Localization of Long-Term Memory Within the Drosophila Mushroom Body

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N-methylglucamine, 20 mM TEA-Cl, 0.2 mM EGTA, 2 mM $MgCl_2$, 20 mM Na_2-creatine phosphate, 5 mM Mg-ATP, and 0.1 mM guanosine triphosphate, and 10 Hepes (pH 7.35). The bathing solution was 105 mM NaCl, 20 mM $CaCl_2$, 1.5 mM $MgSO_4$, 15 mM glucose, 0.003 mM tetrodotoxin, and 5 mM Hepes (pH 7.35).
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26. Cells were permeabilized with 0.3% Triton X100, fixed in paraformaldehyde, blocked in 10% fetal bovine serum (FBS) in phosphate-buffered saline (PBS) for 1 hour, and incubated with primary antibodies diluted in 10% FBS for 1 hour. CFP-tagged protein was detected with rabbit polyclonal antibody to GFP (Panvera). Chromogranin B was detected with a mouse monoclonal antibody (generously provided by W. Huttner). Cells were washed and stained with secondary antibodies (fluorescein-conjugated goat antibody to rabbit for CFP and Texas red–conjugated goat antibody to mouse for chromogranin) for 1 hour. After four washes in PBS, cover slips were mounted and examined with a Bio-Rad MRC 600 confocal system. Subcloning into the pECFP-C1 vector (Clonetech) used Eco RI and Bam HI for synaptotagmin I and Hind III and Kpn I for synaptotagmin IV. Transfection was as in (*15*).
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Localization of Long-Term Memory Within the *Drosophila* Mushroom Body

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The mushroom bodies, substructures of the *Drosophila* brain, are involved in olfactory learning and short-term memory, but their role in long-term memory is unknown. Here we show that the *alpha-lobes-absent* (*ala*) mutant lacks either the two vertical lobes of the mushroom body or two of the three median lobes which contain branches of vertical lobe neurons. This unique phenotype allows analysis of mushroom body function. Long-term memory required the presence of the vertical lobes but not the median lobes. Short-term memory was normal in flies without either vertical lobes or the two median lobes studied.

The organization of the *Drosophila* brain, which shows highly organized and specialized structures despite its small size, in combination with its sophisticated behavioral repertoires and powerful molecular genetic tools render this organism a model of choice for the study of integrative brain functions, such as associative learning and memory. The mushroom bodies (MBs) constitute a prominent bilateral structure of the insect brain. The MBs are formed and rearranged sequentially during embryonic and postembryonic development (*1*–*3*). In adult *Drosophila*, they are composed of about 5000 neurons, which receive, through the calyx, olfactory inputs from the antennal lobes. The MBs are essential for associative learning and memory (*4*–*6*). Several proteins involved in learning and short-term memory are detected at high levels in the MBs (*7*), and chemical ablation of *Drosophila* MBs abolishes olfactory learning (*6*). Synaptic transmission from the MBs is required for retrieval of short-term memories but not for acquisition or storage (*8*, *9*). With intensive and spaced training, *Drosophila* can also display long-term memory (LTM), which depends on protein synthesis after the conditioning paradigm (*10*). We have now tested whether *Drosophila* MBs are involved in LTM formation.

Three categories of MB intrinsic neurons (Kenyon cells), associated with five sets of lobes, have been described (Fig. 1A) (*1*, *11*). Two types of neurons branch to give rise to a vertical and a median lobe (α/β lobes and α′/β′ lobes, respectively). The third type composes the median γ lobe. Uniquely identifiable efferent neurons originate from specific parts of the medial and vertical lobes, and send their axons to characteristic regions of the forebrain (*12*). Afferents from the forebrain also invade specific parts of the lobes (*12*). The implication of this architecture, which also characterizes other insect MBs (*13*), suggests that the lobes are not identical and may support quite distinct functions.

Genetic characterization of the *alpha-lobes-absent* (*ala*) mutant, which shows abnormal MBs, was previously reported (*14*). The original mutation corresponds to the insertion of a P-element and is recessive. Phenotypic revertants as well as new mutant alleles were produced by excision of the P-element (*14*). We reassessed the *ala* brain defect using the *GAL4-OK107* enhancer-trap line (*15*), which labels all five lobes (Fig. 1), and the antibody to FasciclinII (FasII), which strongly labels α/β lobes and weakly labels γ lobes (*11*, *16*). Three batches of brain analyses showed that 10.5 ± 2.8% of *ala* individuals possessed all five lobes in both hemispheres, 36 ± 2.4% lacked the β and β′ lobes in both hemispheres, and 4.5 ± 1.1% lacked α and α′ vertical lobes. The remaining flies showed different combinations of phenotypes in the left and right hemisphere. *ala* flies without vertical lobes also showed a fusion of the left and right β and β′ lobes (Fig. 1C). This fusion phenotype is also observed in a fraction of *ala* flies with all lobes present (3.6 ± 1.4% of total) (*16*). A similar phenotype distribution was observed in *ala/Df(1)4b18* individuals (*17*). γ lobes appeared to be normal in *ala* mutants. This observation was reinforced by the fact that in second instar larvae *ala* mutants possessed normal vertical and median γ projections (*18*).

The *ala* mutant was trained to associate an odor with electric shocks by using three different experimental paradigms (*19*, *20*): (i) a single training cycle protocol to induce short-term memory (*21*); (ii) an intensive spaced conditioning protocol, consisting of 10 individual training sessions with a 15-min rest interval between each session, to induce LTM (*10*); and (iii) a massed conditioning protocol, consisting of 10 consecutive training sessions without rest, to induce 24-hour memory but not protein-synthesis dependent LTM (*10*).

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In spite of the severe brain phenotype, 1-hour, 3-hour, 24-hour spaced, and 24-hour massed memory performances were normal in *ala* mutant (Fig. 2A). We next tested whether the absence of specific MB lobes results in specific memory defects. Several thousand *ala* flies were trained and tested in the three conditioning protocols. These protocols are discriminative because during the test flies tend to avoid the previously shock-associated odor and move toward the second odor (not previously associated with shock). Flies that made the correct choice were collected separately from those that made the wrong choice, and their brains were analyzed by staining with antibody to FasII (*16*). This allowed us to correlate a memory performance index (PI) with each category of *ala* brain defect (Fig. 2B). There are three advantages of such an experiment. First, it allowed a regional analysis of the MB function, which could not be performed with traditional genetic tools. Second, all flies came from the same siblings and were treated equally under conditioning and testing conditions. Third, the experiment was performed blindly with respect to brain defects. We also took advantage of the fact that flies with different memory abilities are known to perform independently of each other when mixed for conditioning and testing (*21*, *22*).

In agreement with our first analysis, 3-hour memory was not reduced in flies lacking either α α′ or β β′ lobes, confirming that these structures are not required for proper odor or shock perception (*6*). LTM was normal in the absence of β β′ lobes but fully abolished in the absence of α α′ lobes, despite the fact that these two sets of lobes originate from the same neurons during development. In contrast, 24-hour memory generated after massed training was not reduced in flies lacking vertical lobes. The LTM deficit associated with the absence of vertical lobes was not detected immediately (Fig. 2A) because these flies represent less than 5% of the *ala* population. The reduction in LTM cannot be attributed to the fused β β′ defect because *ala* flies with all lobes showed normal scores when their β β′ lobes were fused (*23*).

In *Drosophila*, the dendrites of efferent neurons leading from the MBs reside in the lobes or at the junction between the lobes and the pedunculus, but not posteriorly in the pedunculus itself (*12*). The fact that short-term memory was not affected by the lack of α α′ or β β′ lobes has two possible explanations. First, functional redundancy of neural connections may have prevented us from observing short-term memory defects in the absence of two of these four lobes. This view is supported by the fact that memory loss was observed after transient inactivation of neurons that form α/β lobes (*9*). Alternatively, γ lobes, which are normal in *ala* flies, could represent the main neuronal substrate for short-term memory. This interpretation is supported by the fact that the *rutabaga* (*rut*) learning defect can be rescued by tissue-specific rut^{+} expression in γ lobes (*24*).

This study shows that the MBs play an essential role in LTM formation. Furthermore, 24-hour memory after spaced training was fully abolished in the absence of vertical lobes, suggesting that in normal flies a unique molecular and cellular pathway underlies this memory phase. In a previous study, this phase was not completely eliminated after protein synthesis inhibition (*10*). At the time, this observation was interpreted to mean that LTM represented only half of the memory displayed at 24 hours after spaced training. The present results, however, suggest that residual ribosomal activity was the cause of this partial inhibition. In the larva, vertical axon branches of neurons associated with the

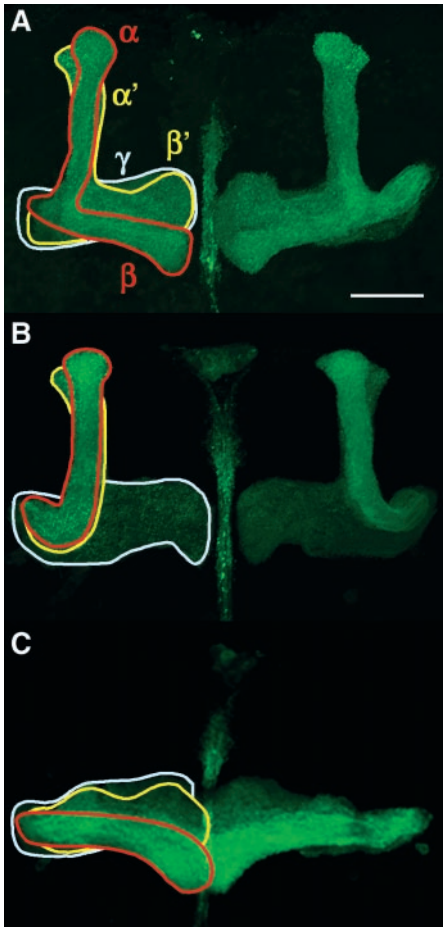

Fig. 1. *ala* mutants show specific MB defects. (**A**) Composite confocal images of wild-type CS adult mushroom body (*25*). Expression of the *UAS-mCD8-GFP* transgene driven by the P insertion *GAL4-OK107* allows visualization of the whole mushroom body. Three sets of neurons generate five axonal lobes. The γ lobe is outlined in white, the α and β lobes are outlined in red, and the α′ and β′ lobes are outlined in yellow. The color code is conserved in (B) and (C). The median bundle is also revealed with *GAL4-OK107*. Scale bar, 40 μm. (**B**) ala^{E13} MBs without β β′ lobes. (**C**) ala^{E13} MBs without α α′ lobes. In these flies, the β and β′ lobes cross the midline.

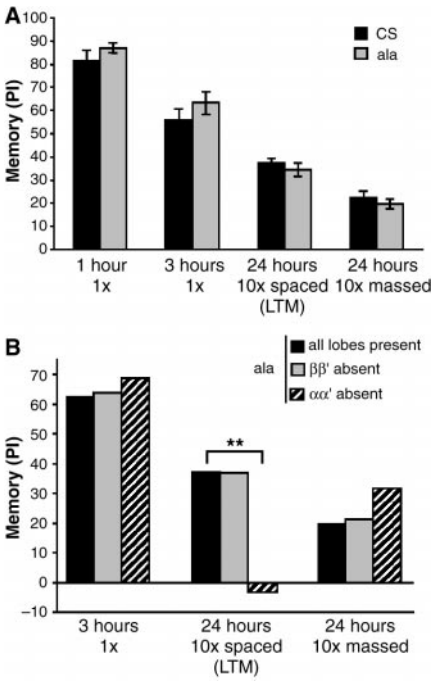

Fig. 2. LTM is abolished in absence of MB vertical lobes. (**A**) *ala* mutant stock has normal olfactory memory. PIs were measured 1 hour and 3 hours after one conditioning cycle, and 24 hours after 10 cycles of training with (spaced) or without (massed) 15-min intervals between each training cycle (*19*, *20*). Memory scores of *ala* flies (gray bars) were not significantly different from wild-type flies (black bars) at all times tested (*t* test). Bars represent means of PIs; errors are SEMs (standard errors of the mean); $n = 8$ to 12 groups. (**B**) Memory scores of three categories of *ala* flies: all lobes present (black bars), β β′ lobes absent (gray bars), and α α′ lobes absent (striped bars). LTM was significantly decreased in flies without α α′ lobes in comparison with flies with all lobes present (χ^2 $P = 0.0012$), shown by asterisks (*26*). On the contrary, flies with β β′ lobes absent were not affected ($P > 0.5$). The 24-hour memory increased after massed training in flies with α α′ lobes absent was not significant ($P > 0.25$) (*27*). 1530 flies were tested at 3 hours ($n = 8$ groups), with a distribution of 96 flies with all lobes present, 599 flies with β β′ lobes absent, and 99 flies with α α′ lobes absent. After spaced training, 1273 flies were tested ($n = 10$ groups), with a distribution of 111 flies with all lobes present, 480 flies with β β′ lobes absent, and 53 flies with α α′ lobes absent. After massed conditioning, 1607 flies were analyzed ($n = 11$ groups), with a distribution of 204 flies with all lobes present, 503 flies with β β′ lobes absent, and 44 flies with α α′ lobes absent.

medial γ lobe might fulfill a similar role to adult vertical projections in long-term information processing. Loss of these projections during metamorphosis could erase some long-term information specific to the larval stage. In adult *Drosophila*, few efferent neurons from the γ lobe extend to around the α α′ lobes (*12*), which could represent a pathway that converts information from short-term to long-term memory. Alternatively LTM may form independently of short-term memory. Further analyses must be performed to resolve that issue and to determine the role of α and α′ lobes in LTM formation.

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16. Supplementary material is available at *Science* Online at www.sciencemag.org/cgi/content/full/294/5544/1115/DC1. See Web fig. 1.
17. A. Pascual, T. Préat, data not shown.
18. A. Pascual, M. Chaminade, T. Préat, unpublished data.
19. The wild-type reference stock was Canton-Special (CS). The *ala*^{1} P-element induced mutant (abbreviated *ala*) and the *ala*^{E13} excision mutant (*14*) were outcrossed in a CS background to prevent modifier accumulation. Flies were conditioned by exposure to one odor paired with electric shocks and subsequent exposure to a second odor in the absence of shock. A barrel-type machine was designed that allowed simultaneous automated conditioning of six groups of flies (*20*). During the tests, flies were exposed simultaneously to both odors in a T-maze (*21*). After 2 min, flies were trapped in either T-maze arm and counted. A reciprocal conditioning experiment was run with different flies of the same genotype, the second odor being associated with electric shock. The performance index (PI) corresponds to an averaged and normalized probability of the correct answer, so that a 50:50 distribution (no memory) yields a PI of zero.
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23. Twenty-four hour PI after spaced training, 36.7 for flies with normal lobes and 35.7 for flies with fused lobes; 24-hour PI after massed training, 18.7 for flies with normal lobes and 21.2 for flies with fused lobes.
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25. The *Drosophila* central nervous system was dissected in 4% paraformaldehyde in phosphate-buffered saline (PBS), fixed for 20 min, mounted in mowiol (Calbiochem, La Jolla, CA), and examined with a Leica TCS SP2 laser scanning confocal microscope (Wetzbar, Germany).
26. Because flies without vertical lobes represent less than 5% of the *ala* population, on average only three to four such flies were present in each conditioning experiment. Calculating a PI for each experiment with so few individuals was meaningless, and therefore *t*- test analyses were not applied here. A global PI was calculated for each category of *ala* flies by adding flies from repeated experiments. This global score represents a mean of individuals' behavior. Statistical analyses were performed with a nonparametric χ^2 test. The reference theoretical value used for χ^2 analysis of *ala* flies with missing lobes was the distribution of *ala* flies with all lobes coming from the same experiments.
27. χ^2 analysis predicts that a population of more than 7000 *ala* flies would have to be run through the massed conditioning protocol and their brain analyzed in order to validate statistically the observed difference.

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SNARE Function Analyzed in Synaptobrevin/VAMP Knockout Mice

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SNAREs (soluble NSF-attachment protein receptors) are generally acknowledged as central components of membrane fusion reactions, but their precise function has remained enigmatic. Competing hypotheses suggest roles for SNAREs in mediating the specificity of fusion, catalyzing fusion, or actually executing fusion. We generated knockout mice lacking synaptobrevin/VAMP 2, the vesicular SNARE protein responsible for synaptic vesicle fusion in forebrain synapses, to make use of the exquisite temporal resolution of electrophysiology in measuring fusion. In the absence of synaptobrevin 2, spontaneous synaptic vesicle fusion and fusion induced by hypertonic sucrose were decreased ~10-fold, but fast Ca^{2+}-triggered fusion was decreased more than 100-fold. Thus, synaptobrevin 2 may function in catalyzing fusion reactions and stabilizing fusion intermediates but is not absolutely required for synaptic fusion.

Intracellular fusion reactions are generally thought to be mediated by SNAREs, a large family of membrane proteins characterized by a common sequence called the SNARE motif (*1*–*6*). During fusion, SNAREs from opposing membranes form core complexes through their SNARE motifs. Different fusion reactions involve distinct sets of SNAREs, although some SNAREs function in multiple reactions. SNAREs have probably been studied in greatest detail at the synapse where the synaptic vesicle SNARE synaptobrevin (also called VAMP) interacts with the plasma membrane SNAREs syntaxin 1 and SNAP-25. Synaptobrevin is a minimal SNARE that consists only of a short NH_2-terminal sequence, a SNARE motif, and a COOH-terminal transmembrane region. Syntaxin 1, in contrast, contains an NH_2-terminal three-helical domain that interacts with multiple other proteins in addition to a SNARE motif and a membrane anchor, and SNAP-25 includes two SNARE motifs besides a membrane anchor (*6*). In spite of substantial progress in the identification of SNAREs as essential components of membrane fusion reactions, their precise role in fusion has remained enigmatic. Three principal hypotheses have been proposed. First, the original SNARE hypothesis posited that SNAREs determine the specificity of fusion reactions (*7*), and recent in vitro fusion reactions have provided support for this idea (*8*). Second, experiments with yeast vacuoles indicated a role for SNARE complexes preceding the actual fusion reaction, suggesting that complex formation catalyzes the subsequent fusion reaction but does not actually execute it (*9*). Finally, it has been proposed that core

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