

Activity-induced targeting of profilin and stabilization of dendritic spine morphology

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Morphological changes in dendritic spines have been implicated in connective plasticity in brain circuitry, but the underlying pathway leading from synaptic transmission to structural change is unknown. Using primary neurons expressing GFP-tagged proteins, we found that profilin, a regulator of actin polymerization, is targeted to spine heads when postsynaptic NMDA receptors are activated and that actin-based changes in spine shape are concomitantly blocked. Profilin targeting was triggered by electrical stimulation patterns known to induce the long-term changes in synaptic responsiveness associated with memory formation. These results suggest that, in addition to electrophysiological changes, NMDA receptor activation initiates changes in the actin cytoskeleton of dendritic spines that stabilize synaptic structure.

Experimental manipulations of brain activity produce changes in the shape and number of dendritic spines that form the postsynaptic contact sites for excitatory synapses in the brain, suggesting that they are the substrate for morphological adaptations in brain circuits associated with long-term memory^{1–5}. Various studies have suggested that changes in dendritic spine morphology are important in experience-dependent sculpting of circuitry in the brain^{6–10}, but the cellular mechanisms underlying such changes have not yet been identified. Morphological plasticity in dendritic spines is driven by dynamic actin filaments concentrated in spine heads, and drugs that inhibit actin dynamics block dendritic spine motility^{11,12} and interfere with the development of long-term potentiation (LTP)^{13,14}. Together, these data suggest that actin may serve as a link between activity-induced modulation of synaptic transmission and long-term changes in synaptic morphology associated with memory consolidation.

Despite this evidence for the importance of actin dynamics in synaptic plasticity, very little is known about the regulation of actin at the synapse. In particular, the molecules that mediate between the activation of postsynaptic receptors and actin-based changes in spine morphology have not been identified. In the experiments reported here, we focused on profilin, a small actin-binding protein implicated in regulating actin polymerization at the cell surface^{15–17}. To examine its possible involvement in regulating actin dynamics in dendritic spines, we recorded profilin distribution and dendritic spine motility in live cultured hippocampal neurons expressing GFP-tagged profilin constructs and GFP-tagged actin.

RESULTS

NMDA receptors trigger profilin targeting to spine heads

Expressed as a GFP-tagged fusion protein in cultured hippocampal neurons, profilin II, the major brain isoform¹⁸, was highly enriched in heads of dendritic spines in ~4% of transfected cells ($n = 239$), whereas in other cells, spine enrichment was weak or undetectable

(Supplementary Fig. 1 online). By comparison, unmodified soluble GFP was always evenly distributed between dendrite shafts and spines (Supplementary Fig. 1 online). The enrichment of profilin II in spines of some cells, but not others, suggested that its cytoplasmic localization might be activity-dependent. To test this hypothesis, we treated transfected neurons with 10 μ M glutamate to stimulate postsynaptic glutamate receptors (Fig. 1 and Supplementary Fig. 2 online). Images captured before and after treatment showed a striking redistribution of profilin II-GFP into the heads of spines that began 5–8 min after glutamate addition and was maximal after 30 min (Fig. 1a). The same treatment did not change the distribution of unmodified GFP (Fig. 1b). In addition to brain-specific profilin II, we studied the ubiquitously distributed isoform profilin I, which is weakly expressed in brain^{18,19}. Compared to profilin II, GFP-tagged profilin I showed partial or no enrichment in dendritic spines before stimulation, and it was less markedly redistributed to spines after stimulation (data not shown).

To determine which glutamate receptor classes were involved in profilin targeting, we treated profilin II-GFP transfected cells with a variety of subtype-selective glutamate receptor agonists and antagonists (Table 1). Blocking NMDA receptors (treated with 10 μ M glutamate) with the antagonists APV or MK-801 greatly reduced the accumulation of profilin II in spines so that in 28 cells tested, none showed strong targeting and 5 showed partial spine labeling (Table 1a,b). By contrast, blocking either AMPA or metabotropic glutamate receptors, or both together, was far less effective in inhibiting glutamate-induced accumulation of profilin II in spines (Table 1b). The NMDA-receptor specificity of this effect was confirmed by treating cells with NMDA itself, which produced strong targeting of profilin II to spines in all cells examined (Table 1c). Stimulating cells with the metabotropic glutamate receptor agonist 3,5-dihydroxyphenylglycine (DHPG) was less effective, and AMPA was ineffective in 14 of 16 cells tested and showed only marginal effects in the remaining 2 cells (Table 1c).

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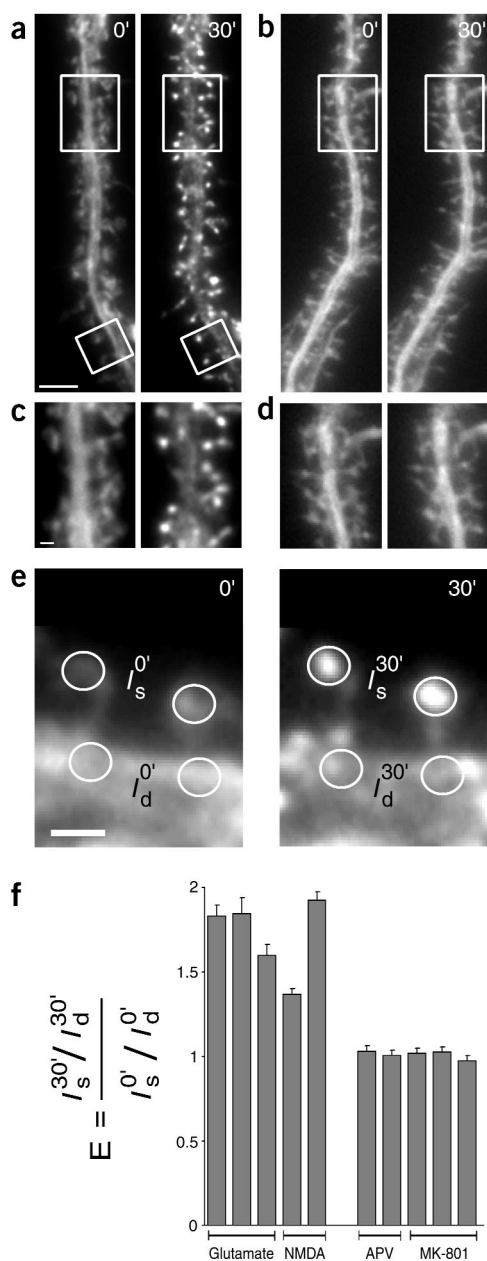


Figure 1 Glutamate receptor stimulation redistributes profilin II to dendritic spines. (a) Profilin II-GFP distribution in a segment of dendrite from a transfected cell before (0') and after (30') exposure to 10 μ M glutamate for 30 min. (b) Dendrite segment from a control cell expressing GFP where glutamate treatment does not induce protein accumulation in spines. (c) Detail of the rectangular area in a. (d) Detail of the rectangular area in b. (e, f) Quantification of profilin enrichment in dendritic spines. (e) Higher-magnification pictures of boxed area near the bottom of a. The circles indicate areas related to two spines where profilin II-GFP fluorescence was measured in the spine head and the underlying dendrite (see Methods for details). The relative enrichment (E) of profilin II-GFP in spines following stimulation was calculated from the fluorescence intensity in the spine head (I_s) and the underlying dendritic shaft (I_d), both before ($I_s^{0'}$, $I_d^{0'}$) and 30 min after ($I_s^{30'}$, $I_d^{30'}$) stimulation. (f) Histograms showing the profilin enrichment before and after stimulation. Left, data for 40 spines on dendritic segments from each of three cells treated with 5 μ M glutamate and two cells treated with 5 μ M NMDA. Right, data for cells treated with glutamate (5 μ M) together with NMDA receptor antagonists APV (500 μ M, 2 cells) or MK801 (50 μ M, 3 cells). Error bars represent s.e.m.; $P < 0.005$ for glutamate- and NMDA-treated cells versus APV- and MK-801 treated controls; single-tailed t -test. Scale bars: (a) 5 μ m, (c, e) 1 μ m.

(Fig. 2b and Table 2). Subsequently treating the same cells with 5 μ M NMDA produced strong targeting of profilin to spines (Fig. 2c; $n = 6$), showing that the postsynaptic receptor-dependent mechanism was intact following botulinum toxin treatment. Taken together, these results indicate that the accumulation of profilin II in spines is triggered by synaptically released glutamate acting on postsynaptic NMDA-type glutamate receptors. Long-term electrophysiological effects of NMDA receptor activation are Ca^{2+} -dependent^{20,21}. This also seems to be the case for profilin II targeting because in cells treated with 10 μ M glutamate in the absence of extracellular Ca^{2+} , no accumulation of profilin II in spines was detectable (data not shown).

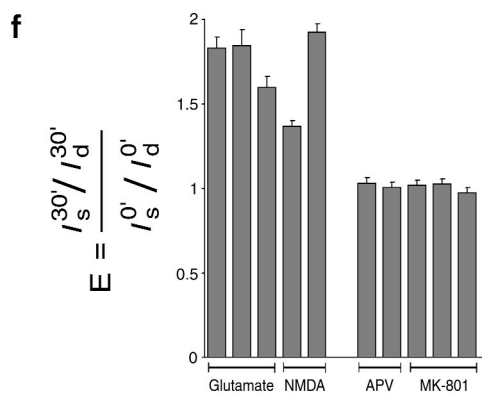
Electrical stimulation induces profilin targeting

Two leading cellular models for memory formation in the mammalian brain, LTP and LTD (long-term depression), are associated with the influx of Ca^{2+} through NMDA receptor channels activated by specific patterns of electrical stimulation^{22–26}. To evaluate their possible relationship to profilin targeting to dendritic spines, we examined profilin II-GFP expressing cells exposed to these stimulation patterns. Tetanic or theta burst stimulation patterns produced strong targeting of profilin to dendritic spines that was maximal after 45 min in 17 of 18 cells examined (Fig. 3). Low-frequency stimulation of 900 pulses at 1 Hz also produced strong targeting of profilin to spines after 45 min in 22 of 24 cells examined. Comparing profilin enrichment in dendritic spines induced by electrical stimulation patterns to that produced by glutamate or NMDA treatment showed that similar levels of profilin enrichment were produced by both electrical and pharmacological stimulation (Fig. 3d).

To control for the specificity of these effects, we exposed cells to a 'neutral' stimulation procedure that does not alter postsynaptic responsiveness²⁵. This neutral pattern involves applying a total of 900 pulses, but instead of the steady 1 Hz used in the low-frequency protocol, they are delivered in bursts of ten at 10 Hz, with an inter-burst interval of 10 s (Fig. 3a). In each of six cells tested using this procedure, there was no change in the distribution of profilin II-GFP between dendrite shafts and spines. As the accumulation of profilin in spines is Ca^{2+} -dependent, a possible reason why this neutral stimulation pattern did not provoke spine targeting could be that it does not raise postsynaptic Ca^{2+} levels enough to trigger the mechanism leading to profilin targeting. To test this possibility, we examined the effects of raising the extracellular Ca^{2+} concentration and found that after increasing it by 50% (from 2 to 3 mM), the previously ineffective

Presynaptic glutamate triggers profilin II targeting

Profilin II targeting to spines could also be induced by depolarizing cells through exposure to 90 mM KCl. This produced reliable accumulation of profilin II in spines, with strong targeting in 3 of 5 cells tested (Table 2). Depolarization of the postsynaptic membrane activates postsynaptic NMDA receptors by relieving the Mg^{2+} block that operates on them at resting membrane potential. To see whether this effect was involved in depolarization-induced profilin targeting, we followed the distribution of profilin II-GFP in transfected cells after removing Mg^{2+} from the medium when spontaneous release of glutamate from presynaptic terminals should be capable of activating postsynaptic NMDA receptors. After 30 min, 11 of 12 neurons examined showed increased concentrations of profilin II in spine heads (Fig. 2a and Table 2). This redistribution did not occur if presynaptic release of neurotransmitter was blocked by pre-incubating neurons ($n = 7$) with botulinum toxin (100 ng/ml) for 12 h preceding the experiment



'neutral' pattern now provoked profilin II targeting to spines in 12 of 14 cells examined (Fig. 3c). Together, these results suggest that activity-induced accumulation of profilin in dendritic spines depends on sustained elevation of postsynaptic Ca^{2+} levels.

Profilin targeting and stabilization of spine morphology

To evaluate the duration of profilin accumulation in dendritic spines following receptor activation, we treated profilin II-GFP expressing cells with 5 μ M glutamate for 30 min to induce spine targeting and examined their dendrites at various times after glutamate was removed (Supplementary Figs. 3 and 4 online). In experiments in which cells were monitored continuously, there was no reduction of profilin II-GFP levels in spine heads 45 min after glutamate withdrawal (Supplementary Fig. 3 online). In other experiments, cells were exposed to glutamate for 30 min and then incubated in culture medium for an additional 4 or 12 h before imaging. Even after these long periods of post-stimulus incubation, profilin II-GFP remained concentrated in spine heads ($n = 10$ cells at 4 h, 4 cells at 12 h). Thus the accumulation of profilin in dendritic spines induced by activating postsynaptic receptors persists for several hours beyond the initiating stimulus.

To assess the effect of profilin targeting on dendritic spine motility, we examined double-transfected neurons expressing profilin II and actin tagged with yellow and cyan fluorescent protein (YFP and CFP), spectral variants of GFP that can be imaged simultaneously in the same cell. This allows the distribution of profilin II in dendrites to be visualized at the same time that actin dynamics, and hence spine motility, are monitored by time-lapse recording (Fig. 4; also see Supplementary Fig. 7 and Supplementary Videos 1 and 2 online). As shown previously, the low-frequency stimulation pattern (900 pulses at 1 Hz) induced targeting of profilin to spine heads (Fig. 4a). Concurrent time-lapse recording of CFP-actin (Fig. 4b) showed that, simultaneously with profilin accumulation, actin dynamics in spine heads were suppressed in each of five cells recorded (Fig. 4c,d).

To determine the time course of this profilin-related blockade of spine motility, we monitored actin dynamics in spines as cells were exposed to the low-frequency regime of 900 pulses at 1 Hz (Supplementary Videos 3 and 4 online). In these experiments, we also controlled for the possibility that expressing exogenous GFP-tagged profilin II might influence spine actin dynamics by using cells expressing only GFP-actin. In this way, we could examine the influence on actin dynamics of the low-frequency stimulation pattern in cells when only endogenous profilin was present. Time-lapse recordings confirmed that spines were motile under control conditions and that no detectable change in motility occurred during the first 6 min of stimulation (data not shown). But after 30 min, when 900 pulses at 1 Hz had been delivered followed by 15 min of post-stimulus recovery, spine motility was blocked in 10 of 11 GFP-actin cells recorded. To establish the time course of spine stabilization, we made further time-lapse recordings of actin dynamics over different periods following the onset of low-frequency stimulation. This showed that spine motility was unchanged 10 min after stimulation began, but then slowed progressively over the next 10 min, at which time motility had ceased (Supplementary Videos 5 and 6 online).

Blocking profilin targeting destabilizes spine structure

Profilin is thought to be recruited to the cell surface via binding to polyproline-rich motifs in plasma membrane-associated proteins such as VASP and Mena^{27,28}. To test whether this mechanism might account for the activity-dependent accumulation of profilin in spine heads, we took advantage of previous experiments showing that a peptide sequence comprising three repeats of the core polyproline

Table 1 Dependence of profilin II targeting to dendritic spines on glutamate receptor subtype

		Number of cells in category		
		++	+	-
a	Construct			
	Agonist			
Profilin II-GFP	Glutamate	22	3	1
GFP	Glutamate	-	-	7
b				
Receptor subtype	Antagonist	++	+	-
NMDA	APV	-	5	17
	MK-801	-	-	6
AMPA	CNQX ($n = 6$) / NBQX ($n = 15$)	6	14	1
mGluR	MCPG ($n = 17$) / Ly341495 ($n = 9$)	10	13	3
AMPA and mGluR	Ly341495 / MCPG / NBQX	6	1	1
c				
Receptor subtype	Agonist	++	+	-
NMDA	NMDA	16	-	-
mGluR I	DHPG	4	4	1
AMPA	AMPA	-	2	14

(a) Distribution of profilin II-GFP and GFP in dendrites after stimulating all receptor subtypes with 10 μ M glutamate for 30 min. (b,c) Effects of subtype-selective antagonists and agonists on glutamate receptor-induced profilin-GFP targeting. Images above the columns illustrate the classification of strong (++) , partial (+) and no (-) targeting of profilin II-GFP. Cells initially lacking high spine profilin concentrations were assessed after stimulation. Arrows indicate spines showing strong profilin II targeting; arrowheads point out spines with no accumulation. Drug concentrations: NMDA, 10 μ M; DHPG, 10 μ M; AMPA, 2 μ M; APV, 500 μ M; MK-801, 50 μ M; CNQX, 20 μ M; NBQX, 20 μ M; MCPG, 100 μ M–1 mM; Ly341495, 30 μ M. $n =$ number of cells examined.

motif from VASP blocks profilin binding to *Listeria monocytogenes* and inhibits its actin-dependent motility in the cytoplasm when microinjected into cells²⁹. In this procedure, we simultaneously transfected hippocampal neurons with profilin-YFP and CFP-G(GP₅)₃, a construct consisting of CFP fused to three repeats of this VASP core polyproline motif GPPPPP (GP₅). This way, we could test the effects of the peptide in competing for profilin targeting to dendritic spines by observing its effect on profilin distribution and NMDA receptor-dependent targeting in dendrites.

Cells expressing CFP-G(GP₅)₃ and profilin-YFP showed striking changes in both dendrite morphology and profilin-YFP distribution (Fig. 5). Instead of the well-formed dendritic spines normally present on hippocampal neurons maintained in culture for >21 d, dendrites in 24 of 26 CFP-G(GP₅)₃ expressing cells examined produced diverse, irregularly shaped protrusions which tended to be abnormally long and in most cases lacked the distinct head characteristic of mature dendritic spines (Fig. 5). In these cells, profilin-YFP was spread throughout the cytoplasm of dendrites, and in 10 of 14 cells tested, this distribution did not change following exposure to 10 μ M glutamate for 30 min, a treatment that induced robust targeting of profilin to dendritic spines in control cells (Fig. 5a,b). In a further four cells, profilin-YFP showed partial redistribution to some dendritic protrusion. In all cells, glutamate treatment did not consolidate the morphology of irregularly

Table 2 Presynaptically released glutamate induces profilin II targeting to dendritic spines

Treatment	Numbers of cells		
	++	+	-
1) 90 mM KCl	3	2	-
2) 90 mM KCl + 100 μ M APV	-	-	7
3) Zero $[Mg^{2+}]_o$	9	2	1
4) Zero $[Mg^{2+}]_o$ + botulinum toxin A	-	-	6

1) Depolarization induced by exposing cells to 90 mM KCl redistributes profilin II to spines. 2) This treatment is ineffective when NMDA-receptors are blocked by the antagonist APV (100 μ M). 3) Removal of external Mg^{2+} and therefore unblocking NMDA-receptors induces profilin targeting to spines. 4) When cells were preincubated with botulinum toxin A (100 ng/ml) for 12 h to block presynaptic glutamate release, removal of external Mg^{2+} did not lead to profilin II targeting. As control, subsequent addition of 5 μ M NMDA to directly stimulate postsynaptic receptors triggered the redistribution of profilin to spine heads. (++, strong targeting; +, weak targeting; -, no targeting; n = number of cells examined).

shaped dendrite protrusions produced by expressing CFP-G(GP₅)₃ so that spine structure remained destabilized (Fig. 5a,b). To ensure that these effects did not arise through interference with dendrite development, we transfected hippocampal neurons with CFP-G(GP₅)₃ after 18 d in culture, when mature dendritic spines have already begun to appear, and imaged them 2–4 d later. Destabilized spine structures were present in all cells expressing the competitor peptide, whereas control cells in the same cultures expressing YFP-actin showed dendritic spines of normal mature morphology (Supplementary Fig. 5 online). Together, these observations implicate surface-associated polyproline-rich proteins in the activity-dependent targeting of profilin to dendritic spine heads and in the consolidation of spine structure.

Profilin distribution *in vivo*

The profilin-GFP construct used in the above cell transfection experiments was also used to raise transgenic mice. Two independently generated lines were analyzed for the distribution of profilin-GFP in tissue sections and for the ability of glutamate receptor activation to induce targeting of profilin to dendritic spines in cultured hippocampal neurons. Profilin II-GFP expression in neurons from these was some three

times lower than in cells transfected *in vitro* (Supplementary Fig. 6 online). Confocal imaging of profilin-GFP fluorescence in stratum radiatum of hippocampal area CA1 from transgenic mouse brain showed accumulations of profilin-GFP corresponding in size and distribution to dendritic spines (Fig. 6a). Individual spines showed varying levels of fluorescence ranging from dull to bright, which is further demonstrated in a rotating z-series stack of this field (Supplementary Videos 7 and 8 online). Furthermore, we found activity-dependent targeting of profilin to dendritic spines in cultures of hippocampal neurons from profilin-GFP expressing transgenic mice (Fig. 6b). The glutamate-induced increase of profilin-GFP fluorescence in spine heads seen in this example was observed in each of eight cells from two independently established cultures after treatment with 5 μ M glutamate. The relative enrichment (E ; see Fig. 1) of profilin-GFP fluorescence in spine heads induced by treatment in these cells was 1.49 ± 0.07 (mean \pm s.e.m.), a value comparable to that found in profilin-GFP transfected neurons (Fig. 1f).

DISCUSSION

Previous studies implicate the actin cytoskeleton of dendritic spines in anatomical plasticity at central synapses, but they do not indicate how the activation of postsynaptic receptors is transduced into morphological change. Our observations implicate brain profilin in this process, showing that it becomes concentrated in dendritic spine heads after activation of postsynaptic NMDA receptors, and that this redistribution is associated with suppression of actin dynamics and stabilization of spine morphology. This result is consistent with evidence that NMDA receptor activation is involved in the maturation of excitatory synapses during brain development^{30–32} and that maturation of brain circuitry is marked by the progressive stabilization of dendritic spines^{7,9,33,34}. It is also consistent with several studies implicating profilins in stabilizing dynamic actin structures. In non-neuronal cells, profilin has been shown to stabilize actin filaments, increasing the density of the submembraneous actin network and suppressing membrane ruffles^{17,35}. Similarly, in cells microinjected with a chemically cross-linked 1:1 complex of profilin-actin, which cannot dissociate but is otherwise functionally intact, actin dynamics at the cell surface are suppressed³⁶. A recently proposed model suggests that profilin may act as a clamp limiting actin filament elongation³⁷. Binding of profilin to both actin and to surface ligands such as VASP, Mena and N-WASP^{27,28,38} suggests a mechanism by which it may regulate cell-surface motility. The destabilizing effect on spine morphology of expressing CFP-G(GP₅)₃, a peptide competitor for the polyproline binding domain of the profilin, suggests further that binding of profilin to polyproline motifs in surface-associated proteins of the Mena/VASP family is instrumental in both the NMDA receptor-dependent targeting of profilin to dendritic spines and the associated stabilization of the actin cytoskeleton in spines.

Several features of the dendritic spine stabilization that accompanies profilin targeting suggest a possible relationship to the electrophysiological changes characteristic of long-term potentiation and depression.

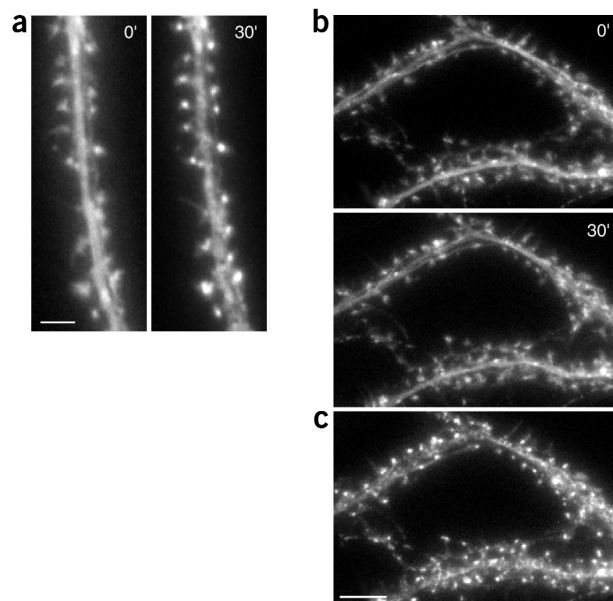


Figure 2 Presynaptically released glutamate triggers targeting of profilin II to dendritic spines. (a) Profilin II-GFP distribution before (0') and after (30') 30 min in zero Mg^{2+} , showing redistribution of profilin II to spines following Mg^{2+} removal. (b) Profilin II-GFP distribution in cells preincubated in botulinum toxin for 12 h before (0') and after (30') removal of Mg^{2+} from the medium. There was no redistribution of profilin II to spines. (c) The same culture following exposure to 5 μ M NMDA for a further 30 min. This induced strong targeting of profilin II to spines, showing that the postsynaptic receptor-dependent targeting mechanism was intact after botulinum toxin treatment. Scale bars: (a) 5 μ m, (c) 10 μ m.

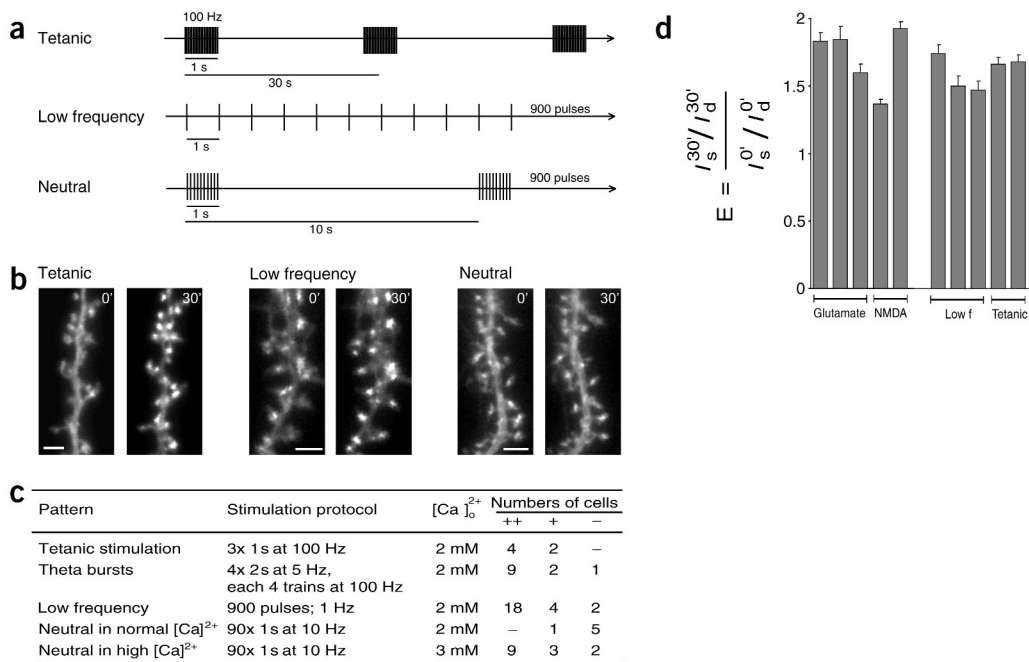


Figure 3 Electric-field stimulation patterns associated with long-term synaptic potentiation and depression induce proflin II targeting to dendritic spines. (a) Schematic diagram of three different stimulation patterns used. Tetanic stimulation, consisting of three trains of pulses at 100 Hz for 1 s. Low-frequency stimulation: 900 pulses applied at 1 Hz (total duration 15 min). Neutral: 900 pulses applied over 15 min in packets of 10 pulses in 1 s. (b) Dendritic segments of proflin II-GFP transfected cells treated with a tetanic, low-frequency or neutral-stimulation pattern (two panels each; before (0') and after (30') stimulation). Scale bars, 3 μ m. (c) Both high-frequency (tetanic and theta burst stimulation patterns) and low-frequency stimulation patterns induce redistribution of proflin to spines 30 min after the onset of stimulation. The neutral stimulation pattern did not trigger proflin targeting at physiological extracellular Ca²⁺ concentration, but proflin targeting was rescued when Ca²⁺ levels were increased by 50% from 2 mM to 3 mM, indicating its dependence on the magnitude of Ca²⁺ influx. (d) Quantification of proflin II-GFP enrichments in dendritic spines following electrical stimulation (for details, see Fig. 1e,f). Error bars represent s.e.m.; there was no significant difference between electrically and pharmacologically treated cells ($P > 0.2$), but electrically stimulated cells show significantly higher proflin enrichment (E) than glutamate-stimulated cells that were blocked with APV and MK-801 ($P < 0.005$).

Both phenomena are triggered by the activation of NMDA receptors, both depend on influx of Ca²⁺ into the postsynaptic cytoplasm, and both endure for hours following the stimulus that provokes them. Similarly, the same electrical stimulation patterns that elicit long-term potentiation or depression also induce proflin redistribution and spine stabilization in cultured neurons. This relationship is stimulus-specific, as a 'neutral' pattern that does not produce long-term changes in synaptic strength²⁵ also failed to induce proflin targeting. However, increasing the extracellular Ca²⁺ concentration, a treatment which influences the

probability of inducing long-duration changes in synaptic signaling³⁹⁻⁴¹ converted the neutral stimulus into one that was effective in promoting proflin redistribution and dendritic spine stabilization.

Initially we were surprised to find that both high- and low-frequency patterns of electrical stimulation induced proflin targeting and spine stabilization, as these are associated with changes of synaptic signal strength in opposite directions, namely LTP and LTD. Two recent studies, however, each using different methods, agree with our results in showing that both low- and high-frequency stimulation patterns stabilize actin in dendritic spines. Using photobleach recovery of GFP-actin fluorescence one group found that the same low-frequency stimulus pattern (900 pulses at 1 Hz), which induced proflin targeting and blocked spine motility in our experiments, suppresses actin turnover in dendritic spines⁴². Using rhodamine-labeled phalloidin staining to

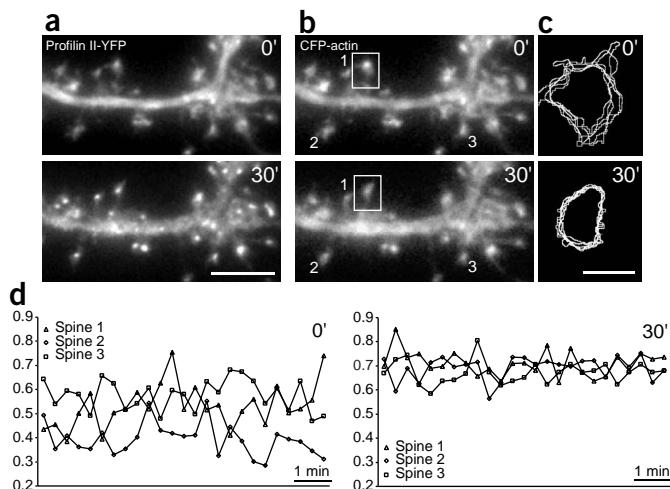


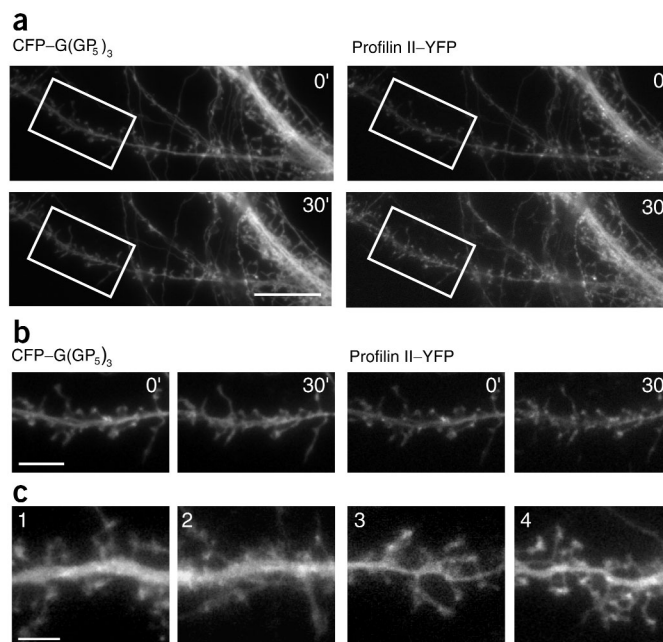
Figure 4 Electrical stimulation redistributes proflin II to dendritic spines and coincidentally stabilizes spine morphology. (a,b) Dendrite segment of a cell double-transfected with proflin II-YFP (a) and CFP-actin (b).

(a) Proflin II-YFP images captured before (0') and after (30') stimulation showing that proflin II becomes enriched in dendritic spines. Scale bar, 5 μ m. (b) Single frames showing CFP-actin from two time-lapse video sequences (see Supplementary Videos 1 and 2 online) captured before (0') and after (30') stimulation. (c) Detail of the spine indicated by the boxed area in b, in which thresholded outlines at five successive time intervals, 15 s apart, were overlaid before (0') and after (30') stimulation. Scale bar, 1 μ m. (d) Shape factor plots for the three spines labeled 1, 2 and 3 in b before (0') and after (30') stimulation.

Figure 5 A peptide competitor of profilin binding to polyproline-rich proteins prevents profilin targeting and destabilizes spine structures. (a) Hippocampal neurons double-transfected to express CFP linked to three repeats of a VASP polyproline motif, CFP-G(GP₅)₃ and profilin II-YFP, before (0') and after (30') treatment with 5 μ M glutamate. (b) Higher-magnification images of the boxed areas in a showing that profilin II-YFP is randomly distributed among dendritic shafts and dysgenic spines in CFP-G(GP₅)₃ expressing cells, and that neither profilin II-YFP distribution nor spine structure changed after stimulation. (c) Examples of abnormally shaped dendritic spines on four independently transfected CFP-G(GP₅)₃ expressing cells. Scale bars: (a) 10 μ m, (b) 5 μ m, (c) 3 μ m.

assess actin filament levels, another group found that various patterns of high-frequency stimulation promote actin assembly in dendritic spines, implying a reduction in actin dynamics⁴³. In these experiments, actin filament stabilization developed over 45 min following high frequency stimulation, a time course similar to that required for profilin targeting and stabilization of actin dynamics in our experiments. The second set of experiments⁴³ were done *in vivo* by giving trains of high-frequency stimulation in the hippocampus, making it possible to assess the duration of actin filament stabilization, which lasts up to several weeks. This result is consistent with our finding that profilin-dependent stabilization of dendritic spine structure, once established, lasted for as long as we were able to maintain and observe the stimulated neurons. These recent results thus agree with our observation in showing a long-duration stabilization of the spine actin cytoskeleton following patterns of electrical stimulation associated with both LTD and LTP.

Our results now add a potential mechanism for activity-dependent stabilization of the spine actin cytoskeleton involving NMDA receptor-dependent recruitment of profilin to dendritic spines via its binding to polyproline-rich proteins. In addition, our data suggest that the electrophysiological and cytoskeletal aspects of activity-induced synaptic plasticity involve distinct cellular mechanisms. First, profilin redistribution and spine stabilization was induced by patterns of electrical stimulation associated with both increases and decreases in



synaptic strength. Second, we tested several antagonists of protein kinases and phosphatases known to be essential for the induction of LTP⁴⁴ or LTD⁴⁵ and found that they had no effect on activity-induced profilin targeting to spines. Third, inhibitors of gene transcription and protein synthesis required for the 'late' phase of LTP^{24,46,47} also failed to affect profilin targeting (Supplementary Table 1 online). Together these data indicate that long-term changes in synaptic strength and profilin-related stabilization of synaptic morphology can be triggered in parallel with NMDA receptor activation, but that the two phenomena are mechanistically independent.

An interesting feature of our results is the extremely long duration of high profilin levels in dendritic spines following NMDA receptor-induced targeting. An outstanding question concerning memory formation is how synaptic specificity is maintained during the interval of hours when mechanisms involving gene activation and protein synthesis convert the initially labile memory trace into an enduring record^{24,48}. It has been suggested that this gap is bridged by a molecular sign that 'tags' activated synapses, marking them out as future recipients of the consolidation process^{24,49,50}. The targeting of profilin into the spine cytoplasm and subsequent stabilization of synaptic morphology over several hours imply that modulation of the actin cytoskeleton may be involved in preserving synaptic structure until the consolidation process is complete.

METHODS

Cell culture and microscopy. Cultures of dispersed hippocampal neurons were transfected with cDNA constructs as described¹¹ and maintained in glia-

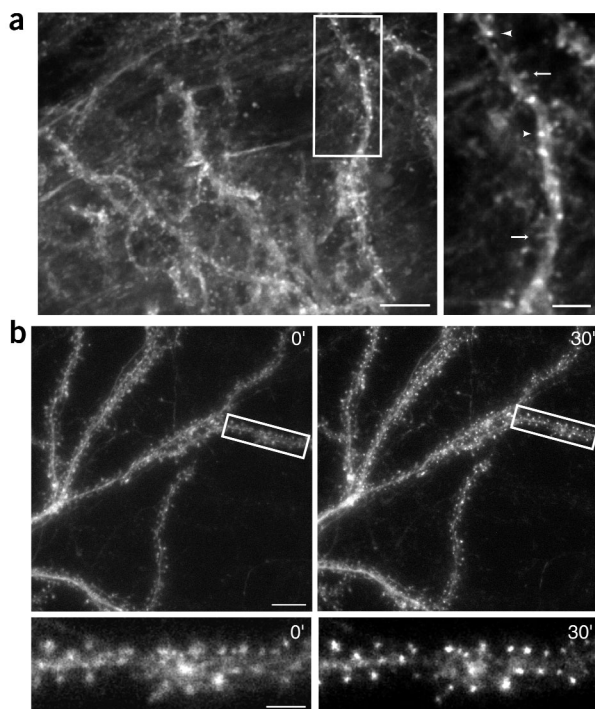


Figure 6 Profilin distribution and dendritic spine targeting in tissue and cells from profilin II-GFP transgenic mice. (a) Left, confocal image of a section from hippocampal region CA1 of an adult profilin II-GFP transgenic mouse (scale bar, 10 μ m). Right, detail of the boxed area (scale bar, 5 μ m) showing profilin II is more strongly enriched in some spines (arrowheads) relative to others (arrows). (b) Dendrites of hippocampal neurons from profilin II-GFP transgenic mice after 21 d *in vitro* showing activity-induced redistribution of profilin-GFP to spine heads before and after treatment with 5 μ M glutamate for 30 min (top panels, scale bar, 10 μ m; bottom panels, scale bar, 5 μ m).

conditioned, serum-free medium for 19–28 d before imaging. They were examined at 37 °C in Tyrode's solution (pH 7.3) using GFP-optimized filters and a cooled CCD camera¹¹. For receptor blockade experiments, cells were preincubated with antagonists for 15 min before stimulation. For depolarization, each cell was given four 3-min pulses of 90 mM KCl at 10-min intervals. Electric field stimulation patterns were programmed using a Master-8 pulse generator (AMPI, Israel) and delivered via platinum electrodes in a custom-built electrically isolated observation chamber (LIS, Switzerland). Transgenic mice expressing profilin-GFP from a chicken β -actin promoter were raised under standard procedures. Brain tissue was fixed by cardiac perfusion with 4% formaldehyde, and sections were examined by confocal microscopy. Animal experiments were performed under a permit from the Basel Cantonal Veterinary Office.

Image analysis and quantification. Activity-induced targeting of profilin-GFP to dendritic spines was classified on a cell-by-cell basis as 'strong' (++) for cells in which all spines showed increased profilin levels after stimulation, 'partial' (+) for cells in which some but not all spines showed increased profilin levels, and 'no targeting' (–) for cells in which no change in profilin distribution was evident. To assess the extent of stimulation-induced profilin-GFP accumulation in dendritic spines, the fluorescence intensities of spine heads and of a circular spot of the same area in the underlying dendrite shaft was measured by integrating pixel intensities using MetaMorph, before and after stimulation. For each cell, 40 spines on a dendritic segment were examined. Spine outlines were generated from threshold images using an edge-detection function of MetaMorph imaging software (Universal Imaging Corp.) and changes in spine shape were assessed using the 'shape factor' algorithm of MetaMorph¹¹.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Crick, F. Do dendritic spines twitch? *Trends Neurosci.* **5**, 44–46 (1982).
- Matus, A., Ackermann, M., Pehling, G., Byers, H.R. & Fujiwara, K. High actin concentrations in brain dendritic spines and postsynaptic densities. *Proc. Natl. Acad. Sci. USA* **79**, 7590–7594 (1982).
- Harris, K.M. & Kater, S.B. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu. Rev. Neurosci.* **17**, 341–371 (1994).
- Halpain, S. Actin and the agile spine: how and why do dendritic spines dance? *Trends Neurosci.* **23**, 141–146 (2000).
- Matus, A. Actin-based plasticity in dendritic spines. *Science* **290**, 754–758 (2000).
- Geinisman, Y., Berry, R.W., Disterhoft, J.F., Power, J.M. & Van der Zee, E.A. Associative learning elicits the formation of multiple-synapse boutons. *J. Neurosci.* **21**, 5568–5573 (2001).
- Lendvai, B., Stern, E.A., Chen, B. & Svoboda, K. Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex *in vivo*. *Nature* **404**, 876–881 (2000).
- Knott, G.W., Quairiaux, C., Genoud, C. & Welker, E. Formation of dendritic spines with GABAergic synapses induced by whisker stimulation in adult mice. *Neuron* **34**, 265–273 (2002).
- Trachtenberg, J.T. *et al.* Long-term *in vivo* imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* **420**, 788–794 (2002).
- Ottersen, O.P. & Helm, P.J. Neurobiology: How hardwired is the brain? *Nature* **420**, 751–752 (2002).
- Fischer, M., Kaech, S., Knutti, D. & Matus, A. Rapid actin-based plasticity in dendritic spines. *Neuron* **20**, 847–854 (1998).
- Dunaevsky, A., Tashiro, A., Majewska, A., Mason, C. & Yuste, R. Developmental regulation of spine motility in the mammalian central nervous system. *Proc. Natl. Acad. Sci. USA* **96**, 13438–13443 (1999).
- Kim, C.H. & Lisman, J.E. A role of actin filaments in synaptic transmission and long-term potentiation. *J. Neurosci.* **19**, 4314–4324 (1999).
- Krucker, T., Siggins, G.R. & Halpain, S. Dynamic actin filaments are required for stable long-term potentiation (LTP) in area CA1 of the hippocampus. *Proc. Natl. Acad. Sci. USA* **97**, 6856–6861 (2000).
- Carlsson, L., Nystrom, L.E., Sundkvist, I., Markey, F. & Lindberg, U. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* **115**, 465–483 (1977).
- Buss, F., Temm-Grove, C., Henning, S. & Jockusch, B.M. Distribution of profilin in fibroblasts correlates with the presence of highly dynamic actin filaments. *Cell Motil. Cytoskeleton* **22**, 51–61 (1992).
- Rothkegel, M. *et al.* Plant and animal profilins are functionally equivalent and stabilize microfilaments in living animal cells. *J. Cell. Sci.* **109**, 83–90 (1996).
- Witke, W., Sutherland, J.D., Sharpe, A., Arai, M. & Kwiatkowski, D.J. Profilin I is essential for cell survival and cell division in early mouse development. *Proc. Natl. Acad. Sci. USA* **98**, 3832–3836 (2001).
- Honore, B., Madsen, P., Andersen, A.H. & Leffers, H. Cloning and expression of a novel human profilin variant, profilin II. *FEBS Lett.* **330**, 151–155 (1993).
- Artola, A. & Singer, W. Long-term depression of excitatory synaptic transmission and its relationship to long-term potentiation. *Trends Neurosci.* **16**, 480–487 (1993).
- Yang, S.N., Tang, Y.G. & Zucker, R.S. Selective induction of LTP and LTD by postsynaptic $[Ca^{2+}]_i$ elevation. *J. Neurophysiol.* **81**, 781–787 (1999).
- Bliss, T.V. & Collingridge, G.L. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31–39 (1993).
- Bear, M.F. & Malenka, R.C. Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.* **4**, 389–399 (1994).
- Martin, S.J., Grimwood, P.D. & Morris, R.G. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu. Rev. Neurosci.* **23**, 649–711 (2000).
- Dudek, S.M. & Bear, M.F. Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. *Proc. Natl. Acad. Sci. USA* **89**, 4363–4367 (1992).
- Carroll, R.C., Lissin, D.V., von Zastrow, M., Nicoll, R.A. & Malenka, R.C. Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. *Nat. Neurosci.* **2**, 454–460 (1999).
- Reinhard, M. *et al.* The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. *Embo J.* **14**, 1583–1589 (1995).
- Gertler, F.B., Niebuhr, K., Reinhard, M., Wehland, J. & Soriano, P. Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics. *Cell* **87**, 227–239 (1996).
- Kang, F., Laine, R.O., Bubbs, M.R., Southwick, F.S. & Purich, D.L. Profilin interacts with the Gly-Pro-Pro-Pro-Pro sequences of vasodilator-stimulated phosphoprotein (VASP): implications for actin-based Listeria motility. *Biochemistry* **36**, 8384–8392 (1997).
- Liao, D., Zhang, X., O'Brien, R., Ehlers, M.D. & Huganir, R.L. Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. *Nat. Neurosci.* **2**, 37–43 (1999).
- Rumpel, S., Hatt, H. & Gottmann, K. Silent synapses in the developing rat visual cortex: evidence for postsynaptic expression of synaptic plasticity. *J. Neurosci.* **18**, 8863–8874 (1998).
- Petralia, R.S. *et al.* Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses. *Nat. Neurosci.* **2**, 31–36 (1999).
- Grutzendler, J., Kasthuri, N. & Gan, W.B. Long-term dendritic spine stability in the adult cortex. *Nature* **420**, 812–816 (2002).
- Roelandse, M., Welman, A., Wagner, U., Hagmann, J. & Matus, A. Focal motility determines the geometry of dendritic spines. *Neuroscience* (in the press).
- Finkel, T., Theriot, J.A., Dize, K.R., Tomaselli, G.F. & Goldschmidt-Clermont, P.J. Dynamic actin structures stabilized by profilin. *Proc. Natl. Acad. Sci. USA* **91**, 1510–1514 (1994).
- Hajkova, L., Nyman, T., Lindberg, U. & Karlsson, R. Effects of cross-linked profilin:beta/gamma-actin on the dynamics of the microfilament system in cultured cells. *Exp. Cell. Res.* **256**, 112–121 (2000).
- Dickinson, R.B. & Purich, D.L. Clamped-filament elongation model for actin-based motors. *Biophys. J.* **82**, 605–617 (2002).
- Suetsugu, S., Miki, H. & Takenawa, T. The essential role of profilin in the assembly of actin for microspire formation. *Embo J.* **17**, 6516–6526 (1998).
- Christofi, G., Nowicky, A.V., Bolsover, S.R. & Bindman, L.J. The postsynaptic induction of nonassociative long-term depression of excitatory synaptic transmission in rat hippocampal slices. *J. Neurophysiol.* **69**, 219–229 (1993).
- Mulkey, R.M. & Malenka, R.C. Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. *Neuron* **9**, 967–975 (1992).
- Artola, A., Hensch, T. & Singer, W. Calcium-induced long-term depression in the visual cortex of the rat *in vitro*. *J. Neurophysiol.* **76**, 984–994 (1996).
- Star, E.N., Kwiatkowski, D.J. & Murthy, V.N. Rapid turnover of actin in dendritic spines and its regulation by activity. *Nat. Neurosci.* **5**, 239–246 (2002).
- Fukazawa, Y. *et al.* Hippocampal LTP is accompanied by enhanced f-actin content within the dendritic spine that is essential for late LTP maintenance *in vivo*. *Neuron* **38**, 447–460 (2003).
- Soderling, T.R. & Derkach, V.A. Postsynaptic protein phosphorylation and LTP. *Trends Neurosci.* **23**, 75–80 (2000).
- Isaac, J. Protein Phosphatase 1 and LTD. Synapses are the architects of depression. *Neuron* **32**, 963–966 (2001).
- Frey, U., Krug, M., Reymann, K.G. & Matthies, H. Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region *in vitro*. *Brain Res.* **452**, 57–65 (1988).
- Nguyen, P.V., Abel, T. & Kandel, E.R. Requirement of a critical period of transcription for induction of a late phase of LTP. *Science* **265**, 1104–1107 (1994).
- McGaugh, J.L. Time-dependent processes in memory storage. *Science* **153**, 1351–1358 (1966).
- Frey, U. & Morris, R.G. Synaptic tagging: implications for late maintenance of hippocampal long-term potentiation. *Trends Neurosci.* **21**, 181–188 (1998).
- Martin, K.C. & Kosik, K.S. Synaptic tagging—whose it? *Nat. Rev. Neurosci.* **3**, 813–820 (2002).