Integrin-mediated short-term memory in *Drosophila*

Michael S. Grotewiel, Christine D. O. Beck, Kwok Hang Wu*, Xin-Ran Zhu* & Ronald L. Davis

Department of Cell Biology and the Department of Neurology, Baylor College of Medicine, Houston, Texas 77030, USA

Volado **is a new memory mutant of** ***Drosophila*****. The locus encodes two isoforms of a new α-integrin, a molecule that dynamically mediates cell adhesion and signal transduction. The** ***Volado*** **gene is expressed preferentially in mushroom body cells, which are neurons known to mediate olfactory learning in insects.** ***Volado*** **proteins are concentrated in the mushroom body neuropil, brain areas that contain mushroom body processes in synaptic contact with other neurons.** ***Volado*** **mutants display impaired olfactory memories within 3 min of training, indicating that the integrin is required for short-term memory processes. Conditional expression of a** ***Volado*** **transgene during adulthood rescues the memory impairment. This rescue of memory is reversible, fading over time along with expression of the transgene. Thus the** ***Volado*** **integrin is essential for the physiological processes underlying memory. We propose a model in which integrins act as dynamic regulators of synapse structure or the signalling events underlying short-term memory formation.**

Behavioural and cellular studies have revealed two broad phases of memory: short-term and long-term memory. Short-term memory, which lasts from minutes to hours, is thought to occur through changes in synaptic efficacy produced by rapid and transient biochemical alterations in the relevant neurons[1–5]. In contrast, long-term memory, which lasts from days to years, is thought to occur through changes in synaptic efficacy produced by the restructuring of synapses as a result of altered gene expression[6–10]. The formation of long-term, but not short-term, memory has therefore been thought to rely upon morphological restructuring of synapses using mechanisms similar to those involved in brain development.

In *Drosophila*, the formation of olfactory memories is scripted in cyclic AMP signalling in neurons of the mushroom bodies[4,11,12]. Studies linking cAMP signalling, mushroom bodies, and olfactory learning demonstrated the preferential expression in mushroom bodies of three genes required for normal learning: *dunce* (*dnc*), *rutabaga* (*rut*) and *DCO* (the genes encoding cAMP phosphodiesterase, adenylyl cyclase and the catalytic subunit of protein kinase A (PKA), respectively)[11]. Moreover, the characterization of two other learning genes (*amnesiac* and *DCREB2*) of *Drosophila* is consistent with a dominant role for cAMP in modulating the physiology of neurons that mediate behavioural plasticity. The *amnesiac* gene encodes a peptide similar to pituitary adenylyl cyclase-activating peptide[13] and *DCREB2* encodes a transcription factor that may mediate cAMP-dependent gene expression[14]. These studies suggest that mushroom bodies function as the integration and memory centre for olfactory learning by using the cAMP signalling system[4,11].

Despite the evidence pointing to the cAMP signalling system, many different types of molecules must be engaged during learning to bring about the overall physiological changes in the relevant neurons. Indeed, the involvement of an assortment of protein kinases, transcription factors, enzymes involved in neurotransmitter biosynthesis, neuropeptides and other factors have been suggested[5,15–19]. Here we report the isolation of a new *Drosophila* memory gene, *Volado* (*Vol*). (*Volado* is a Chilean colloquialism with no English counterpart, but is loosely translated as 'forgetful' or 'absent-minded'. In Chile, it is often used in reference to professors and scientists.) *Vol* encodes a new α-integrin, a type of cell-surface receptor known to dynamically mediate cell adhesion and signal transduction[20]. Lesions in *Vol* have a dominant effect upon short-term memory following olfactory conditioning. Remarkably, conditional expression of *Vol* just before training rescues the memory deficit of *Vol* mutants. This rescue is reversible, supporting a dynamic role for integrins in neuronal and behavioural plasticity. These data indicate that integrin-mediated signalling or synaptic restructuring underlie the formation, stability or retrieval of short-term memory.

The *Vol* locus encodes new α-integrins

The importance of mushroom bodies in olfactory learning had previously prompted us to construct and screen ~6,000 enhancer detector lines for preferential expression of the *lacZ* reporter in these brain structures[21]. About 100 lines with preferential mushroom body expression were isolated, including insertions at the *dnc*, *rut*, *DCO* and *leonardo* (*leo*) genes[11,22]. Line 1,116 (Vol^1) from this screen also expressed *lacZ* in mushroom bodies; the enhancer detector element in this line was mapped to cytological position 51E.

We isolated the region flanking the enhancer detector element along with wild-type genomic and cDNA clones for the locus. The locus is organized into two transcription units, *Vol*-long (*Vol*-l) and *Vol*-short (*Vol*-s), which encode RNAs of 4.6 kilobases (kb) and 4.4 kb, respectively (Fig. 1b). The *Vol*-l RNA is expressed selectively in heads, whereas *Vol*-s is expressed in both head and body tissues (not shown). Mapping experiments showed that the Vol^1 enhancer detector element resides within the first intron of *Vol*-l and within the 5′ flanking region of *Vol*-s (Fig. 1a). Imprecise excision of the element led to the isolation of Vol^2, an allele with a deletion of 816 nucleotides of genomic sequence that removes the first exon of *Vol*-s (Fig. 1a). Reverse-transcription-polymerase chain reaction (RT-PCR) analyses of head RNA revealed that expression of the *Vol*-l transcript was greatly reduced in Vol^1, but the *Vol*-s transcript was unaffected (Fig. 1c). Conversely, the Vol^2 lesion eliminated the *Vol*-s transcript without discernible changes in *Vol*-l (Fig. 1c). Neither allele affected the expression of PKA, the internal control in these experiments (Fig. 1c). The effects of the alleles on expression of the two transcripts, as confirmed by RNA blotting experiments (not shown), are consistent with the nature of the physical lesions at the gene (Fig. 1a). Thus the Vol^1 and Vol^2 alleles disrupt the expression of *Vol*-l and *Vol*-s, respectively.

The cDNAs for *Vol*-l and *Vol*-s predict new α-integrins of 1,115 amino acids, differing only in the first 63 amino acids (Figs 1a, 2). Vol proteins contain many characteristics of other α-integrins[20]. Vol proteins are 23–28% identical in amino-acid sequence with known α-integrins and contain a single transmembrane domain near the carboxy terminus. The proteins begin with 24 residues of a hydrophobic, putative signal peptide, have 11 potential glycosylation sites [NXT(S)] in the extracellular region, and have three repeats in the

* Present addresses: Department of Biological Sciences, Columbia University, New York, New York 10027, USA (K.H.W.); Zentrum fur Molekulare Neurobiologie, Institut fur Neurale Signalverarbeitung, D-20246 Hamburg, Germany (X.-R.Z.)

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extracellular region that match the consensus DX(D/N)X(D/N)GXXD, a domain found in proteins that bind divalent cations[23]. Moreover, the Vol sequence has a cleavage recognition site (RKRR) in the extracellular domain[24], a site required for signal transduction by some α-integrins. After cleavage at these sites, the amino-terminal and carboxy-terminal integrin fragments are held together by disulphide bonds[20,25]. Furthermore, the cytoplasmic domain of *Vol* contains the consensus sequence KXFF[K/R]R, which binds calreticulin[26] and regulates integrin affinity for ligand.

Expression of *Vol* in mushroom bodies

The *Vol*[1] mutant preferentially expressed the *lacZ* reporter in the nuclei of mushroom body neurons (Fig. 3a). To determine whether the enhancer detector reflected authentic Vol expression, we performed immunohistochemical analyses with an antiserum made against the C terminus of the protein. The Vol antigen was found to be concentrated in the mushroom body perikarya and calyces (Fig. 3b), peduncles (Fig. 3c) and α, β and γ lobes (Fig. 3d). The calyces, peduncles and lobes contain the mushroom body dendrites, axons and axon terminals, respectively[11]. The distribution of the antigen was not noticeably altered in the *Vol*[1] or *Vol*[2] mutants (data not shown), suggesting that both the *Vol-l* and *Vol-s* isoforms are globally coexpressed in the mushroom bodies. Enriched expression was also observed in the ellipsoid body (not shown), a region of the central complex thought to be involved in the coordination of motor behaviours[27]. The distribution of Vol in the mushroom body calyces and lobes (regions in which mushroom body neurons form synapses with other neurons) suggests that the *Vol* integrins may regulate synapse function.

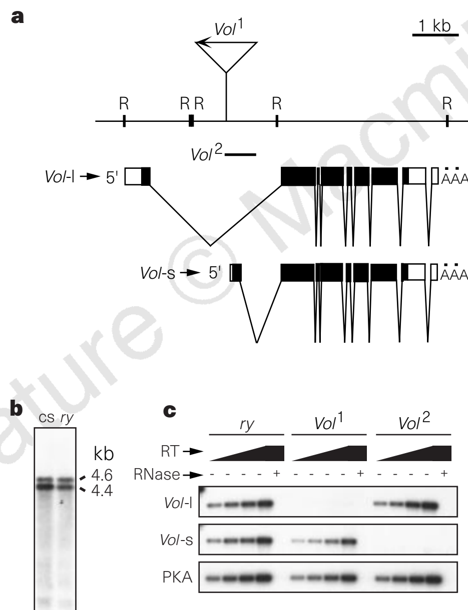

Figure 1 The *Vol* gene structure, transcripts and mutations. **a**, An *Eco*RI (R) restriction map of the locus is shown with the position of the *Vol*[1] enhancer detector element indicated by a triangle. The direction of transcription of the *lacZ* reporter in the enhancer detector element is indicated by the arrow. Two transcription units, *Vol*-long (*Vol*-l) and *Vol*-short (*Vol*-s), were deduced by comparing cDNA sequences with genomic sequences. The first exon of each transcription unit is spliced to a common 2nd exon. Filled boxes represent the open reading frame. The 816 base-pair deletion in *Vol*[2] is indicated by the line spanning the first exon of *Vol*-s. **b**, Blots of adult head RNA showing the 4.6- and 4.4-kb transcripts of *Vol*-l and *Vol*-s, respectively, in Canton-S (cs) and *ry* animals. **c**, RT-PCR analyses of total head RNA from *rosy* (*ry*), *Vol*[1] and *Vol*[2] adults. Each graded bar represents increasing amounts (from left to right) of a single RT reaction added to the subsequent PCR. Both *Vol*-l and *Vol*-s were present in *ry*; however, the expression of *Vol*-l was dramatically reduced in *Vol*[1] and expression of *Vol*-s was undetectable in *Vol*[2]. The internal control using PKA primers allowed quantitative comparisons to be made between the various RT-PCR reactions. RNase (+) added before the RT reaction abolished all signals.

Vol mutations produce a memory deficit

The expression pattern of *Vol*, coupled with preliminary behavioural experiments, suggest that the gene is important for olfactory memory. To test this hypothesis, we assayed *Vol* mutants for aversive olfactory classical conditioning[4]. Populations of animals were administered electric shock (unconditioned stimulus; US) in the presence of one odour, the conditioned stimulus (CS^+), and were subsequently presented with a second odour (CS^-) without shock. To evaluate discriminative avoidance behaviour, the trained animals were allowed to distribute between converging CS^+ and CS^- odours carried in air currents within a T-maze.

Animals homozygous for the *Vol*[1] insertion or the *Vol*[2] deletion performed poorly relative to *rosy* (*ry*) control flies at all time points after training (Fig. 4a; genotype, $P = 0.0001$; retention interval, $P = 0.0001$; genotype $\times$ retention interval, n.s.; see Supplementary Information for the complete statistical treatment for these and

Vol-l

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MNAESTMFPH IFLALLALIS HIEAFNFMPR PSRVINSPKH LKFHINQTRS SYFGYTLVIR
QTSIIVGAPR AQSTLESQRT INETGAIYRC SLTNGVCSPY VLDSRGNVDA PYSEYTFDSE
RKDFQWLGGS MDGGTKDTDK LLVCAPRFYA PSSRDNHLHG VCYWVNNTVA STPQHVTRIS
PLRLKSEQVK EEDNGNKASF FYIMGELGLS AHVADDNTKF LIGAPGINTW RGSVILYRQV
DPVDNPTASR RDTSKALRRT YRDVDSNDYT PEHYAPEIPT PGLWGQEEDS YFGYAVSSGF
FDSSNPTKLL YVATAPQANK QSGEAYIFDV RGKSIHKYHV FRGEQFGEYF GYSVLAEDLN
GDGKTDVIVS APQHALEDSH DNGAIYVFIN KGFFNFERQI LRSPVETMAR FGTALSRLGD
INHDGYNDVA VGAPFAGNGT VFIYLGSENG LRDQPSQRLD APSQQPSKYG SHMFGHGLSR
GSDIDGNGFN DFAIGAPNAE AVYLYRAYPV VKVHATVKSE SREIKPEQEK VKITACYRLS
TTSTDKLVQE QELAIRIAMD KQLKRVKFTQ TQTNEISFKV NANFGEQCRD FETQVRYSEK
DIFTPIDLEM HYELTKKVPD SEEFCETCAV VDPTEPKVST QNIIFSTGCA TDVCTADLQL
RSKNVSPTYI LGSADTLRLN YEITNIGETA YLPQFNVTST SRLAFAQVPG NCKVVDAVMV
CDLNRGRPLA KGDTDSVTIS FDVSQLSGQS LISHAEVFST GYEQNPTDNR QTNVIGLKEF
TEIDASGGQT NRQIDLEHYS NSAEIVNNYE IKSNGPSVIE QLTVSFYIPI AYKVAGSTAI
IPIINVTSLK MQASYDSQLL SIDLYDQNNT MLVVDPVEVT TTLSGGLERT VITQNRQSYD
IHTSGHVHQT MEVLDTSMVA TASMSRKRRD LKALTANREQ YARISNVKAH DLLSDDFKGK
LPVNRTIVFN CRDPEMTICV RAEMRVHFRP EKSINLNMRY SVDLNEVNAI LVDPWEYFVI
LTDLKLQKKG DPTSTSFSIN RRIEPNIISK HQETGLPIWI IIVSVIGGLL LLSAISYLLY
KFGFFNRTKK DELDRLVQQN PVEPEAENLN SGGNN
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Vol-s

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MVGQDRDFWA LLVLGLWCLS SHCNAFNLSP LPNRQILDPQ FATNLPKVRA SYFGFTMSLR
PNG
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Figure 2 *Vol* encodes two forms of an α-integrin. The complete predicted amino-acid sequence for *Vol*-l and the first 63 amino acids of *Vol*-s are shown. Triangles above the sequence indicate where the coding sequence is separated by introns. The putative transmembrane domain and cleavage site are indicated by single and double underlines, respectively.

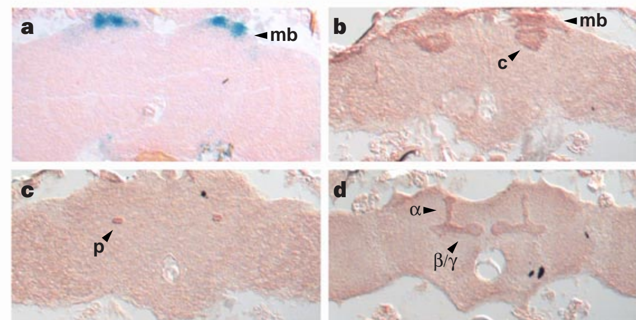

Figure 3 *Vol* is preferentially expressed in mushroom bodies. **a**, Frontal section of a *Vol*[1] adult head stained for β-galactosidase activity. Staining (blue) was observed within the mushroom body perikarya (mb). The β-galactosidase encoded by the enhancer detector element carried a nuclear targeting sequence which explains the nuclear localization of the histochemical stain. **b–d**, Frontal sections of Canton-S adults after immunostaining with an affinity-purified antiserum raised against the C terminus of *Vol*. Expression (dark brown) was observed in: **b**, the cell bodies (mb) and calyces (c); **c**, the peduncle (p); and **d**, the α, β and γ lobes (α, β, γ).

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other data presented below). The effects of these mutations on memory were indistinguishable, suggesting that the two integrin isoforms are functionally redundant. We have shown previously that neither the enhancer detector itself nor the expression of *lacZ* in mushroom bodies has any significant effect upon performance (ref. 22 and K.H.W. and R.L.D., unpublished). The performance deficits in *Vol* mutants were present at the earliest testable time point after training (3 min), indicating that the formation, stability or retrieval of short-term memory is dependent on integrin function.

To examine further the effects of the *Vol* alleles on early memory, and to investigate their recessive or dominant nature, we trained and tested the performance of animals heterozygous or homozygous for the two lesions of the gene. The Vol^1 and Vol^2 animals exhibited memory deficits at both 3 and 15 min after training (Fig. 4b; 3 min, $P = 0.0001$; 15 min, $P = 0.0001$), confirming the results in Fig. 4a. The performance indices of the Vol^1/+ and Vol^2/+ heterozygous animals were similarly reduced relative to *ry*, but were not significantly different from the corresponding homozygous mutants (Fig. 4b; Vol^1 versus *ry* at 3 min, $P = 0.0001$; at 15 min, $P = 0.0001$; Vol^2 versus *ry* at 3 min, $P = 0.00015$; at 15 min, $P = 0.0001$; Vol^1 versus Vol^1/+ at 3 min, n.s.; at 15 min, n.s.; Vol^2 versus Vol^2/+ at 3 min, n.s. at 15 min, n.s.). Trans-heterozygous animals, Vol^1/Vol^2, also exhibited a performance index equivalent to Vol^1/+ or Vol^2/+ (not shown). Thus, as with *dnc*, *rut*, *turnip*, *radish* and *cabbage*, mutations in *Vol* have a dominant effect on memory[4]. The dominant effect is particularly noteworthy for *Vol* alleles, as three of the four transcription units were preserved in animals heterozygous for *Vol*-l or *Vol*-s lesions. These data support the existence of a threshold requirement for *Vol* expression in the processes underlying memory, making them acutely sensitive to decreased expression of this gene.

To eliminate the possibility that the poor performance of *Vol* mutants was due to defects in sensorimotor processes, we tested their ability to sense and avoid electric-shock pulses and the odours used for conditioning[4]. The avoidance behaviour of *Vol* mutants and control animals to electrified grids and odours used for conditioning at multiple strengths of these stimuli was indistinguishable. For example, the avoidance indices to 0.8 ml octanol were 63 ± 4, 68 ± 4 and 65 ± 5 for *ry*, Vol^1 and Vol^2, respectively. (See Supplementary Information for the complete olfactory and electroshock avoidance data.) We also explored the morphology of the brain, with particular emphasis on mushroom bodies, to determine whether the poor performance was attributable to defects in brain structure. Serial paraffin sections of control and mutant brains failed to reveal any discernible differences in morphology when stained with haematoxylin and eosin, an antibody against the nuclear antigen D-Mef2, which reveals a subset of mushroom body cell nuclei (J. Crittenden and R.L.D., unpublished); an antibody against the *leo* gene product, which delineates the mushroom body calyces, cell bodies, peduncles and lobes[22]; or an antibody against FasII, which reveals a subset of the mushroom body lobes (Fig. 5 and data not shown). Therefore, neither sensorimotor nor gross neuroanatomical defects can account for the memory deficit of *Vol* mutants.

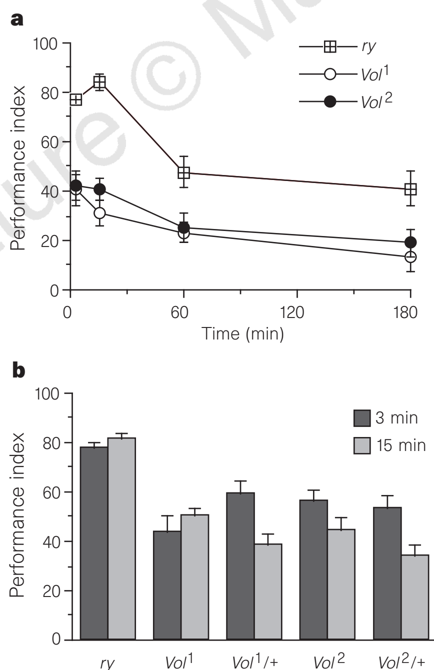

Figure 4 Memory deficits in *Vol* mutants. **a**, Decay curve of conditioned odour avoidance for two *Vol* mutants (Vol^1 and Vol^2) and the control strain (*ry*); $N = 8$–9 for all groups. The mean performance index ± s.e.m. is shown for each genotype at several time points after training. The performance of Vol^1 and Vol^2 was significantly less than *ry* at all time points. **b**, Performance of homozygous and heterozygous *Vol* mutants at 3 and 15 min after training; $N = 8$–11 for all groups. There were no significant differences between the homozygous mutant strains and the corresponding heterozygous strains at either time point.

Conditional rescue of the *Vol* memory deficit

We reasoned that direct evidence for a role of the integrin in physiological processes underlying memory might be obtained by the conditional expression of a *Vol* transgene. Four transgenic lines were generated that contained the *Vol*-s cDNA under the control of the heat-shock promoter (hsp70) in the Vol^2 background (*Vol*-s mutant). Animals were heat-shocked for 15 min at 37 °C, rested for 3 h to allow recovery and expression of the transgene, and were trained and tested for 3-min memory. Two of the transgenic lines failed to show any evidence of heat-dependent rescue in pilot experiments, presumably owing to genomic position effects, and were not analysed further. Two other lines, VS-T2 and VS-T3, were analysed extensively for olfactory memory.

Normal olfactory memory of *ry* control animals and the residual memory in Vol^2 mutants was unaffected by heat shock (Fig. 6a; for *ry*, no heat shock versus heat shock, 3 h, n.s., for Vol^2, no heat shock versus heat shock, 3 h, n.s.). In the absence of heat shock, VS-T3 transgenic animals exhibited mutant levels of performance, but VS-T2 transgenic animals showed partial rescue of memory, possibly

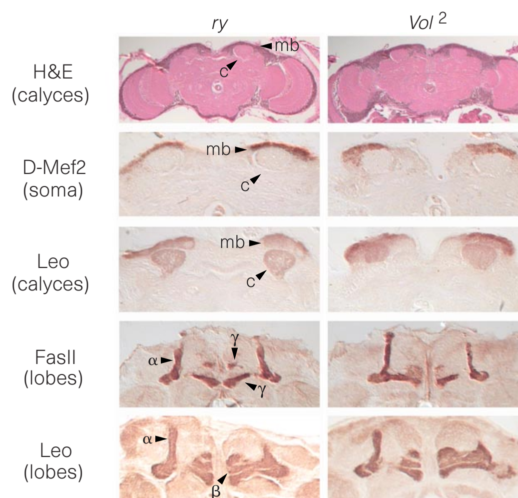

Figure 5 Lack of neuroanatomical defects in *Vol* mutants. Frontal sections from *ry* and Vol^2 adult flies are shown at the level of the mushroom body perikarya (mb) and calcyes (c) after staining with haematoxylin and eosin (H&E) or with an antibody against the nuclear antigen D-Mef2, and at the level of the mushroom body lobes (α, β and γ) after staining with anti-FasII or anti-Leonardo antisera. No differences were observed between *ry* and either mutant (Vol^1 not shown). Slight differences seen here were due to the plane of sectioning. The posterior to anterior arrangement of sections is from top to bottom.

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owing to elevated basal expression of the transgene in mushroom bodies (Fig. 6a; no heat shock, VS-T3 versus *ry*, $P = 0.0001$; VS-T3 versus Vol^2, n.s.; VS-T2 versus *ry*, $P = 0.0008$; VS-T2 versus Vol^2, $P = 0.0003$). However, the 3-min memory of VS-T2 and VS-T3 animals when trained 3 h after heat shock was significantly greater than after no heat shock, and was indistinguishable from the *ry* control (Fig. 6a; heat shock versus no heat shock, VS-T2, $P = 0.0045$; VS-T3, $P = 0.005$; with heat shock, *ry* versus VS-T2, n.s.; *ry* versus VS-T3, n.s.). Therefore, conditional expression of *Vol*-s just before behavioural training was sufficient to fully rescue the mutant phenotype. This rescue cannot be attributed to altered sensorimotor abilities because avoidance behaviour to electric shock and odours by the control and transgenic animals was indistinguishable, with or without heat shock (see Supplementary Information). These data argue that the defective α-integrin expression in *Vol* mutants causes the memory deficits, and that the *Vol* integrin participates in the physiological processes underlying memory.

To determine whether the behavioural rescue was paralleled by the induction of the *Vol* transgene, we assayed *Vol* RNA and protein levels before and after heat shock. As assayed by RT-PCR, heat shock had no effect on the quantity of *Vol*-s RNA in *ry* control animals (Fig. 6b), but produced ~100-fold and ~1,000-fold increases in the level of *Vol*-s RNA in the VS-T2 and VS-T3 transgenic lines, respectively (Fig. 6b). The level of PKA RNA served as an internal control and was unaffected by *Vol* mutation (Fig. 1b), *Vol* transgene expression, or heat shock (Fig. 6b). To measure Vol protein, we performed western blotting experiments using an affinity-purified antiserum raised against the C terminus of Vol that recognized the intact Vol protein (relative molecular mass 125,000; M_r ~125 K) and the C-terminal cleavage fragment produced by proteolysis (M_r ~21 K). This antiserum identified a band (sometimes a doublet) of 26 K in *ry* that was not found in Vol^2 mutants or in non-heat-shocked transgenic animals (Fig. 6c and data not shown). This band represents the C-terminal cleavage fragment. The full-length protein was not detected in *ry* extracts, presumably owing to reduction of the disulphide bond that links the heavy and light chains. In contrast to the *ry* control, a large increase in the expression of both the *Vol* full-length protein and light chain was found in VS-T3 extracts obtained 3 h after heat shock (Fig. 6c). Detection of the intact molecule suggests that the protease is limiting after over-expression of *Vol*. Induction of Vol was also observed in VS-T2 (not

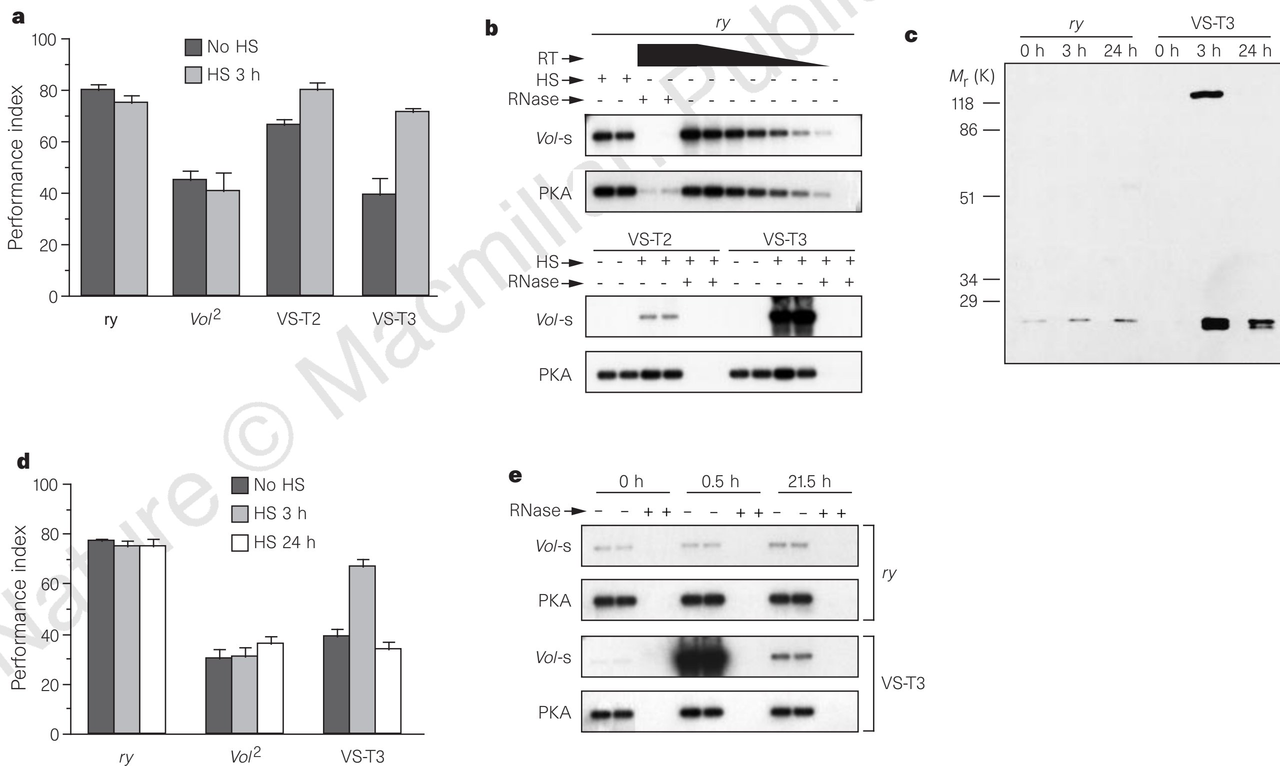

Figure 6 Rescue of the *Vol* memory defect by conditional expression of *Vol*-s. **a**, Memory after 3 min, without heat shock (No HS) or with training 3 h after heat shock (HS 3 h) in *ry*, Vol^2, VS-T2 and VS-T3 flies. Heat shock was for 15 min at 37 °C; $N = 6$ for all groups. Rescue of the mutant phenotype was exhibited by both VS-T2 and VS-T3; VS-T2 exhibited some constitutive rescue. **b**, RT-PCR analyses of *Vol*-s expression. RT-PCR for *ry*, VS-T2 and VS-T3 without (–) or 30 min after (+) HS. Top, *ry* control: HS had no effect on expression of *Vol*-s or PKA in *ry* animals (compare duplicate lanes 1 and 2 with lane 7, all of which are from PCR reactions containing equivalent amounts of input cDNA). Quantification using a BetaGen blot analyser demonstrated that signals for both *Vol*-s and PKA were linear with the mass of input cDNA amplified by PCR. Bottom, *Vol* transgenics: *Vol*-s RNA was nearly undetectable in both VS-T2 and VS-T3 in the absence of HS (–). There was a marked induction of the transgene 30 min after HS (+). Lanes 1, 2 and 7 in the top panels and all lanes in the bottom panels are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment (+) before RT eliminates all signals. Data are representative of 3 independent experiments. **c**, *Vol* protein was induced after HS in VS-T3. Immunoblotting was performed on extracts from whole flies without (0 h) or 3 and 24 h after HS. Western blots containing 0.5 fly equivalents per lane were incubated with an affinity-purified antiserum generated against the C-terminus of the Vol integrin. This antiserum recognizes both the full-length protein (~135K) as well as the light chain (doublet at ~26K). These data, confirmed by detection with an antiserum generated against the extracelluar domain (not shown), are representative of 2 experiments. **d**, Memory at 3 min without HS (No HS) or 3 (HS 3 h) and 24 h (HS 24 h) after HS; $N = 6$ for all groups. VS-T3 showed a behavioural deficit without HS, normal performance with HS 3 h before training, but a deficit again when HS was given 24 h before training. **e**, RT-PCR analyses of *Vol*-s RNA expression without (0 h) or 0.5 and 21.5 h after HS. Top, Expression of *Vol*-s was not changed after HS in *ry* animals. Bottom: *Vol*-s RNA was dramatically elevated in VS-T3 0.5 h after HS, and returned to a low level at 21.5 h after HS. As in **b**, PKA expression was not changed by HS in either strain. All lanes are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment before RT eliminates all signals. Data, from a single experiment performed in duplicate, are representative of 2 independent experiments.

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shown). Thus there was a marked elevation of the *Vol* α-integrin in the VS-T2 and VS-T3 transgenics 3 h after heat shock. These RNA and protein analyses demonstrated that *Vol* was conditionally expressed at the time of behavioural assay, confirming that replacement of the *Vol* integrin in adulthood rescued the memory deficit.

Our data point to a physiological role for *Vol*; however, it seemed plausible that the α-integrin might be required for a final step in synapse formation that occurs normally during development, and that the induced expression of the integrin during adulthood simply allows completion of this terminal step. In other words, the integrin might be required for synapse formation but not for synapse stability. If this were the case, the induction of *Vol* expression might cause a long-lasting or permanent rescue of memory. However, if *Vol* participates in a non-developmental, acute aspect of neuronal function, the rescue of memory produced by induction of the *Vol* transgene would be expected to be transient and reversible, persisting only as long as adequate levels of the *Vol* integrin were present.

To distinguish between these possibilities, we explored whether induction of *Vol* produced a permanent or a reversible restoration of memory. As before (Fig. 6a), heat-shock treatment 3 h before training and testing dramatically improved the performance of VS-T3 animals (Fig. 6d; for VS-T3, no heat shock versus heat shock, 3 h, $P = 0.0001$). This rescue was completely reversible. The memory in heat-shocked VS-T3 transgenic animals returned to mutant levels when the animals were trained and tested 24 h after heat shock (Fig. 6d; for VS-T3, no heat shock versus heat shock, 24 h; n.s.; heat shock 3 h versus heat shock 24 h, $P = 0.0001$; for heat shock 24 h, *ry* versus VS-T3, $P = 0.0001$; *Vol*^{2} versus VS-T3, n.s.). *Vol* RNA and protein expression in the transgenic animals, which reflect abundance in all cells, were markedly elevated at early time points after heat shock (0.5 and 3 h, respectively), and decreased to low levels at late time points (21.5 and 24 h, respectively) (Fig. 6c, e). Thus the induction and ensuing decline of *Vol* expression correlated well with the behavioural rescue and subsequent return to a state of memory impairment. The temporal parallels in RNA level, protein expression, and memory argue strongly that *Vol* mediates a physiological process that is critical to memory formation, stability or retrieval.

Taken together, our data support three important points. First, reduced expression of the *Vol* integrin produces an impairment in memory without altering sensorimotor abilities or neuroanatomy. Second, this phenotype is rescued by the expression of the integrin just before training in the adult animal, demonstrating a role in adults for this adhesion molecule. Third, the reversibility of the memory rescue indicates that the *Vol* integrin mediates a dynamic process underlying memory.

Role of integrins in plasticity

The identification, isolation and characterization of *Vol* follows previous studies of four other learning genes with similar expression patterns: *dnc*, *rut*, *DCO* and *leo*[11,22,28–30]. The discovery of another memory mutant in which the underlying gene is expressed preferentially in mushroom bodies reinforces the conclusion that these cells are required for olfactory learning and memory. Mushroom bodies have been the centre of physiological, anatomical and behaviour studies in large insects[11,12], and have since been shown to be important in *Drosophila* behaviour[11]. One attractive model has mushroom bodies as centres for the reception and integration of many different forms of sensory information, including information about odours and electric shock presented during olfactory classical conditioning. To encode memory, the converging sensory information is thought to alter the physiology of mushroom body cells, using the cAMP signalling system as well as other types of molecules[4,11,22,31]. Our results demonstrate that integrins should now be included in the family of molecules required for memory formation.

Integrins have diverse biological roles in apoptosis, cell-cycle regulation, cell migration, blood clotting and leukocyte function[20,25,32,33]. They function as $\alpha\beta$ heterodimers, mediating adhesive interactions of cells with the extracellular matrix or with counter-receptors displayed by other cells. They also dynamically transduce information across cell membranes bi-directionally[20,25,32,33]. Ligand binding to integrins induces a variety of signalling events within cells, and agonist activation of classical signal-transduction pathways can alter the affinity of integrins for their ligands within just a few minutes[25].

The dynamic adhesion role for integrins suggests how the *Vol* integrin, and integrins in general, may underlie alterations in synaptic plasticity and behaviour. We envisage that release of a modulatory neurotransmitter on a mushroom body neuron might mobilize the intracellular events altering the binding of integrins displayed at another synapse made by that cell. For example, protein kinase C or Ras activation[20,32,34] is known to activate integrin binding. This could produce a rapid (within minutes) alteration in the structure and efficacy of that synapse. The modulation of integrin affinity for ligands might also underlie the construction or pruning of existing synapses, or the activation of silent synapses during learning or memory encoding. Thus the formation of short-term memory may use synaptic rearrangements like long-term memory, but through an integrin-dependent and protein synthesis-independent mechanism. Alternatively, integrins might modulate neuronal function through ligand binding followed by activation of intracellular signalling events. For example, integrins are known to stimulate several signal-transduction pathways in many types of cells, including Ca^{2+} mobilization, tyrosine kinase activation, and induction of protein kinase C[33]. Integrin-dependent stimulation of these pathways in the relevant neurons may be fundamental to learning and memory.

Our results demonstrating a role for integrins in behavioural plasticity fit well with studies showing integrin-dependent modulation of synaptic plasticity. Notably, peptide inhibitors of integrin binding have no effect on the formation of long-term potentiation, but block the maintenance of this form of synaptic plasticity[35,36]. In addition, the enhancement of neurotransmitter release from motor-neuron terminals owing to muscle stretch is blocked by peptide inhibitors[37]. These physiological studies[38], coupled with our behavioural studies, support a model in which integrins mediate dynamic processes at synapses underlying memory formation or stability.

Note added in proof: A paper reporting on another aspect of the same novel integrin subunit is in press (Stark, K. A., Yee, G. H., Roote, C. E., Williams, E. L., Zusman, S. & Hynes, R. O. A novel α integrin subunit associates with βPS and functions in tissue morphogenesis and movement during *Drosophila* development. *Development* (in the press)). □

Methods

Cloning, mutagenesis and transgenic animals. Genomic sequences flanking the *Vol*^{1} insertion were isolated by plasmid rescue[39]. Wild-type genomic clones were isolated from a Canton-S library made in lambda DASHII (provided by M. Eberwine); cDNA clones were isolated from libraries prepared from *Drosophila* head RNA (provided by C. Hall). The 4.6-kb *Vol*-l RNA sequence is represented by a cDNA of ~4,600 residues. The 4.4-kb *Vol*-s RNA is represented by 3,366-bp cDNA. The *Vol*^{2} excision was isolated after dysgenesis. Briefly, flies carrying the *Vol*^{1} enhancer detector element were crossed to X^{cs}; *CyO/2*^{cs}*; ry Sb* P[ry^+,Δ2-3,99B]/*TM6*, *Tb* ('cs' denotes chromosomes derived from a wild-type Canton-S stock). Dysgenic progeny carrying *CyO* were crossed to X^{cs}; *CyO/leo*^{1375}; *ry*$^{506\text{-iso}}$ flies. *CyO*; *ry*$^{506\text{-iso}}$ progeny were selected for stocks; *ry*$^{506\text{-iso}}$ is an isogenic *ry*^{506} chromosome. Excision derivatives were characterized by Southern blotting, extensive PCR analyses, and sequencing of PCR products that cross deletion break points. Because of the nonspecific behavioural effects of mini-*white* vectors[40,41], a new P-factor vector (pCy-20-dbhsp) for driving genes behind the hsp70 promoter was constructed with ry^+ as the selectable marker (provided by G. Roman). This vector,

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containing an *Mlu*I–*Kpn*I fragment of the *Vol*-s cDNA was injected into Vol^2 embryos. Chromosomal localization of the transgenes and the generation of homozygotes for the transgenes were performed by standard crosses. The presence of the Vol^2 allele in the transgenic animals was confirmed by PCR analyses of genomic DNA. The *Vol* transgene resides on the X and 2nd chromosome, respectively, in VS-T2 and VS-T3. Flies were collected in clean food vials, transferred to pre-warmed food vials, and immersed in a water bath at 37 °C for 15 min. Following heat-shock, flies were transferred to room-temperature food vials and stored until testing.

RNA blots and RT-PCR analyses. For RNA blots, poly(A)$^+$ RNA was isolated after tissue homogenization in guanidinium-isothiocyanate, banding in CsCl gradients, and by batch adsorption to oligo-(dT) cellulose. Poly(A)$^+$ RNA was fractionated (10 μg per lane) by formaldehyde–agarose gel electrophoresis. For RT-PCR experiments, total RNA from heads or whole flies was extracted using Trizol (Gibco-BRL) according to the manufacturer's instructions. Each RT reaction contained 3 μg total RNA, 500 ng oligo-(dT), and 200 U SuperScript II (Gibco-BRL) in a total volume of 20 μl. The reactions were incubated at 42 °C for 50 min and digested with 10 U *Sau*3AI and 10 U *Aci*I at 37 °C for 3 h. RNase A treatments (10 μg) prior to RT reactions were for 1 h at 37 °C. Portions of 0.2–5.0% of the RT reactions were amplified using PCR for 20 cycles. For amplification of *Vol* first-strand cDNAs, an antisense primer than anneals to the common 2nd exon of *Vol* (857 nucleotides 3′ of the translation start site) was used in combination with sense primers specific for the first exon of either *Vol*-l or *Vol*-s (84 and 118 nucleotides 5′ to translation start site, respectively). For amplification of PKA, primers that anneal to the 2nd exon of *DCO* were used. PCR products (942, 975 and 356 bp for *Vol*-l, *Vol*-s and PKA, respectively) were electrophoresed in agarose gels, blotted and hybridized to ^{32}P-labelled probes.

Histology, generation of antisera, and immunoblotting. β-Galactosidase staining and haematoxylin and eosin staining were performed as described[21,22,29–31]. For generation of antisera, rabbits were injected with a purified glutathione *S*-transferase (GST)–Vol fusion protein containing either Vol amino-acid sequence 1087–1115 (C terminus) or 358–496 (extracellular domain). For immunohistochemistry using anti-Vol antisera and the anti-FasII monoclonal antibody 1D4, adult heads were fixed in 4% paraformaledhyde at 4 °C for 2 h and incubated in 25% sucrose in Ringer's solution at 4 °C overnight. Serial cryosections (10 μm) were incubated with affinity-purified anti-Vol or anti-FasII antibody at 4 °C overnight. For anti-D-Mef2 and anti-Leonardo staining, adult heads were fixed in Carnoy's solution for 4 h, embedded in paraffin, sectioned and incubated with the appropriate antiserum overnight at 23 °C. In all cases, the antigen–antibody complexes were visualized using the Elite Vectastain ABC kit (Vector Laboratories). For immunoblotting, protein extracts were prepared by homogenizing whole flies in 2× Laemli's sample buffer containing 1% β-mercaptoethanol at 75 °C for 30 min. Fly extracts (0.5 fly equivalents per lane) were electrophoresed on SDS–polyacrylamide gels and blotted onto PVDF membranes (Millipore). Blots were incubated with affinity-purified anti-Vol sera overnight at 4 °C, HRP-conjugated goat-anti-rabbit IgG (Jackson Laboratories) for 1 h at 23 °C, and visualized with SuperSignal Chemiluminscent substrate (Pierce).

Behavioural analyses. The differential olfactory conditioning method[4], pairing the presentation of one odour with aversive shock and a second odour with the absence of shock, was used to assess learning and memory performance. Training and testing were performed blind to strain under dim red light at 23–25 °C and 63–68% relative humidity using procedures described[22]. In each group, a performance index was calculated as the fraction of flies that avoided the CS+ minus the fraction of flies that avoided the CS–, and multiplied by 100. In practice, performance-index scores ranged from 0 (naive behaviour) to 100 (perfect performance). Because the minimum possible time between training and testing is 3 min (owing to handling and recovery of flies after transfer), 3-min memory reflects the earliest testable time point. To test longer-term memory, the flies were returned as a group to their collection vials for the appropriate retention interval and then tested as above. Odour avoidance was calculated as the fraction of flies that avoided the odour in one arm minus the fraction of flies that avoided fresh air provided in the control arm, and multiplied by 100. Electroshock avoidance was calculated similarly.

Statistics. Statistical analyses were performance using Statview 2.0 (Abacus Concepts, Berkeley, CA). Overall analyses of variance (ANOVA) were followed by planned comparisons contrasting the relevant groups. Error rate arising from multiple comparisons was controlled by dividing the alpha level by the number of comparisons being performed on a given set of data[42]. Only statistical results pertinent to the discussion of result are presented in the text; the complete statistical analysis is available as Supplementary Information.

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Correspondence and requests for materials should be addressed to R. L. Davis (e-mail: rdavis@bcm.tmc.edu). The sequence has been deposited in EMBL/GenBank/DDBJ with accession numbers from AF034199 and AF034200.

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