Protocols for the 38-l bioassays are detailed in Supplementary Information. Water was taken from an aquarium previously amended with an environmental water sample from Slocum Creek, North Carolina, and verified by cell counts, SEM and molecular analyses to contain high densities of *P. shumwayae*.

Water from the actively killing tank was used as a positive ('whole water') control. An enriched 'dinoflagellate' fraction was produced by filtering, rinsing and resuspending 3 l of whole water. Viability of dinoflagellates in this fraction was confirmed microscopically. A 'bacteria' fraction was obtained by centrifuging 3 l of whole water at 8,500 *g* for 45 min at 10 °C, resuspending the pellet in 12‰ ASW, filtering through a 5-μm filter to remove dinoflagellates and other protozoa, and bringing the filtrate to 3 l using 12‰ ASW. A 'cell-free' fraction was obtained by removing the supernatant from the centrifuged bacterial pellet and filtering through a 5-μm and then a 0.45-μm filter to a volume of 3 l. Filter-sterilized 12‰ ASW was used as a negative control. We also used a 'high ammonia' control consisting of 12‰ ASW with ammonia and pH adjusted to that of the whole water.

Electron microscopy

Fish killed with tricaine methanesulphonate (MS-222) were fixed in 4% glutaraldehyde with 5% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, at room temperature for ~2 h. Samples were washed with three changes of 0.1 M sodium cacodylate buffer, 15–30 min each, and stored overnight at 4 °C in a third change of buffer. They were postfixed with 1% OsO_4 in 0.1 M buffer, pH 7.2, at room temperature for 1 h and then washed with buffer in three changes of 0.1 M cacodylate, pH 7.2, 15–30 min each. The caudal peduncle was cut off the fish with a single-edged razor blade and processed for TEM analysis, and the body of each fish was processed for SEM by standard methods detailed in Supplementary Information.

Other methods

Larval fish sources, water quality measurements and cell count protocols are detailed in Supplementary Information.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Protein phosphatase 1 is a molecular constraint on learning and memory

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Repetition in learning is a prerequisite for the formation of accurate and long-lasting memory. Practice is most effective when widely distributed over time, rather than when closely spaced or massed. But even after efficient learning, most memories dissipate with time unless frequently used[1,2]. The molecular mechanisms of these time-dependent constraints on learning and memory are unknown. Here we show that protein phosphatase 1 (PP1) determines the efficacy of learning and memory by limiting acquisition and favouring memory decline. When PP1 is genetically inhibited during learning, short intervals between training episodes are sufficient for optimal performance. The enhanced learning correlates with increased phosphorylation of cyclic AMP-dependent response element binding (CREB) protein, of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and of the GluR1 subunit of the AMPA receptor; it also correlates with CREB-dependent gene expression that, in control mice, occurs only with widely distributed training. Inhibition of PP1 prolongs memory when induced after learning, suggesting that PP1 also promotes forgetting. This property may account for

ageing-related cognitive decay, as old mutant animals had preserved memory. Our findings emphasize the physiological importance of PP1 as a suppressor of learning and memory, and as a potential mediator of cognitive decline during ageing.

One feature of many forms of vertebrate learning is that practice determines whether learned material is remembered or forgotten, and whether the retained information is precise or vague. During practice, the pattern of distribution of training sessions is critical, and long intervals between sessions allow better encoding and more robust memory than do short intervals[1]. A possible explanation for this interval-dependent phenomenon is that with frequent and repetitive training, the processing of information from a trial is attenuated because information from the previous trial is still being processed, suggesting that a minimal delay is required for complete input integration[2]. An additional temporal limit on learning and memory is that much of the information that is stored after learning gradually dissipates with time unless regularly retrieved and re-consolidated[3]. These time-dependent constraints on learning and memory are poorly understood.

Several reports have suggested the existence of endogenous molecular suppressors that negatively control the efficacy of neuronal transmission and memory formation[4]. One proposed category of such memory suppressors is constituted by protein phosphatases. These molecules, together with protein kinases, regulate many cellular processes by the reversible phosphorylation/dephosphorylation of specific substrates[5]. For instance, the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (PP2B) was recently reported to block learning, memory storage and memory retrieval[6–8]. Here we examine the role of the protein phosphatase 1 (PP1), a phosphatase thought to act downstream from calcineurin and to negatively regulate synaptic plasticity[9,10] in the mechanisms that constrain learning efficacy and that underlie forgetting.

To determine whether delays between training sessions influence learning in rodents, we tested mice in an object recognition task[11]. In this task, memory traces of different strength can be elicited by varying the number and duration of training sessions. Memory for objects is inferred from the animal's ability to distinguish a novel

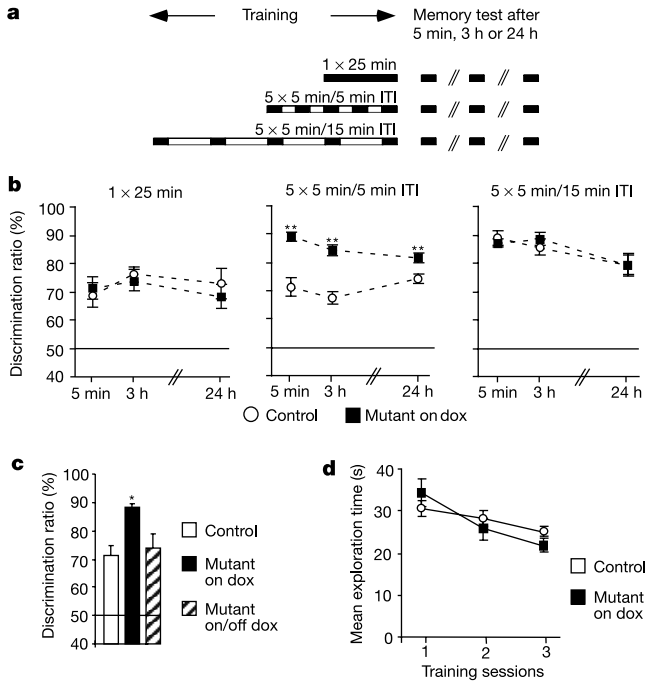

Figure 1 Distributed training improves performance in the object recognition task. **a**, Scheme of training and testing protocols. ITI, inter-trial interval. **b**, Discrimination ratios in control mice 5 min ($F(1,23) = 10.296$, $P < 0.01$, for 5-min versus 15-min ITI), 3 h ($F(1,9) = 22.976$, $P = 0.001$ for 5-min versus 15-min ITI) or 24 h after training with either one of the protocols described in **a**. Discrimination ratios in mutant mice 5 min ($F(1,13) = 16.565$, $P = 0.001$ for 1 × 25-min versus 5 × 5 min/5-min ITI), 3 h ($F(1,13) = 5.217$, $P < 0.05$ for 1 × 25-min versus 5 × 5 min/5-min ITI), or 24 h ($F(1,20) = 14.823$, $P = 0.01$ for 1 × 25-min versus 5 × 5 min/5-min ITI) after training. Lines at 50 indicate chance level. Dox, doxycycline. **c**, Reversibility of the enhancement in performance. Mutant mice ($n = 9$) were treated with dox, trained with a set of objects for five 5-min sessions with 5-min ITIs and tested 5 min later (Mutant on dox). Dox was withdrawn and the animals were trained 2 weeks later with five 5-min sessions/5-min ITIs using a second set of objects, then tested 5 min later (Mutant on/off dox; $F(1,14) = 6.816$, $P < 0.05$ mutant on dox versus on/off dox). Control mice $n = 22$. **d**, Representative graph of total exploration during training in control ($n = 22$) and mutant ($n = 7$) mice. Decrease in total exploration per object across training sessions (1 to 3) indicates habituation to the objects. Control groups in these experiments and the following ones were treated or not treated with dox. Results from these groups were similar and therefore were pooled.

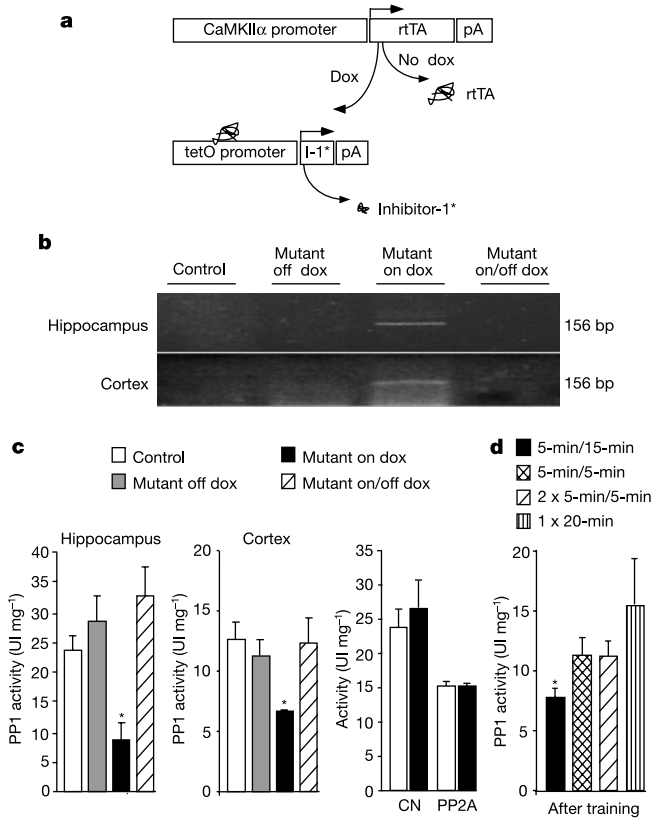

Figure 2 Genetic or training-dependent inhibition of PP1. **a**, Schematic representation of the transgenes used to achieve dox-dependent *I-1** expression. Heterozygous mice expressing rtTA with the CaMKIIα promoter were crossed with heterozygous mice carrying the *tetO* promoter fused to the *I-1** gene. In double transgenic animals, dox induces *I-1** expression while no expression occurs in the absence of dox. pA, polyadenylation signal. **b**, Dox-dependent and reversible *I-1** mRNA expression. A 156-base-pair (156 bp) *I-1** band was detected by RT–PCR in hippocampus and cortex from mutant mice on dox. There was no band in the absence of dox (Mutant off dox) or two weeks after dox removal (Mutant on/off dox). **c**, Dox-dependent and reversible inhibition of PP1. PP1 activity is reduced in hippocampus and cortex of mutant mice on dox ($n = 3$) compared to control mice ($n = 6$), mutant mice off dox ($n = 4$) or mutant mice on/off dox ($n = 3$) ($P < 0.05$ for all). Right panel, calcineurin (CN) and PP2A activity in control and mutant mice. UI, nmol phosphate released per min per mg protein. **d**, Training-dependent inhibition of PP1. PP1 activity is reduced in cortex of control mice trained for 5 min followed by a 15-min delay ($n = 6$, versus naive or all other trained groups, $P < 0.05$) but not in mice trained once for 5 min followed by a 5-min delay ($n = 4$) or twice for 5-min followed by a 5-min delay (20 min in total, $n = 3$), or trained once for 20 min ($n = 3$).

object from familiar objects after learning. Mice were trained on a massed (a single 25-min trial) or a distributed protocol with brief intervals (five 5-min trials with 5-min inter-trial intervals, ITIs) then tested for their memory for objects (Fig. 1a). In control mice, memory performance was moderate when tested 5 min ($n = 7$), 3 h ($n = 9$) or 24 h ($n = 8$) after massed training (open circles in left panel, Fig. 1b). Similarly, distributed training with brief ITIs elicited average discrimination ratios after 5 min ($n = 10$), 3 h ($n = 6$) or 24 h ($n = 13$) ($P > 0.1$ versus massed training, middle panel, Fig. 1b), denoting the limited efficacy of massed or brief-interval training. To test whether longer delays between training trials would be more efficient, ITIs were extended from 5 to 15 min. These longer intervals led to enhanced performance whether the animals were tested 5 min ($n = 7$), 3 h ($n = 6$) or 24 h ($n = 6$) after training ($P < 0.01$ versus 5-min ITIs, right panel, Fig. 1b).

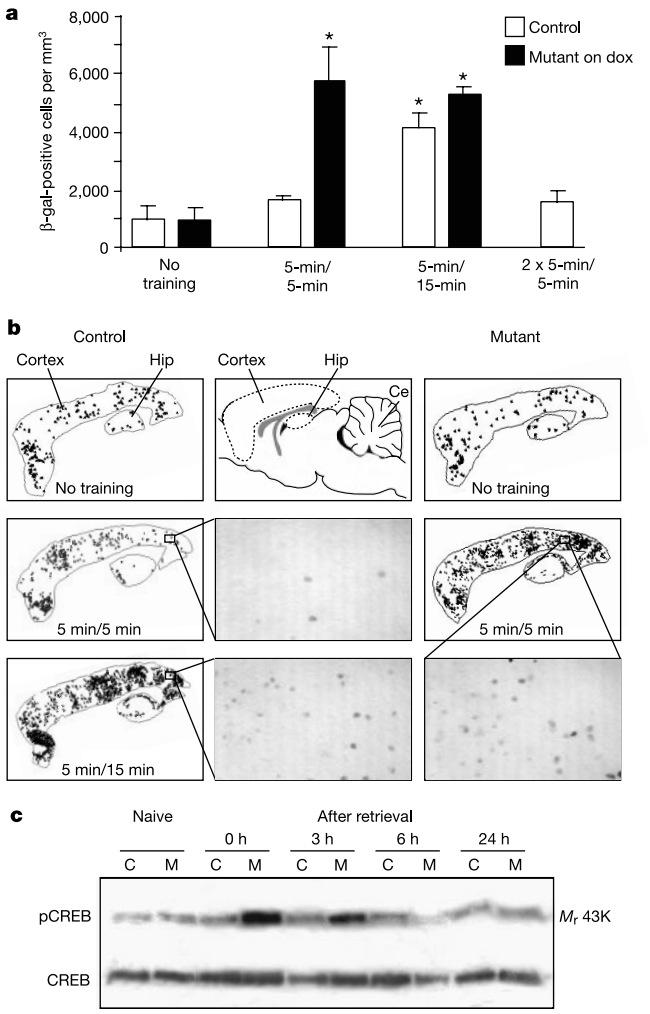

Figure 3 Enhanced CREB activity and phosphorylation. **a**, CREB transcriptional activity during training. Bar graphs are mean number of β-galactosidase positive cells per volume of cortex before training (no training, control, $n = 4$; mutant on dox, $n = 3$), and either after 5-min training followed by a 5-min delay (5-min/5-min, control, $n = 3$; mutant on dox, $n = 3$), 5-min training followed by a 15-min delay (5-min/15-min, control, $n = 3$; mutant on dox, $n = 3$), or two sessions of 5-min training followed by a 5-min delay (2 × 5-min/5-min, control, $n = 3$). **b**, Representative diagrams and corresponding close-ups of cell counts in control (left panels) and mutant (right panels) mice before training (no training), or after 5-min training followed by a 5-min delay or 5-min training followed by a 15-min delay (only control). Middle top panel, scheme of a mouse brain with cortex and hippocampus (Hip) lined up such as displayed in left and right diagrams. Ce, cerebellum. **c**, Time course of CREB phosphorylation after retrieval. Representative western blot of pCREB in control (C) and mutant mice on dox (M) not trained (naive) or trained on five sessions of 5-min with 5-min ITIs and tested for retrieval 24 h later. The levels of pCREB and total CREB (relative molecular mass $M_r = 43{,}000$) were examined either immediately, 3, 6 or 24 h after retrieval (both control and mutant mice, $n = 2$ for each time point). In the mutant mice, pCREB level was high immediately after, and 3 h after, memory retrieval, then dropped back to baseline after 6 h. The pCREB level was only slightly increased in control mice after 3 and 6 h. The level of total CREB protein was similar in control and mutant animals.

To assess if this effect may result from the relief of a memory suppressor and examine whether PP1 could be such a suppressor, PP1 activity was genetically reduced in the brain of transgenic mice and the effect on performance was evaluated. Mutant mice expressing inducibly a constitutively active form of inhibitor 1 (*I-1**)[12], an endogenous inhibitor of PP1 (ref. 13), were generated using the reversible tetracycline-controlled transactivator (rtTA) system under the control of the Ca^{2+}/calmodulin-dependent protein kinase α (CaMKIIα) promoter[7,14] (Fig. 2a). In double transgenic animals, *I-1** messenger RNA was expressed in hippocampus and cortex (Fig. 2b) and PP1 activity was reduced by respectively 67.7 ± 12% and 45.5 ± 1% in these brain areas upon doxycycline (dox) treatment ($P < 0.05$, Fig. 2c). No mRNA expression (Fig. 2b) and no reduction in PP1 activity (Fig. 2c) were detected in mutant animals not treated with dox. Moreover, transgene mRNA expression (Fig. 2b) and PP1 inhibition (Fig. 2c) were fully reversible and could be suppressed in both adult hippocampus and cortex after dox removal. Phosphatase inhibition was specific to PP1 as calcineurin or PP2A activity was not changed by transgene expression in the mutant mice (Fig. 2c).

In *I-1** mutant mice, massed training in the object recognition task led to moderate performance 5 min ($n = 8$), 3 h ($n = 8$) or 24 h ($n = 8$) after training, similarly to control mice (filled squares in left panel, Fig. 1b). By contrast with closely spaced sessions, *I-1** mutant mice had significantly enhanced memory for objects at all intervals after training (5 min and 3 h, $n = 7$; 24 h, $n = 14$, $P < 0.01$ versus control, middle panel, Fig. 1b). Indeed, performance was similar to that achieved in control mice trained with widely spaced sessions and was optimal, as widely distributed training in *I-1** mutant mice did not further increase discrimination (5 min, $n = 9$; 3 h, $n = 8$; 24 h, $n = 8$; right panel, Fig. 1b). This improvement was directly due to PP1 inhibition and not to any permanent alteration resulting from *I-1** transgene expression, as it was fully reversed by suppressing transgene expression and retraining the animals with different objects (Mutant on/off dox, Fig. 1c). Moreover, this reversion was not due to prior learning as two consecutive sessions of training led to similar performance on both sessions in control mice. Furthermore, in mutant mice, transgene expression induced only after the second session (mutant off/on) enhanced performance to a similar extent as when induced before the first session (not shown). Finally, total exploration of objects during training was similar in all groups, reflecting comparable motivation (Fig. 1d). These results therefore indicate that both the genetic inhibition of PP1 and long ITIs in training favoured learning and memory in a similar and mutually occlusive manner. To confirm this effect, we examined whether long ITIs in control mice correlated with PP1 inhibition. PP1 activity was consistently and significantly reduced in control animals trained for 5 min followed by a 15-min delay, but not in animals trained for one or two sessions of 5 min followed by a 5-min delay or trained on a massed 20-min session (Fig. 2d), supporting the hypothesis that widely distributed training is more efficient because it relieves a PP1-dependent constraint. This profound effect of training on PP1 may reflect the need for rapid neutralization of PP1, possibly together with activation of opposing kinases, for optimal signal transduction.

To identify the signalling pathways involved and determine whether PP1 inhibition recruits similar mechanisms whether induced genetically or with long ITIs, we examined several targets of PP1. As the formation of long-term memory requires protein

synthesis and involves primarily the transcription factor CREB protein[15,16], a PP1 substrate[17], we first examined CREB activity during learning. Hence, we crossed the *I-1** animals with reporter mice carrying CREB-responsive elements (Cre) fused to the *lacZ* gene in which CREB-mediated gene expression can be monitored by β-galactosidase staining[18]. Before training, β-galactosidase expression was low in *I-1** control and mutant mice carrying the *lacZ* gene (Fig. 3a, b), indicating minimal CREB activation in resting conditions. By contrast, in mutant but not in control mice, 5-min training followed by a 5-min delay strongly increased CREB-dependent gene expression in both cortex ($F(1, 4) = 13.41$, $P < 0.05$, Fig. 3a, b) and hippocampus (bar graph not shown but see Fig. 3b). Likewise, a 15-min delay following training induced an intense signal in mutant mice ($F(1, 4) = 153.62$, $P < 0.01$). A similarly strong activation was achieved in control mice only when training was followed by a 15-min delay ($F(1, 4) = 28.828$, $P < 0.01$ versus 5-min delay, Fig. 3a, b). This activation was directly dependent on training distribution rather than on the sole passage of time after training, as two consecutive sessions of 5-min training/5-min delay (20 min total) did not induce more gene expression than a single such session in control mice (Fig. 3a). Finally, memory retrieval also appeared to be constrained by PP1, because the levels of phosphorylated CREB (pCREB), a marker for CREB activation[19], were significantly increased in the mutant mice after retrieval (Fig. 3c). Overall, these results indicate that both the genetic inhibition of PP1 and long delays during training increase CREB transcriptional activity in the brain, suggesting that PP1 and short intervals constrain learning by preventing gene expression. These data are consistent with previous studies in *Drosophila* and mice showing that CREB is important for determining the number of trials and the trial intervals necessary for the formation of long-term memory[16].

Once learned and stored, information must be maintained in memory over time for later recall. To assess whether PP1 can also influence the persistence of memory, we tested spatial memory in the *I-1** mice using a water maze[20]. In this maze, animals have to find an escape platform hidden in a tank of opaque water by using spatial cues placed in the experimental room. Trial after trial, new spatial information must be learned, remembered and retrieved to accurately navigate to the platform. Memory for the platform was tested at various delays after training. Consistent with the improvement in the object recognition test, spatial performance in the mutant mice was enhanced on the water maze. During training, the mutant mice located the platform faster and reached minimal escape latencies sooner than control mice (five versus seven training blocks, $F(1,274) = 10.96$ $P < 0.01$ overall, Fig. 4a), indicating that the *I-1** transgene enhanced spatial learning and memory.

In an attempt to relate this effect to a biochemical modification,

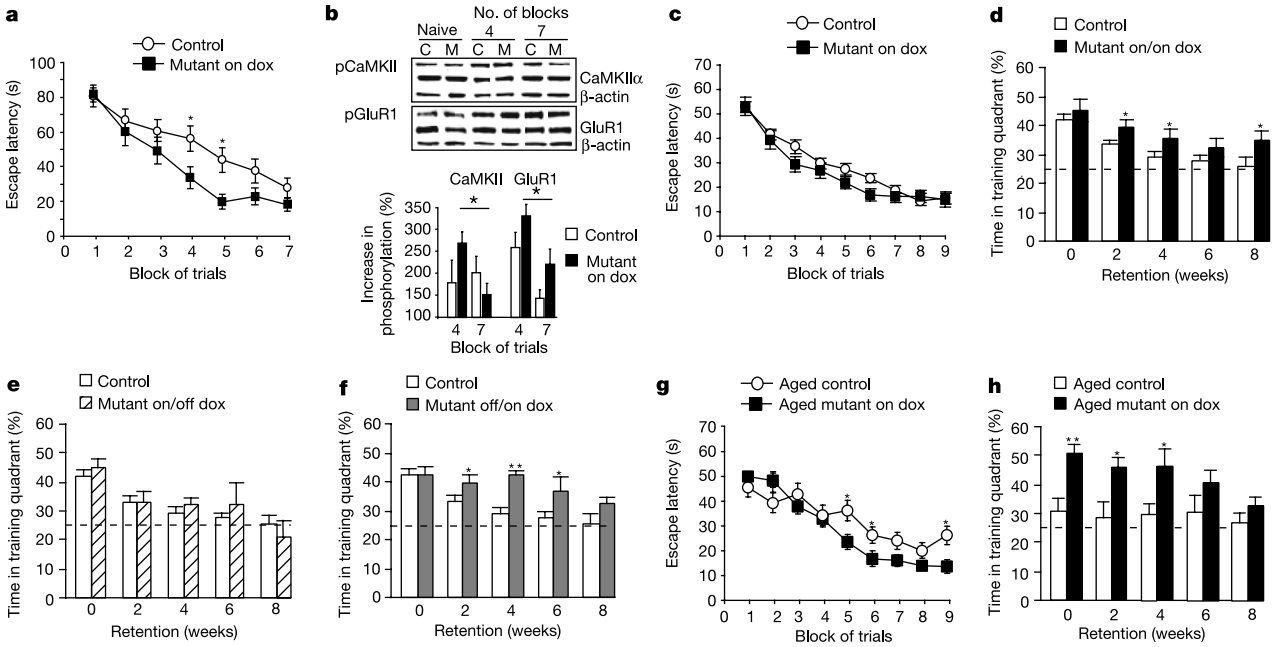

Figure 4 Improved spatial learning and memory in *I-1** mutant mice. **a**, Spatial learning in control mice ($n = 10$) and mutant mice on dox ($n = 10$) trained on seven consecutive blocks of two trials (90 seconds each) separated by 30–60 min. **b**, CaMKII and GluR1 phosphorylation in spatial training. Control and mutant mice on dox were trained as in **a**, and the levels of pCaMKII and pGluR1 were analysed by western blotting of membrane-enriched hippocampal preparations either before training (naive, CaMKII: control (C), $n = 2$; mutant (M), $n = 2$; GluR1: C, $n = 4$; M, $n = 3$) or 10 min after 4 training blocks (CaMKII: C, $n = 2$; M, $n = 3$; GluR1: C, $n = 4$; M, $n = 3$) or 7 training blocks (CaMKII: C, $n = 3$; M, $n = 2$; GluR1: C, $n = 3$; M, $n = 3$) (top panels). Total CaMKIIα and GluR1, and β-actin were measured for quantification. Per cent increase in CaMKII and GluR1 phosphorylation in naive mice versus trained mice after 4 and 7 training blocks (bar graphs). **c**, Spatial acquisition in control mice ($n = 28$) and mutant mice on dox ($n = 27$) trained on nine consecutive blocks of three trials (60 seconds each) separated by 24 h. This training phase was followed by a 7-day training period using another platform position (not shown). **d**–**f**, Spatial memory in control and mutant mice treated with dox either **d**, during and after training (on/on dox); **e**, only during training (on/off); or **f**, only after training (off/on) and probe trials were performed 3 h (time point 0, control, $n = 28$; mutant on dox, $n = 28$; mutant off/on dox, $n = 13$; mutant on/off dox, $n = 6$), 2 weeks (control, $n = 28$; mutant on dox, $n = 19$; mutant off/on dox, $n = 13$; mutant on/off dox, $n = 6$), 4 weeks (control, $n = 28$; mutant on dox, $n = 16$; mutant off/on dox, $n = 11$; mutant on/off dox, $n = 6$), 6 weeks (control, $n = 28$; mutant on dox, $n = 15$; mutant off/on dox, $n = 13$; mutant on/off dox, $n = 6$), and 8 weeks (control, $n = 24$; mutant on dox, $n = 14$; mutant off/on dox, $n = 13$; mutant on/off dox, $n = 5$) after training. In mutant on/off dox, behavioural testing was performed two weeks after dox removal. Dashed lines indicate chance level (25%). **g**, **h**, Spatial performance in aged mice. Learning curve (**g**), and probe trials (**h**), in aged control ($n = 9$) and mutant ($n = 9$) mice trained on nine consecutive blocks of three trials separated by 24 h (no platform transfer) and treated with dox during and after training. Probe trials were performed 1 day (time point 0), 2 weeks, 4 weeks, 6 weeks and 8 weeks after training. For all experiments, the time spent in other quadrants of the maze was similar in all groups and not significantly different from chance (not shown). Asterisk, $P < 0.05$; double asterisk, $P < 0.01$.

we examined the levels of phosphorylation of another two targets of PP1, CaMKII and the GluR1 subunit of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor[21–24]. Both are present in synapses and therefore are more likely to be activated during short training trials (like the water maze trials) than are late components of the transcriptional machinery in the nucleus. Western blot analyses revealed that the levels of both CaMKII phosphorylated at Thr 286 (pThr286CaMKII, referred to as pCaMKII) and GluR1 phosphorylated at Ser 845 (pSer845GluR1, referred to as pGluR1) increased after training in control and mutant mice ($P < 0.05$, Fig. 4b). In the mutant mice, the increase in both pCaMKII and pGluR1 was larger than in control mice after training (genotype effect, $P \leq 0.05$). These results suggest a correlation between CaMKII and GluR1 phosphorylation and performance, and a possible involvement of these PP1 targets in the observed improvement.

In order to examine long-term spatial memory on the water maze, we elicited robust learning in all groups. For this, we used a more intensive training protocol (3 trials per day over 9 days). With such protocol, both control and mutant groups learned similarly well and reached minimal escape latencies after 8–9 days ($P < 0.05$ overall, Fig. 4c). Likewise, both groups rapidly learned a second platform position (not shown). Similar learning in control and mutant mice suggested here that the transgene effect was occluded by sustained training. We then tested spatial memory on several successive probe trials. One day after training, memory for the platform position was high in both groups. After 2 weeks, however, it gradually decayed in control mice ($F(1,54) = 10.861$, $P < 0.01$) and was fully extinguished by 6 weeks (Fig. 4d). But in *I-1** mutant mice, performance declined only slightly and remained significantly higher than in control mice and than baseline up to 4 weeks after training ($P < 0.05$ versus control at 2, 4 and 8 weeks), indicating that PP1 inhibition prolonged memory (Fig. 4d). We determined more precisely the temporal window of this effect by taking advantage of the inducibility of the transgene expression and inducing it either during or after training. Although *I-1** expression only during training did not prevent the weakening of memory (Fig. 4e), its induction only after learning was sufficient to maintain memory for up to 6 weeks after training ($P < 0.05$ versus control at 2 and 6 weeks, $P < 0.01$ at 4 weeks, Fig. 4f). This indicated that independent of its function during learning, PP1 specifically acts after learning to promote forgetting. It is not known whether PP1 is also involved in extinction, a form of memory erasure that, unlike forgetting, is associated with re-learning of new information.

To further investigate the physiological relevance of the PP1-dependent mechanism to forgetting, in particular in ageing-related memory decline[25], we tested old animals (15–18 months) in the water maze. Although both aged control and mutant mice were able to learn the platform position when intensively trained ($P < 0.05$ overall), performance was significantly lower in the control mice ($P < 0.05$ versus aged mutant, overall), suggesting a slight deficit in spatial learning (Fig. 4g). Spatial memory was also impaired in aged control mice one day after learning ($P > 0.1$ versus baseline, Fig. 4h) or later, whereas it was still robust in the mutant mice 24 h ($F(1,16) = 16.243$, $P = 0.001$ versus aged control) or even 4 weeks after learning ($P < 0.05$ versus aged control at 2 and 4 weeks, Fig. 4h), indicating again that PP1 inhibition prevents memory decline.

The present findings demonstrate the existence of a molecular constraint operated by PP1 that regulates both the acquisition and the retention of information. This constraint may preserve synaptic circuits from saturation in young individuals, but its dysregulation in old animals may precipitate memory decay[26,27]. These data further strongly suggest that forgetting in ageing may not necessarily result from an irreversible rundown of molecular components but rather from the active intervention of PP1, possibly through mechanisms that depend on NMDA[28] (*N*-methyl-D-aspartate) receptor, opening new perspective for therapeutic treatment of aged individuals. The possibility that I-1* may partly mediate its effects through an unknown function independent of PP1 inhibition cannot, however, be excluded. Finally, our findings shed new light on the function of I-1, a PP1 inhibitor thought to be critical for synaptic plasticity[29]; we suggest that the function of I-1 should be further investigated by complementary electrophysiological analyses. □

Methods

Mice

We produced *tetO-I-1** mice by microinjection of the *tetO* promoter (pMM400 plasmid[30]) fused to the *I-1** gene[12] into F_2 C57Bl/6J × CBA fertilized eggs, and backcrossed for 4–6 generations to C57Bl/6J background. CaMKIIα promoter-rtTA and Cre-*lacZ* mice were also backcrossed to C57Bl/6J background. Double (*I-1**/rtTA) and triple (*I-1**/rtTA/*lacZ*) transgenic mice were obtained by heterozygous crossings. Control mice are littermates carrying no transgene or either one of the transgenes. Before testing, mice were fed dox (Westward Pharmaceuticals Corp.) at 6 mg per g food when indicated. For all experiments, control mice were treated or not treated with dox and results were pooled. Male mice 2.5–6 months old were used for behaviour. Aged males were 15–18 months old.

Assays

Polymerase chain reactions after reverse transcription of RNA (RT–PCRs) were performed as previously described[8] using a Superscript kit (GibcoBRL) and oligonucleotides specific for the *I-1** transgene. For phosphatase assays, hippocampi and cortex were removed from adult brain and homogenized with a syringe in lysis buffer (50 mM Tris-HCl buffer at pH 7.5, 200 mM NaCl, 12 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.05% NP-40, 1 mM $CaCl_2$) containing a cocktail of protease inhibitors (Sigma). Cellular debris were removed by ultracentrifugation (150,000*g*) for 1 hour at 4 °C then supernatants were desalted by gel filtration. Phosphatase activity was measured using a Biomol green assay kit (BioMol) in the presence or absence of either EGTA to block calcineurin activity, okadaic acid to block PP1 and PP2A activity, or inhibitor-2 (Bioconcept) to block PP1 activity.

Western blotting

For CREB, crude extracts were prepared from cortex by homogenization in lysis buffer (50 mM Tris-HCl pH 7.5, 20 mM EGTA, 200 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40) containing a cocktail of protease and phosphatase inhibitors. For CaMKII and GluR1, membrane-enriched preparations were obtained from hippocampi by homogenization in ice-cold buffer (0.32 M sucrose, 50 mM Tris-HCl pH 7.5, 4 mM EGTA, 10 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 10 mM NaF, 1 mM phenylmethylsulphonyl fluoride, PMSF) containing protease inhibitors, centrifugation then resuspension in buffer without sucrose. All samples were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) before electroblotting. Membranes were incubated either with anti-pSer 133 CREB (1:1,000), anti-CREB (1:1,000), anti-pThr 286 CaMKII (1:1,000), anti-CaMKIIα (1:2,000), anti-pSer 845 GluR1 (1:1,000), anti-GluR1 (1:1,000) or anti-β-actin (1:1,000) antisera (Upstate Biotechnology), and visualized by chemiluminescence before densitometric quantification (NIH Image).

β-galactosidase staining

Brains were extracted immediately after testing, frozen on dry ice then kept at −80 °C until used. 40-μm cryostat sagittal sections were fixed for 10 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS, washed, then incubated in X-gal solution (5 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 3 mM $MgCl_2$) at 37 °C overnight. The number and distribution of X-gal-positive cells were determined on nine sections cut across one hemisphere (with six intervening sections) by stereology using the optical fractionator method (Stereoinvestigator software, MicroBrightfield Corp.). Sampling frames (300 × 300 × 20 μm) were used to count cells in 30–50 randomly selected areas within each tissue section.

Behaviour

The object recognition task was performed as previously described[8] using an automated tracking system (Viewpoint). Briefly, all mice were habituated to an empty arena (63 × 51 × 25 cm) three times for 5–10 min then trained in the presence of three objects placed in fixed locations according to the protocol described in Fig. 1a. At various intervals following training, one of the familiar objects was replaced with a novel object and memory was tested in a 5-min session. Independent groups of animals were used for each time point. Discrimination ratio corresponds to the time spent exploring the novel object divided by the total time of exploration of all objects. The water maze task was performed as described previously[8] with 60-s or 90-s training trials. Learning was evaluated by monitoring escape latencies during training with an automated tracking system (Viewpoint). For probe trials, the platform was removed from the maze and the animals were allowed a 60-s search. The same groups of mice were successively tested on each probe trial. The time (%) spent in each quadrant of the maze was recorded. Animals were left on the platform for 30 seconds after each training trial and each probe trial. Swimming speed was similar in control and mutant animals (not shown). The experimenter was blind to genotype for all behavioural tests.

Data analysis

One- or two-way analyses of variance (ANOVAs) with genotype as between-subject factor and session, block, day or quadrant as within-subject factor, followed by Fisher post-hoc tests (when necessary) were performed. Data are mean ± s.e.m.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Chromosomal clustering of muscle-expressed genes in *Caenorhabditis elegans*

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Chromosomes are divided into domains of open chromatin, where genes have the potential to be expressed, and domains of closed chromatin, where genes are not expressed[1]. Classic examples of open chromatin domains include 'puffs' on polytene chromosomes in *Drosophila* and extended loops from lampbrush chromosomes[2,3]. If multiple genes were typically expressed together from a single open chromatin domain, the position of co-expressed genes along the chromosomes would appear clustered. To investigate whether co-expressed genes are clustered, we examined the chromosomal positions of the genes expressed in muscle of *Caenorhabditis elegans* at the first larval stage. Here we show that co-expressed genes in *C. elegans* are clustered in groups of 2–5 along the chromosomes, suggesting that expression from a chromatin domain can extend over several genes. These observations reveal a higher-order organization of the structure of the genome, in which the order of genes along the chromosome is correlated with their expression in specific tissues.

We developed a method called messenger RNA tagging to isolate muscle mRNA, because this tissue is difficult to isolate in *C. elegans*. The basis of the technique is to use a characterized promoter to express an epitope-tagged mRNA-binding protein, such as poly(A)-binding protein (PAB-1), in cells or tissues of interest (Fig. 1). Because poly(A)-binding proteins bind tightly to the poly(A) tail of mRNAs[4], mRNAs from specific tissues can be enriched by cross-linking them to the tagged PAB-1, and co-immunoprecipitating the complex of mRNA and tagged PAB-1 using an anti-epitope monoclonal antibody[20]. DNA microarrays can then be used to identify which mRNAs have been enriched by co-immunoprecipitation, indicating that the corresponding gene is expressed in the same cells as the tagged PAB-1.

To isolate the mRNA expressed in muscle, we first generated animals that express Flag::PAB-1 in non-pharyngeal muscles from an integrated transgene using the *myo-3* promoter[5] (*myo-3*p; see Methods) (Fig. 2a). The mRNA–Flag::PAB-1 complex was co-immunoprecipitated from cell lysate using anti-Flag monoclonal antibodies. About 55% of the Flag::PAB-1 was immunoprecipitated from the lysate (Fig. 2b). Experiments using dot blot techniques and polymerase chain reaction with reverse transcription (RT–PCR) show that *unc-54*, which is specifically expressed in muscle[5], was co-immunoprecipitated with the muscle-expressed Flag::PAB-1 but that *gld-1*, which is specifically expressed in the germ line[6], was not (Fig. 2c and data not shown).

Next, we used DNA microarrays to analyse the ratio of the mRNA enriched by co-immunoprecipitation with Flag::PAB-1 relative to the mRNA present in the starting cell-free extract. Fluorescently labelled probes (see Methods) were then hybridized to DNA microarrays[7] containing 90% of the 19,733 genes currently estimated in the *C. elegans* genome[8]. We repeated the mRNA-tagging experiment six times to assess statistically which genes are enriched.

We found that the rank order of genes that are enriched in each immunoprecipitation experiment is more consistent than their absolute level of enrichment. This indicates that the immunoprecipitation procedure enriches genes consistently relative to each