but is apparently excluded from the nucleus. This distribution differs from that for the gene products of *dunce*, *rutabaga*, and *DC0*, which are found exclusively in the neuropil of the mushroom bodies. An additional difference is the presence of LEO in the neuropil and perikarya of the ellipsoid body. The presence of LEO in thoracic ganglia and ovaries suggests that these two tissues probably account for most of the protein detected in bodies by Western blots.

The neuronal expression of *leonardo* is in accord with its vertebrate homologs that are found predominately in the brain, especially in the cerebellum, hippocampus, olfactory bulb, and motor neurons in the brainstem and spinal cord (Boston et al., 1982a; Watanabe et al., 1991, 1993; Patel et al., 1994; Aitken, 1995). In fact, the ζ isoform is the most abundant 14-3-3 protein in avian brains (Patel et al., 1994). Expression of 14-3-3 proteins in neurons suggests that they are integral components of the physiology and function of these cells. The preferential expression of LEO in centers for insect olfactory learning and memory and the behavioral phenotype upon its disruption indicate that at least some of the vertebrate isoforms may similarly participate in information processing and storage.

Mutations in *leonardo* Affect Mushroom Body Expression

Mutations in *leonardo* appear to define two broad categories. Lethal mutations disrupt all expression of the gene as exemplified by the two P-element insertions, leo^{P1188} and leo^{P1375}. The viable excisions disrupt preferential expression of *leonardo* in the mushroom bodies, but do not seem to result in observable viability decrements. These alleles complement leo^{P1188} and leo^{P1375} for lethality, yet leo^{P1375} fails to complement the viable alleles for the behavioral deficit. One interpretation for this is that the first intron of the gene contains elements essential for expression in the mushroom bodies. The $leo^{P1.3H}$ allele carries a P-element in the second intron of the gene, away from these putative regulatory elements. Interestingly, in addition to a mild behavioral deficit and decrement in mushroom body expression, $leo^{P1.3H}$ animals complement the leo^{P1375} lethality poorly, suggestive of a mild, albeit general, effect on gene expression. Moreover, the alleles $leo^{2.3}$ or leo^{X1} in combination with leo^{P1375} exhibit LEO immunoreactivity in the mushroom bodies comparable with that of the $leo^{2.3}$ or leo^{X1} animals. In addition, the behavioral phenotype of these heteroallelics is no more severe than that of either $leo^{2.3}$ or leo^{X1}. These results suggest that the leo^{P1375} insertion disrupts mushroom body expression at least as much as the $leo^{2.3}$ and leo^{X1} excisions. Thus, we speculate that the first intron of *leonardo* contains elements required for specific expression in the mushroom bodies.

Deficits in Olfactory Learning/Memory of *leonardo* Mutants

Mutations that compromise *leonardo* expression in the mushroom bodies result in deficits in associative olfactory learning and memory proportional to this reduction. Though sensitive to the level of LEO in the mushroom bodies, a near 50% reduction in leo^{P1375} heterozygotes is not enough to compromise 3 min memory. This suggests

that the amount of LEO in the mushroom bodies of mild behavioral alleles is less than 50% of the controls. Though highly reduced, mushroom body expression is not completely abolished in the most severe behavioral alleles that we have characterized. The remaining LEO protein may represent a basal level of expression essential for viability that is controlled by elements different from those responsible for mushroom body–specific expression. It appears unlikely, then, that any of the mutant lines tested are completely null for LEO expression in the mushroom bodies. Thus, the 30% reduction in performance may be a conservative estimate of the contribution of *leonardo* to processes essential to normal olfactory learning and memory. Since mutations that nearly abolish cAMP signaling in the mushroom bodies result in 60% reduction in olfactory learning (Han et al., 1992; Skoulakis et al., 1993b), it is tempting to speculate that LEO participates in processes that contribute part of the remaining behavioral readout. Collectively, the results of this study establish a critical role in neuroplasticity for a member of the 14-3-3 protein family almost 30 years after its discovery (Moore and Perez, 1968).

The role of LEO in mushroom body physiology, although unknown, may prove highly revealing for learning mechanisms because of known biochemical functions assigned to 14-3-3 proteins. The 14-3-3 isoforms, especially ζ, interact with RAF-1 in the mitogen-activated protein kinase (MAPK) signal transduction cascade (Fantl et al., 1994; Freed et al., 1994; Fu et al., 1994; Irie et al., 1994; Aitken, 1995). This interaction appears to occur via the phosphorylated second serine in the motif Arg-Ser-X-Ser-X-Pro present in RAF-1 (Muslin et al., 1996). In addition to RAF-1, a small number of other interesting proteins contain one or more of these motifs and are known to bind 14-3-3. These include PKC, CDC25, and Polyoma virus middle T. Other proteins are predicted to bind 14-3-3 because they possess this motif, such as KSR-1 (Muslin et al., 1996). These observations raise the intriguing possibility that LEO may participate in RAS/RAF/MAPK signal transduction pathway(s) operant in mushroom body–mediated learning and memory processes. An equally interesting possibility is the participation of LEO in PKC-mediated processes, because their disruption is known to result in learning and memory deficits (Choi et al., 1991). In either case, the identification of *leonardo* as a Drosophila 14-3-3 homolog essential for olfactory learning provides a new avenue for investigating novel signaling systems that serve learning and memory.

Experimental Procedures

Drosophila Culture

Fly stocks were raised on standard cornmeal food at 22°C–25°C and 40%–60% relative humidity. The *leo*^{P1188}*/CyO; ry*^{506} and *leo*^{P1375}*/CyO; ry*^{506} were isolated in the enhancer detector screen for genes expressed preferentially in the mushroom bodies (Han et al., 1996). Both strains harbor noncomplementary homozygous lethal insertions. The genetic background of all strains used was normalized to the Cantonized isogenic *ry*^{506} background used in the enhancer detector screen, by backcrossing females of a given stock to isogenic *ry*^{506} males for seven generations. An analysis of the lethal phenotype of *leonardo*, which occurs in late embryogenesis, will be presented elsewhere (Skoulakis and Davis, unpublished data).

Viable *leonardo* alleles were generated by mobilization of the P-element in *leo*^{P1375}. X^{CS}; *leo*^{P1375}*/CyO; ry*^{506} males were crossed to X^{CS}; *Sp/CyO*; *Sb*, P[*ry*+ Δ*2*-3](99B)/TM2, *Ubx* females en masse (X^{CS} denotes a Canton-S-derived X chromosome). From the resulting progeny, individual X^{CS}; *leo*^{P1375}*/CyO*; *Sb*, P[*ry*+ Δ2-3](99B)/*ry*^{506} males were selected and crossed to X^{CS}; *SSpBl/CyO; ry*^{506} females. Two sets of strains were established from the progeny of these crosses. First, individual *ry*$^{-}$ males were selected as putative excision-bearing animals and backcrossed to X^{CS}; *SSpBl/CyO; ry*^{506} virgin females. In addition, individual *ry*$^{+}$ males were selected from the progeny of crosses that yielded putative excisions and crossed to X^{CS}; *SSpBl/CyO; ry*^{506} females to obtain stocks harboring putative reinsertions of the P-element elsewhere in the gene ("local hops") that allow for viability. All stocks obtained were tested with the *leo*^{P1188} and *leo*^{P1375} alleles for complementation of lethality. Putative viable local hop stocks were further screened for similarity in the β-galactosidase staining pattern with *leo*^{P1375} to ascertain reinsertion in the locus.

The 267P[*ry*$^{+}$], *ry*^{506} strain (referred to as P267) utilized as a control in the behavioral experiments was isolated in the mushroom body screen. The enhancer detector P-element is inserted on chromosome 3 and there is no β-gal expression in the mushroom bodies. This strain is homozygous viable, does not exhibit any visible phenotype, and appears normal behaviorally.

The MB2487 P[*ry*$^{+}$]; *ry*^{506} strain (referred to as MB2487) utilized as a control in the behavioral experiments was isolated in the mushroom body screen. The enhancer detector P-element is inserted on chromosome 2 and the *lacZ* gene is expressed in the mushroom bodies. This strain is homozygous viable and appears normal behaviorally.

Antibody Preparation

To prepare antibodies against LEO, a DNA fragment containing the protein coding (starting at amino acid 2) and 3′ untranslated sequence was subcloned from one of the cDNA clones (c15), into the pGEX-KT1 expression vector (Pharmacia). Clone c15 is similar to clone c2 shown in Figure 1, except that it lacks the 5′ most untranslated exon. The glutathione-S-transferase–LEO fusion vector (GST–LEO) was induced into Escherichia coli and purified from lysates as recommended by the supplier and the modifications of Frangioni and Neel (1993). Affinity-purified GST–LEO fusion protein was used to immunize two New Zealand White female rabbits using standard methods. Sera obtained from both rabbits after the second boost contained high titers of anti-LEO, whereas preimmune sera were devoid of such antibodies judged by Western blotting. The crude anti-sera was used in all immunohistological procedures and Western blots, since affinity purification of the antibodies did not affect the results. Furthermore, the anti-sera appeared devoid of anti-GST antibodies.

Histology

As described in Han et al. (1992), 10 μm sections of fly heads were obtained on glass slides and stained for *lacZ* expression.

For RNA in situ hybridization, digoxigenin-labeled RNA probes were generated by in vitro transcription of the SalI–HindIII fragment from cDNA clone c15 subloned into the pBluescript (Stratagene) vector as recommended by the digoxigenin-UTP supplier (Boehringer Mannheim). After two ethanol precipitations, the final RNA products were resuspended in 200 μl 5× SSC/50% formamide at 4°C for 10–14 hr and then frozen at −20°C until use. The probes were further diluted in hybridization buffer (5× SSC, 50% formamide, 5% dextran sulfate, 1× Denhardt's, 20% SDS, and 650 μg/ml sonicated, phenol-extracted salmon sperm DNA) 5- to 15-fold, prior to use. After hybridization and following a few brief washes in 2× SSC/50% formamide, the sections were treated with RNAse A for 30 min at room temperature, followed by washes in 2× and 0.1× SSC. All subsequent steps were as described in the Genious kit (Boehringer Mannheim). Antisense and sense probes were generated from the same template simultaneously and used under identical conditions in parallel. Sections probed with sense probes were devoid of signal in all experiments.

Whole-mount RNA in situ hybridization to embryos was performed using random primed digoxigenin-labeled DNA probes derived from

the SalI–HindII fragment of c15 as described previously (Skoulakis et al., 1993a).

Immunohistochemistry on adult heads was performed on frozen and paraffin sections. We obtained 12 μm frozen sections from animals fixed in 4% paraforlmadehyde/0.4 M lysine in 0.01 M phosphate buffer (pH 7.2) with 0.120 M NaCl (PBS) for 3–4 hr at 4°C, washed in PBS, and equilibrated for 10–14 hr at 4°C in PBS containing 25% sucrose. The sections were blocked with 5% serum in 0.1 M phosphate buffer (pH 7.4) containing 1 M NaCl and incubated with a 1:3000 dilution of the anti-LEO anti-serum for 10–15 hr at room temperature. All subsequent steps were as described by the manufacturer of the ABC kit (Vector Laboratories). For the immunohistochemical screen of viable mutants, four control ry^{506} flies and four excision derivatives were mounted side-by-side in a fly collar (Han et al., 1996) and sectioned. The primary antibody was used at a 1:5000 dilution and the effect of each allele was examined in a minimum of four independent experiments.

For paraffin sections, the animals were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid) for 4 hr at room temperature, treated with methylbenzoate for 12 hr, and embedded in paraffin. We obtained 5 μm sections, deparaffinized them in xylene baths, rehydrated them through 100%–30% ethanol series, and then blocked and challenged them with the anti-LEO antibody as described above. Hematoxylin/Eosin staining was performed using standard methods. The D-MEF2 antibody (Lilly et al., 1995) was used at a 1:1000 dilution, whereas the FASII antibody (Grenningloh et al., 1991) was used at 1:25 in the standard buffers described above.

Molecular Biology

Genomic fragments adjacent to the P-element insertions in leo^{P1188}/*CyO*, leo^{P1375}/*CyO* were isolated from HindIII-digested genomic DNA by plasmid rescue (Bellen et al., 1989; Wilson et al., 1989). All nucleic acid manipulations were performed using standard methods. Poly (A)+ RNA isolation and Northern blots were as described by Davis and Davidson (Davis and Davidson, 1984). For one-dimensional Western blotting, one female fly was homogenized in 50 μl Laemli buffer (0.125 M Tris (pH 6.8), 3% SDS, 5% β-mercaptoethanol, 15% glycerol). The homogenates were boiled for 5 min, spun at 16,000 × g for 5 min, and 4 μl were loaded per lane. Under these conditions, equal amounts of protein were loaded per lane. The gels were blotted onto PVDF membrane (Millipore) and the results visualized with ECL (Amersham). For two-dimensional electrophoresis, 12 fly heads or 2 bodies were homogenized in 200 μl 9M urea, 4% NP-40, 2% β-mercaptoethanol, and 2% ampholytes (3.5–10 pI range). The samples were solubilized at room temperature for 2 hr, spun at 200,000 × g for 2 hr, and equal amounts of protein for both heads and bodies were loaded on the IEF gel. The gels were run and blotted according to Dunbar (1987). The results were visualized with ^{125}I-protein A.

Behavioral Assays

Behavioral tests were performed under dim red light at 23°C–25°C and 65%–70% humidity. All animals were 2–6 days old, collected under light CO_2 anesthesia one day prior to testing, and kept in food vials in groups of 120–160 at 21°C–23°C. They were transferred to fresh vials 1–1.5 hr before testing. The olfactory classical conditioning paradigm (Tully and Quinn, 1985), which couples aversive olfactory cues (conditioned stimulus) with electric shock (unconditioned stimulus) was used to assess learning and memory. The aversive odors used were benzaldehyde (BNZ) and 3-octanol (OCT). For training, ~150 flies were placed into a tube lined with an electrifiable grid and presented with air (650 ml/min) for 30 s, the shock-associated odor carried in the air current for 1 min concomitant with 11 1.25 second shocks at 90 V delivered every 5 s. This was followed by delivery of air for 40 s, the control odor in the air current for 1 min, and air again for 40 s. Two groups of animals of the same genotype were trained simultaneously, one to avoid BNZ, the other OCT, while the complementary odorant was used as the respective control. The animals were transferred to a T-maze apparatus immediately (90–100 s to complete the transfer) and allowed to choose between the two odors converging in the middle for 2 min. Since the time between testing and the coupling of the conditioned with the unconditioned stimulus is 3 min, the initial tests are of 3 min memory. To measure longer-term memory, the animals were retained in food vials in the dark after training, transferred to the T-maze, and tested at the indicated times. Performance was measured by calculating an index (PI), which ranges from 100 to 0, for perfect to no learning/memory, respectively. The fraction of flies that avoided the shock-associated odor minus the fraction that avoided the control odor reflected learning due to one of the conditioning stimuli and represented half of the performance index. One performance index was calculated as the average of the half-learning indexes for each of the two groups of animals trained to complementary conditioning stimuli. Since the performance of the three control strains was indistinguishable (see also Wu et al., submitted), all subsequent comparisons were to ry^{506}, which constitutes the most appropriate control, being the source of all chromosomes in the mutants with the exception of $leo^{P1.3H}$, which is phenotypically ry^{+}.

To assess olfactory avoidance, naive animals were given 2 min to choose between one of the odors and air. The air flow in both arms of the maze was kept constant and equal to that used for testing conditioned animals. Avoidance to both odors was tested simultaneously for each strain and all strains used were tested in a given session. Avoidance is represented by a performance index, which is calculated as the fraction of flies that avoid the odorant minus the fraction of flies that do not.

To assess the avoidance of animals to electric shock, the arms of the T-maze were lined with electrifiable grids. Naive flies were placed at the choice point and given 2 min to choose between an electrified and an inert grid. Through the choice period, 1.25 s shocks at various voltages were used and air was passed through the arms at the standard flow rate. Avoidance is measured by a performance index calculated as the fraction of flies that avoid the electrified grid minus the fraction of flies that do not. Again, all strains were tested in a given session.

Statistics

All statistical analyses were performed on untransformed data with the JMP3.1 statistical software package (SAS Institute Inc., Cary, NC). Following the initial ANOVA, planned comparisons (contrast analysis) were performed and the significance level was adjusted for the experimentwise error rate (Sokal and Rohlf, 1981).

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Table 1. Results of Planned Comparisons

	Genotype	Time	Mean ± SEM	t-Ratio	P
Initial Learning					
	ry	0	70.8 ± 1.2		
	MB2487	0	68.8 ± 1.4	0.82	0.413
	P267	0	68.9 ± 1.2	1.00	0.318
	leo^{P1188}/+	0	62.3 ± 3.1	3.00	0.003
	leo^{P1375}/+	0	61.2 ± 4.3	2.98	0.003
	leo^{R1}	0	63.6 ± 3.0	2.43	0.017
	leo^{R2}	0	62.3 ± 2.3	2.60	0.011
	$leo^{5.9}$	0	55.6 ± 3.1	3.99	<0.001#
	$leo^{9.8}$	0	52.1 ± 2.2	6.46	<0.001#
	$leo^{P1.3H}$	0	51.6 ± 2.3	6.95	<0.001#
	$leo^{2.3}$	0	53.6 ± 1.8	5.95	<0.001#
	leo^{7B}	0	47.2 ± 3.1	7.00	<0.001#
	leo^{X1}	0	45.4 ± 2.1	9.41	<0.001#
Retention					
	ry	0	71.3 ± 1.8		
	leo^{R1}	0	68.9 ± 2.6	0.64	0.527
	$leo^{P1.3H}$	0	58.6 ± 1.8	3.39	<0.001#
	$leo^{2.3}$	0	53.5 ± 1.8	4.54	<0.001#
	ry	45	63.4 ± 2.8		
	leo^{R1}	45	64.9 ± 3.8	−0.32	0.751
	$leo^{P1.3H}$	45	49.8 ± 3.9	3.10	<0.001#
	$leo^{2.3}$	45	42.8 ± 3.0	4.71	<0.001#
	ry	90	56.5 ± 2.1		
	leo^{R1}	90	64.0 ± 2.5	−1.56	0.527
	$leo^{P1.3H}$	90	52.0 ± 2.9	1.68	0.095
	$leo^{2.3}$	90	36.9 ± 4.4	4.54	<0.001#
	ry	240	49.2 ± 4.6		
	leo^{R1}	240	56.2 ± 2.8	−0.72	0.475
	$leo^{P1.3H}$	240	42.5 ± 3.3	1.54	0.012
	$leo^{2.3}$	240	34.4 ± 3.2	3.41	<0.001#
Complementation for the Behavioral Deficit					
	ry	0	74.1 ± 1.2		
	leo^{P1375}/+	0	67.5 ± 1.6	2.82	0.113
	$leo^{2.3}$	0	54.2 ± 1.9	8.40	<0.001#
	$leo^{2.3}/leo^{P1375}$	0	56.3 ± 1.7	8.08	<0.001#
	leo^{X1}	0	57.0 ± 2.3	6.71	<0.001#
	leo^{X1}/leo^{P1375}	0	52.7 ± 2.5	6.94	<0.001#

The scores of all genotypes were compared with those of ry^{506} homozygotes. Significant differences are denoted by the pound sign. The level of significance was adjusted for the experimentwise error rate, with $P \leq 0.001$ indicating that the mean is different from that of the control. Initial Learning (Figure 5A); Retention (Figure 5B); and Complementation for the Behavioral Deficit (Figure 5C).

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