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Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology

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Memory storage in mammalian neurons probably depends on both biochemical events and morphological alterations in dendrites. Here we report an activity-dependent stabilization of the MAP kinase (MAPK) pathway, prominent in hippocampal dendrites. The longevity of the signal in these dendrites was increased to hours when multiple spaced stimuli were used. Likewise, spaced stimuli and MAPK activation were critical for protrusion of new dendritic filopodia that also remained stable for hours. Our experiments define a new role for stimulus-specific responses of MAPK signaling in activity-dependent neuronal plasticity. The local biochemical signaling in dendrites complements MAPK signaling in gene expression. Together, these processes may support long-lasting behavioral changes.

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Although a vast array of activity-dependent biochemical changes have been observed in mammalian neurons, it remains unknown which pathway or pathways control activity-dependent morphological changes in dendritic spines, the principal target of excitatory synapses. Electrical or chemical stimulation, or behavioral stimulation such as rearing of animals in enriched environments, can produce changes in dendritic spine structure in hippocampal neurons[1–5]. Understanding the molecular mechanisms controlling the morphological plasticity of dendritic spines may be important in determining the cellular processes that underlie long-lasting neural plasticity and memory storage.

In *Aplysia*, MAPK signaling is critical for synaptic facilitation[6] and the internalization of an axonal cell adhesion molecule, apCAM[7]. The MAPK signaling pathway could, in principle, mediate persistent structural changes in mammalian neurons, where MAPK is activated in a stimulus-dependent fashion[6,8–17], and helps support neuronal excitability[8], synaptic potentiation[8–10], nuclear signaling[6,10–12,18] and memory formation[6,7,13,14,19]. Accordingly, we focused on MAPK signaling in dendrites of cultured hippocampal pyramidal cells and explants of dentate gyrus. We investigated steps leading to MAPK phosphorylation, the dynamics of its activation, and the possible participation of MAPK signaling in long-lasting structural alterations. Our results revealed a persistent activation of the MAPK pathway produced by multiple spaced membrane depolarizations, but not by the prolonging of a single stimulus, a phenomenon reminiscent of the specific memory-stabilizing consequences of spaced training in behavioral experiments[20–23]. This signaling combined information from voltage-gated Ca^{2+} entry, cAMP and Ras, key players in signal transduction pathways previously implicated in establishment of synaptic changes. We found that MAPK activation was critical for the formation of stable dendritic filopodial extensions, of the kind associated with enduring remodeling of synapses and induction of long-term synaptic changes. The formation and stability of these morphological changes also depended on the stimuli arriving in a spaced, repeated fashion. Thus, the pattern of activity not only determined the persistence of the biochemical changes but also controlled the subsequent changes in dendritic architecture.

Results

Persistent MAPK signaling evoked by repeated stimuli

MAPK signaling in dendrites[8,10,14] was detected with immunocytochemistry in cultured hippocampal neurons (**Fig. 1**). To investigate the functional importance of this pathway without complications from MAPK regulation of neuronal excitability (ref. 8 and D. Sweatt, personal communication), we bypassed spike-generating mechanisms by directly imposing membrane depolarizations with hyperkalemic solution (90 mM K^+). This clamped the membrane potential at ~0 mV, at which voltage-gated calcium influx is maximal. Potassium depolarization for three minutes gave rise to intense dually phosphorylated ERK1/ERK2 (referred to here as MAPK) immunoreactivity, which was widespread in both dendrites and cell bodies. Immunoreactivity peaked at 10 minutes and returned to close to baseline by 30 minutes (**Fig. 1a** and **b**). A different result was obtained when the pattern of stimulation was varied. Administration of four spaced depolarizing pulses greatly increased the persistence of phosphorylated MAPK (**Fig. 1a–c**). The effect of repetitive depolarization was specific to the duration of the MAPK phosphorylation; the initial peak was virtually the same for single and repeated stimulation. The presence of spacing between individual stimuli was critical; massing the four pulses together failed to generate the prolongation of MAPK phosphorylation. Application of a single very long stimulus was not as effective as application of four spaced pulses, despite intracellular Ca^{2+} remaining elevated during these stimuli. Similar results were seen in hippocampal pyramidal neurons in disso-

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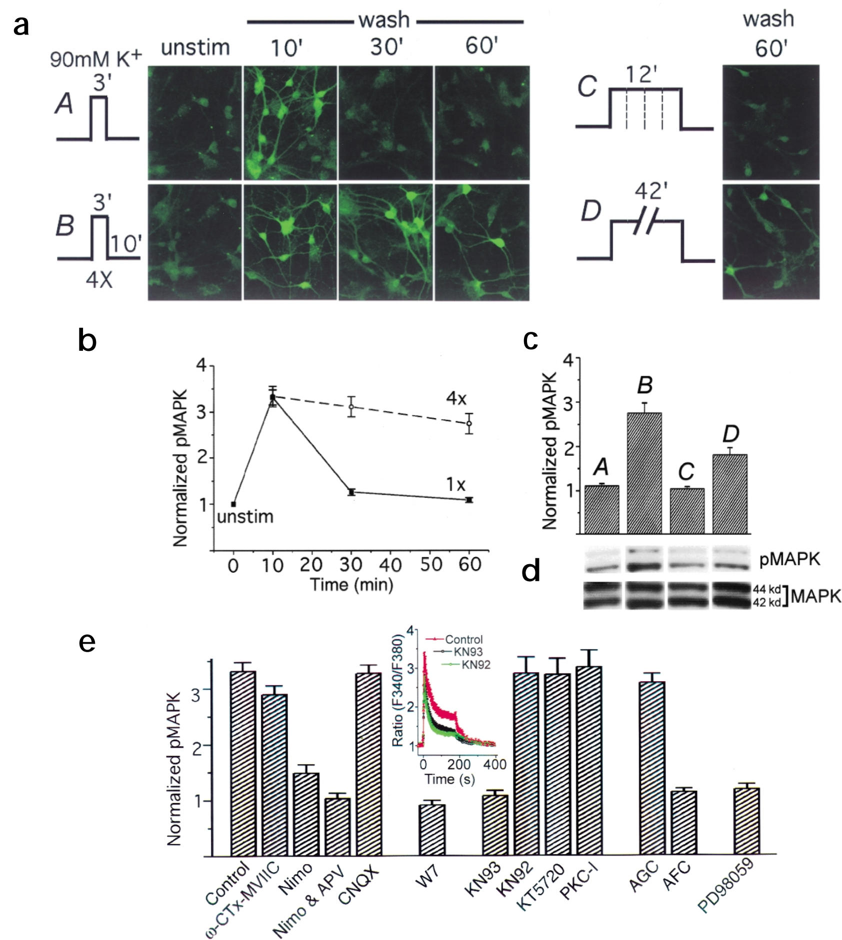

Fig. 1. The duration of activation of MAPK in hippocampal neurons is critically dependent on the pattern of membrane depolarization. (**a**) Confocal immunocytochemical images for dually-phosphorylated ERK1/ERK2 (pMAPK) showing the distinct kinetics of pMAPK in response to (*A*) a single stimulus of 3-min 90 mM K^+ depolarization, (*B*) four repeated 3-min depolarizations with 10-min intervals (*C*) a 'massed' stimulus of 12-min depolarization or (*D*) a prolonged 42-min depolarization. MAPK phosphorylation (shown green) was transient after a single stimulus (*A*) but highly stable after four spaced stimuli (*B*). Massed stimuli were largely ineffective at stabilization (*C* and *D*). (**b**) Pooled data showing time course of dendritic pMAPK after single (3-min depolarization) or spaced repetitive stimuli. Peak levels of pMAPK were indistinguishable in the two cases, but dynamics after the stimulus were very different. (**c**) Quantification of dendritic pMAPK at 60-min after the stimulus, after the different stimuli defined in (**a**). (**d**) pMAPK immunoblot done after the different stimuli defined in (**a**), showing results similar to those obtained via confocal immunocytochemical quantification. Bottom, equal amounts of cell extracts were probed with ERK1/2 antibody in parallel. (**e**) Pharmacological profile of the initial peak (10-min after the stimulus) response of pMAPK. Effects of the channel inhibitors nimodipine (10 μM), APV (100 μM), CNQX (20 μM), ω-CTx-MVIIC (1 μM); actions of inhibitors of signaling molecules, W7 (20 μM), KN-93 (2 μM), AFC (50 μM), KT5720 (1 μM), PD98059 (50 μM) as well as low-potency analogs of KN-93 (KN-92, 10 μM) and of AFC (AGC, *N*-acetyl-S-trans-geranyl-L-cysteine, 100 μM). The PKC-I bar represents pooled data from experiments with either chelerythrine (10 μM) or calphostin C (1 μM). All experiments were replicated at least three times. Two or three coverslips per condition were used in each experiment. Quantification was based on three fields chosen at random from each coverslip. Inset, ratiometric fura-2 Ca^{2+} imaging done in the presence or absence of KN-93 or KN-92 at the doses used here. KN-93 inhibited 22% of peak and 38% of total integrated Ca^{2+} during the stimulus; KN-92 inhibited 23% of peak and 47% of total integrated Ca^{2+}.

ciated cultures and in hippocampal dentate gyrus explants (data not shown). Data were normalized to the mean intensity value for the unstimulated case. Immunoblots of pMAPK/MAPK revealed a similar pattern to that obtained via immunocytochemical analysis (**Fig. 1d**; summary of quantified data, **Fig. 1c**).

Peak MAPK phosphorylation in dendrites displayed a distinctive pharmacological profile (**Fig. 1e**). The initial MAPK phosphorylation depended on voltage-gated Ca^{2+} influx through L-type Ca^{2+} channels, and when nimodipine, an L-type antagonist, was applied in combination with the NMDA receptor antagonist D-APV, the MAPK phosphorylation was completely blocked (**Fig. 1e**). In contrast, inhibition of N- and P/Q-type Ca^{2+} channels (ω-CTx-MVIIC) or AMPA receptors (CNQX) had no effect. The MAPK phosphorylation was prevented by inhibitors of calmodulin (W7) and CaM kinases (KN-93), but blockers of PKA or PKC had no effect (**Fig. 1e**). This selective CaM kinase dependence has been noted previously[24] in another signaling pathway completely dependent upon L-type channels and NMDA receptors (fast, MAPK-independent[10,46] CREB phosphorylation). As previously reported, KN-93 partially inhibited the depolarization-induced Ca^{2+} influx as determined with fura-2 ratiometric Ca^{2+} imaging (inset). However, at the doses used here, the related molecule KN-92 gave rise to a similar (and if anything, greater) side effect. Because KN-92 did not affect pMAPK formation at all (**Fig. 1e**), this side effect of KN-93 could not account for the blockade of pMAPK. *N*-acetyl-S-trans-farnesyl-L-cysteine (AFC), an inhibitor of carboxyl methylation of Ras and related proteins, or PD98059, a specific inhibitor of MEK, eliminated the MAPK phosphorylation. Taken together, these data indicate that the initial phase of dendritic MAPK phosphorylation was supported by a Ca^{2+}-, CaM kinase-[25,26], Ras-[11,27] and MEK-dependent pathway (**Fig. 1e**).

Distinctive features of sustained MAPK signaling

We then asked whether the properties of the sustained MAPK phosphorylation were different from those of the peak phosphorylation. Delayed inhibition of MEK with PD98059 caused the persistent MAPK phosphorylation to return promptly to

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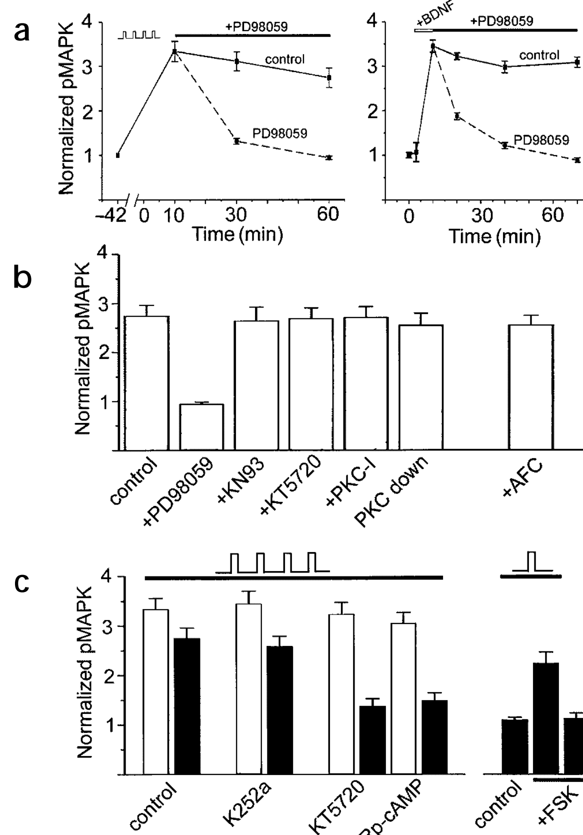

Fig. 2. PKA gates a persistent MEK activation to stabilize dendritic MAPK phosphorylation. (**a**) Effect of the MEK inhibitor PD98059 (50 μM) on pMAPK previously induced by 4 spaced depolarizing stimuli (left) or by 10-min application of BDNF (20–30 ng/mL, right). (**b**) In contrast to PD98059, sustained pMAPK was not abolished by delayed application of KN-93, KT5720, AFC or PKC inhibitors, or by PKC downregulation via 48-h exposure to phorbol ester (200 nM). (**c**) Relative to control, inhibition of receptor tyrosine kinase activity with K252a (200 nM) during stimulation had no effect. Inhibition of PKA with either of the structurally distinct PKA inhibitors KT5720 (1 μM) or Rp-cAMPS (100 μM) during the stimulus largely prevented appearance of the stabilized state, and did not affect the transient peak. Application of forskolin to elevate intracellular cAMP conferred pMAPK stabilization after only a single stimulus. White bars, 10 min post-stimulus time point; black bars, 60 min post-stimulus time point.

baseline levels (**Fig. 2a**). This indicated that the stabilization of dendritic MAPK phosphorylation was supported by persistent kinase activity rather than activity-dependent inhibition of MEK phosphatase or MAPK phosphatase[28]. A similarly sustained but Ca^{2+}-independent MAPK response could be elicited by a single application of the neurotrophin BDNF, and was also eliminated by the delayed application of PD98059 (**Fig. 2a**). In contrast to these effects of MEK inhibition, post-stimulus inhibition of CaM kinases, PKA, PKC or Ras did not affect the sustained MAPK phosphorylation (**Fig. 2b**). Thus, the early peak and sustained phase of MAPK phosphorylation displayed very different pharmacological profiles.

Considering the possible importance of sustained phosphorylation of dendritic MAPK, we closely examined the signaling events that led up to its initial establishment. Depolarization-induced release of BDNF or another neurotrophin, and consequent continuous tyrosine kinase signaling by trk receptors was considered as one possible mechanism. However, exposure to K-252a, a potent inhibitor of trk receptor tyrosine kinase activity, failed to reduce either the initial or sustained MAPK phosphorylation produced by depolarizing stimuli (**Fig. 2c**), even though this agent abolished BDNF-induced MAPK phosphorylation (data not shown). Sustained elevation of Ca^{2+} influx was likewise not involved, as fura-2 Ca^{2+} imaging revealed a prompt return to basal levels after each stimulus (data not shown). Hence, the stable dendritic pMAPK seemed to depend on an autonomously active intracellular biochemical signaling pathway.

PKA signaling was critical for stabilization of dendritic pMAPK, even though PKA was not involved in its initial formation (**Figs. 1e** and **2c**). The sustained MAPK phosphorylation was abolished by inhibition of PKA during stimulation with the mechanistically distinct agents Rp-cAMPS or KT5720. To find out if PKA signaling was sufficient to account for the stimulus pattern-dependent kinetics of pMAPK, we paired single depolarizations with a brief application of forskolin to elevate cAMP (**Fig. 2c**, right). Indeed, this pairing gave rise to sustained dendritic MAPK phosphorylation, whereas brief forskolin treatment alone gave rise to only a brief increase in pMAPK but no sustained phase (see also refs. 6, 10 and 18). Thus, PKA is specifically involved as a gate[29] controlling the persistence, rather than the initial induction of MAPK phosphorylation in hippocampal dendrites.

Involvement of small GTP-binding proteins

There is no consensus on the identity of the small GTP-binding protein that supports sustained MAPK signaling in neurons. In PC12 cells, sustained MAPK can involve Rap1 (ref. 30) or Ras[27]. In our system, transfection with dominant-negative Ras (RasN17) completely abolished both the transient and the sustained pMAPK signal (**Fig. 3**). In contrast, we found no effect of dominant-negative Rap1 (Rap1N17) on MAPK phosphorylation in hippocampal neurons (data not shown). Thus, our data support the idea that Ras signaling is involved in both phases of dendritic pMAPK. However, involvement of Rap1 and other small GTP-binding proteins cannot be completely excluded. Dominant-negative Rap1 may not function well in all systems[31], and because RasN17 acts through sequestration of guanine nucleotide exchange factors (GEFs), it may affect other related pathways.

Nature of persistent MAPK signaling

We next asked how the activation of dendritic MAPK might be sustained (**Fig. 4**). Given that sustained MEK is involved, as shown by delayed application of PD98059 (**Fig. 2**), the main possibilities are as follows. First, MEK itself might become constitutively activated. Second, MEK, or a downstream effector such as MAPK, might participate in a positive feedback cycle. Third, upstream stimulation of MEK might be sustained. The first scenario is highly unlikely, because PD98059 at 50 μM blocks MEK activation, rather than inhibiting MEK's ability to phosphorylate substrates[32,33]. The remaining possibilities differ only in their dependence on MEK activity itself. To distinguish between them, we made use of U0126, a MEK antagonist[32] that has effects readily reversed by washing, unlike PD98059. Delayed application of U0126 after the peak MAPK phosphorylation caused a prompt drop to baseline levels, as expected if MEK activity continued during the sustained phase. Subsequent removal of U0126 in the absence of any further stimulation led to a rapid recovery of

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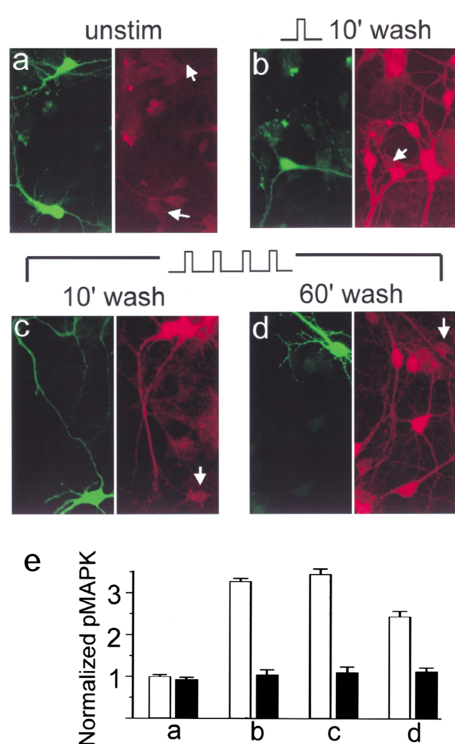


Fig. 3. Contrasting effects of dominant negative forms of Ras and Rap1. MAPK activation in hippocampal neurons from 7 d.i.v. cultures, transfected with dominant-negative H-Ras (RasN17), then stimulated and fixed for immunocytochemistry 24 h later. EGFP-transfected neurons appeared green, and pMAPK-positive neurons appeared red (Texas Red or Cy3 secondary antibody). Cotransfection of dominant-negative Ras (RasN17) abolished both transient and sustained pMAPK signals at all time points tested (**a–d**). In contrast, RapN17 had apparently no effect at any time point in these cells (data not shown). (**e**) Pooled data from experiments with dominant negative Ras. Black bars, transfected cells; white bars, control cells.

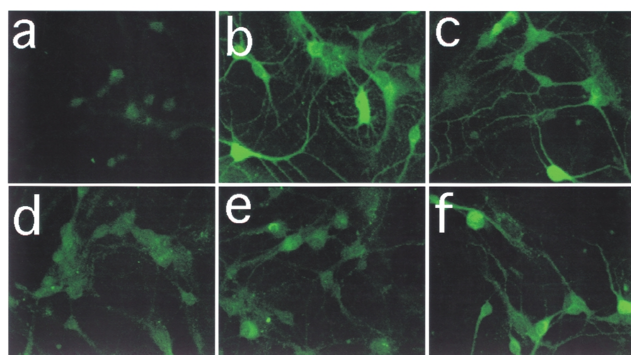
MAPK phosphorylation, to the level expected if MEK had never been inhibited. Contrary to the second scenario, this result established that the persistent signal did not require continuous operation of a feedback loop involving MEK, MAPK or any other downstream effector. The persistence evidently lies upstream of MEK, in a form of biochemical memory that causes its sustained activation.

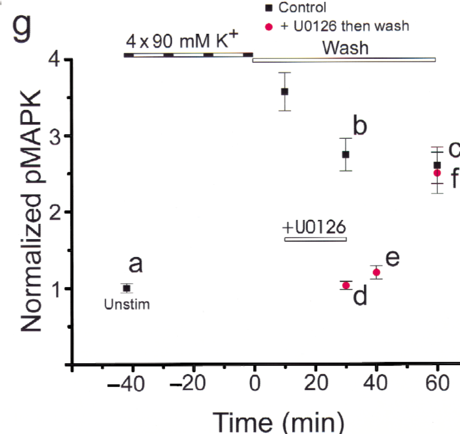
Changes in dendrite structure linked to MAPK pathway

The prominence of activated MAPK in dendrites suggests this pathway may be well suited for implementing local changes on a long-term basis. Indeed, MAP kinase pathways have been implicated in axonal structural changes in *Aplysia*[7] and in long-term modulation of synaptic function in mammalian neurons[8–10], in addition to supporting a slow phase of signaling to nuclear CREB phosphorylation[10–12,18] (unpublished observations). Activity-dependent, long-term structural changes in dendrites have been widely reported[2–4,34,35], resulting from electrical, chemical or even behavioral stimulation. To find out whether the MAPK pathway contributes to these changes, we examined dendritic morphology in the explant cultures of hippocampal dentate gyrus transfected with green fluorescent protein (GFP) as a cellular marker. This system preserved well the native cellular geometry and dendritic spine development, thus allowing spatially detailed and temporally extended investigations of spine and dendrite structure.

Under control conditions (spontaneous activity blocked with tetrodotoxin; TTX), dendritic structure was stable on the time scale of hours (**Fig. 5a**, left), with only very subtle and transient deformations of spines[36,37]. Single stimuli failed to give rise to stable morphological changes (**Fig. 5a**, right and **e**; single 'massed' stimulus). However, spaced repeated application of high-K^+ stimuli caused two distinct forms of stable robust morphological changes in dendritic spines (**Fig. 5b**). First, new structures were formed on the dendritic shaft, some displaying the morphology of filopodial processes, others with the head-neck appearance of mature spines, in line with morphological changes induced with electrical stimulation[2–4]. Second, some of the existing spines put forth filopodial extensions, a structural change seen in other

Fig. 4. Continuous upstream activation of MEK during the post-stimulus stabilized state. (**a–c**) Typical time course of pMAPK after four spaced depolarizing stimuli (graphed in **g**). (**d**) Effect of the MEK inhibitor U0126 (10 μM) applied after stimulation, showing the same prompt reduction of pMAPK as found with PD98059. Removal of this MEK inhibitor allowed a prompt return of pMAPK from baseline to its stabilized level (**e**, **f**), pointing to a continuous upstream activation of MEK.





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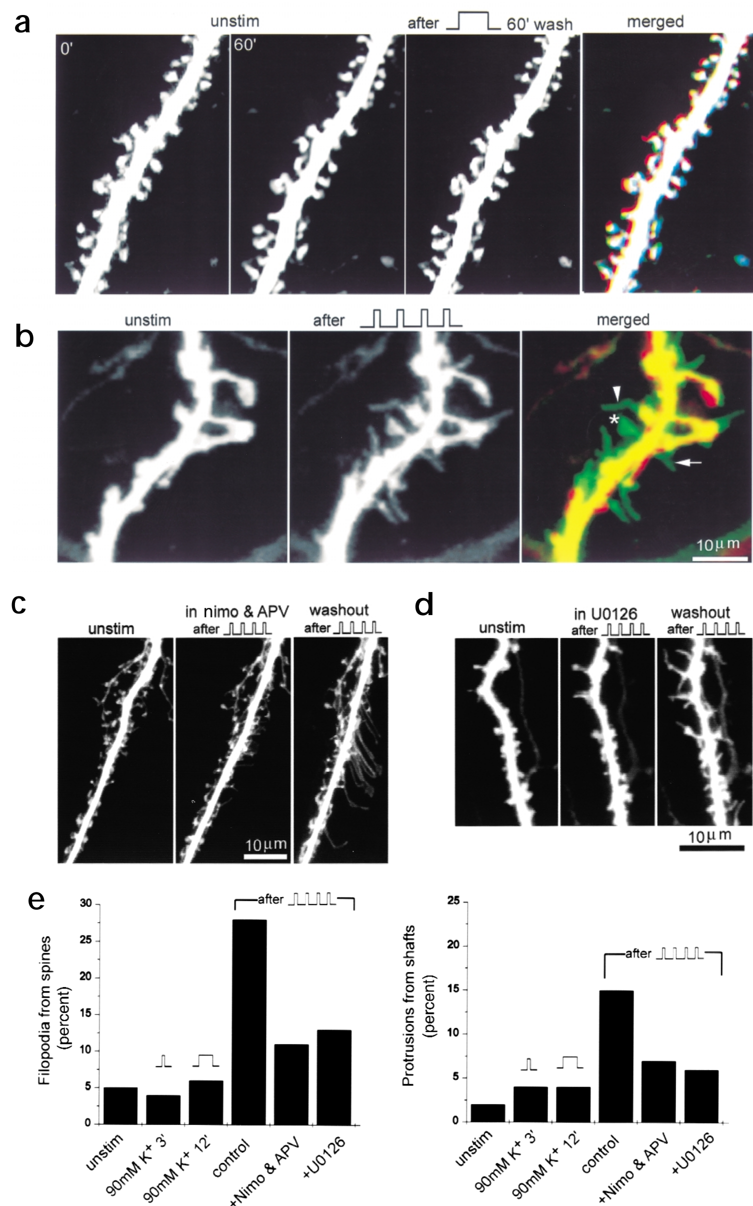

Fig. 5. Activity-dependent structural plasticity in hippocampal neurons depends on MAPK signaling. (**a**) High-resolution dendritic image of a GFP-transfected neuron in hippocampal dentate gyrus explant culture. In the control condition (TTX, left two panels), the morphology was highly stable over a time scale of hours. Typically, less than 5% of spines show filopodial structures. A single 'massed' 12-min depolarizing stimulus failed to give rise to significant morphological plasticity (right two panels). Far right, image generated by merging the first three panels, represented in turn by red, green and blue, to highlight any changes. In this merged image, white indicates an area of no change (that is, complete superposition of all three images), whereas colors indicate the small areas of dynamic behavior over the imaging period. (**b**) After four spaced depolarizing stimuli, numerous filopodia were seen protruding from the spines and the dendritic shaft (middle). Arrowhead, filopodium emerging from the head of a spine. Arrow, filopodium emerging from the dendritic shaft. Asterisk, new spine-like structure with bulbous head. (**c**) L-type calcium channels and NMDA receptors are required for activity-dependent morphological plasticity. Left, control condition. Middle, same dendrite after four depolarizing stimuli in the presence of nimodipine and APV. Little plasticity was seen. Right, striking filopodial formation in same dendrite after washout of the antagonists and subsequent re-stimulation. (**d**) MAPK signaling is involved in activity-dependent morphological plasticity. Left, control condition. Middle, same dendrite after four depolarizing stimuli in the presence of the MEK antagonist U0126, showing only subtle changes. Right, prominent filopodial formation in same dendrite after washout of U0126 and re-stimulation. (**e**) Quantitative summary of morphological changes. Number of filopodia emerging fom spine heads (left) and number of filopodia emerging from the dendritic shaft (right) under the conditions described above. Massed or single stimuli did not give rise to stable changes, whether or not additional time (60 min) was allowed to make the elapsed time from the beginning of stimulation comparable to that in the four-stimulus case.

preparations[37,38], but not in direct association with stimulation. Both types of new structures persisted beyond 30–60 minutes after stimulation ended (**Figs. 5e**, **6** and **7**). In contrast, massed (**Fig. 5a** and **e**) or single (**Fig. 5e**) exposures to high K^+ failed to produce persistent morphological changes (see also ref. 37), even with allowance of additional time (60 minutes) to make the total duration from the beginning of stimulation comparable to that seen with four spaced stimuli (**Fig. 5e**).

The differential structural response to individual depolarizations and spaced trials was reminiscent of the contrasting effects of these stimuli on dendritic MAPK phosphorylation, raising the possibility that the MAPK pathway might be involved in morphological plasticity. This hypothesis was tested by blocking Ca^{2+} entry and by inhibiting MAPK activation. Indeed, the activity-dependent morphological changes in hippocampal dendrites were inhibited by antagonists of NMDA receptors and L-type calcium channels (**Fig. 5c** and **e**), or of CaM kinases (KN-93; data not shown). Furthermore, the specific MEK antagonist U0126 prevented the dendritic protrusions (**Fig. 5d** and **e**). Therefore, each of the manipulations that prevented Ca^{2+} entry or downstream dendritic MAPK signaling inhibited the persistent morphological changes.

Tracking changes in dendritic morphology

The progressive development of dendritic structural changes and the requirement for repetitive stimulation could be observed in real time in single dendritic branches (**Fig. 6a** and **b**). Tracking of length changes in individual filopodia demonstrated a range of behavior illustrated by the examples analyzed quantitatively in Fig. 6b. A typical response consisted of prominent filopodial extension after the third or fourth stimulus, in some cases followed by a post-stimulation consolidation phase in which the extensions became shorter and wider, with an appearance suggestive of new spine formation[2,39].

MEK inhibition does not collapse preformed filopodia

Because MEK/MAPK signaling was found to be important for formation of filopodia, we next asked whether ongoing MEK activity was likely to be important for long-term stability of the

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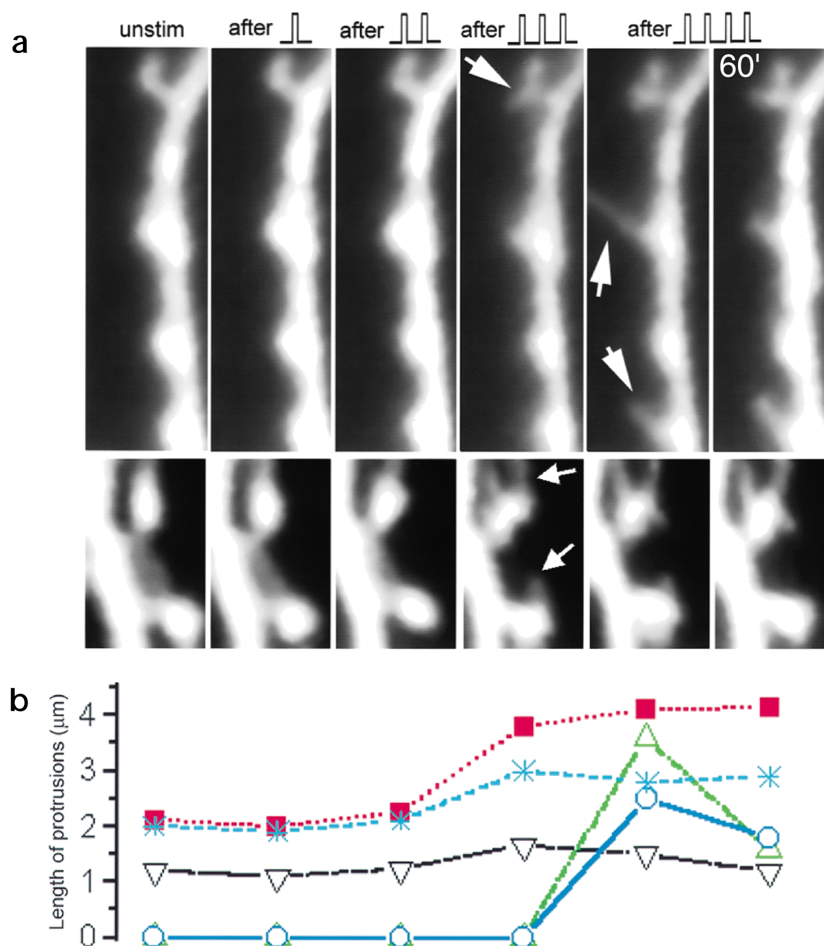

Fig. 6. Dynamics of structural plasticity. Evolution of filopodium formation over the course of the stimulation period. Little change is seen in after the first two stimuli. However, after the third and fourth stimulation periods, prominent filopodial formation was seen (arrows). A subsequent phase of consolidation, in which the new filopodium shortens, widens, and begins to assume a spine-like structure, was seen in some cases (rightmost panels). (**b**) Quantification of filopodium length over the course of the stimulation period for some representative examples.

new filopodia. To test this hypothesis, we acutely applied U0126 after the filopodial formation, which caused pMAPK to return to basal levels within 30 minutes (**Fig. 4**). U0126 did not cause collapse of previously formed new structures (**Fig. 7**). The robust stability of the new structures up to two hours after the end of stimulation was apparently unaffected by acute MEK inhibition, suggesting that at least on the time scale we investigated here, persistence of the filopodia is not temporally tightly linked to ongoing MAPK activity. It remains to be seen whether these structures, once formed by spaced repeated stimulation and MAPK signaling, are maintained by the persistent signaling upstream of MEK or by some other ongoing biochemical process.

Discussion

We have described a persistent biochemical change (MAPK activation) produced by repeated stimulation of hippocampal neurons, and have established that the same type of stimulation, also acting through MAPK, helps control dendrite shape. We found that establishment of the persistent dendritic signaling via MAPK depends upon PKA, but its maintenance relies on continued activation of MEK. The players involved in setting up the sustained kinase activity, including Ras, PKA, MEK and MAPK, serve well-characterized roles in supporting LTP[9,10,40,41] and signaling to the nucleus[6,10,12,18]. However, it was unexpected that these molecules would be critical for a sustained dendritic signal specific to spaced repeated stimulation. These findings provide new perspective on mechanisms of long-term memory, possibly tying together previous data showing that memory formation depends on repetitive stimulation through spaced training, MAP kinases, PKA and dendritic structural changes[7,13,19–22].

The finding that the MAPK pathway helps control dendrite shape (**Figs. 5–7**) has additional implications for activity-dependent neural development[42]. The link between Ca^{2+} entry, dendritic signaling and filopodial extension could be relevant in the activity-dependent bifurcation of existing spines[4,34] as well as in the formation of new spines from dendritic shafts[38]. The same mechanisms may also support the activity-dependent clustering of synapses[43], which can show a striking dependence on electrical activity, L-type Ca^{2+} channels, NMDARs and cAMP signaling. In development as well as in memory formation, stable morphological changes are likely to arise from the synergistic effects of local changes and gene products that depend on signaling to the nucleus[20,44–46]. The pMAPK stabilization mechanism described here could cause a prolonged MAPK-dependent activation of the spine translation apparatus through an effector such as eIF4e[47,48], which could promote local protein synthesis from globally distributed mRNAs. Another possibility is that the MAPK-dependent change in dendritic morphology might itself represent a local event that enables effective interaction with a nucleus-derived structural protein. The breakdown of the fixed,

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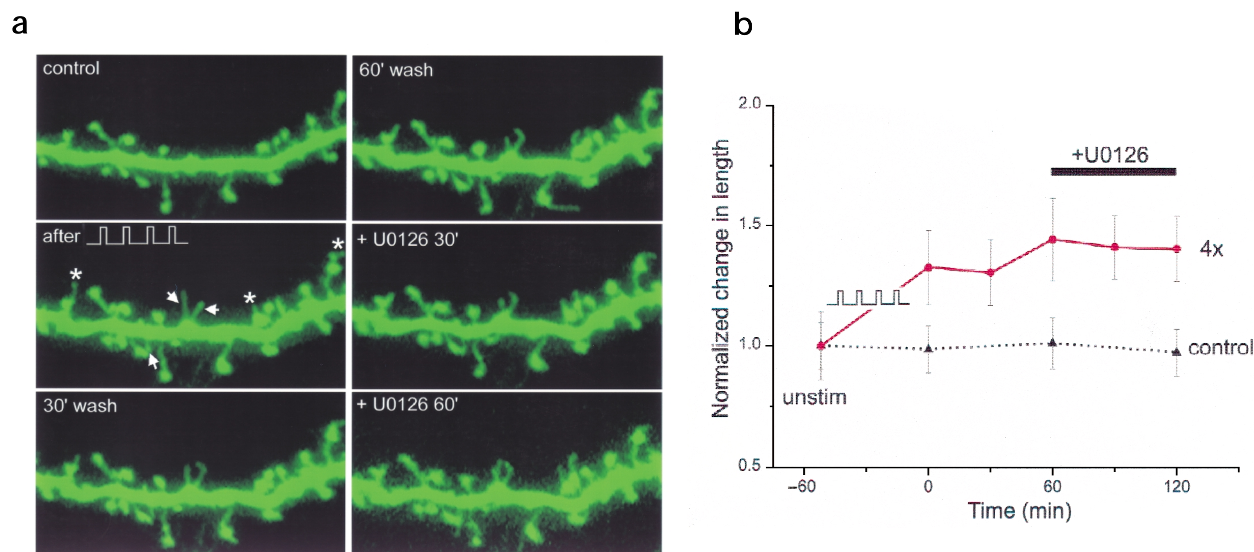

Fig. 7. Stability of structural plasticity. (**a**) Sequence showing long-term stability of protrusions generated by spaced repeated stimuli. Delayed application of U0126 (10 μM) cannot cause acute collapse of the new structures on this time scale. Arrows, filopodia emerging from dendritic shaft; asterisks, filopodia emerging from bulbous heads. (**b**) Summary of experiments using delayed application of U0126. New structures are robustly stable on the time scale of at least two hours.

mature structure caused by spaced repeated stimulation leads to Ras/MAPK-dependent filopodium formation, which could be translated into functional consequences in conjunction with other factors, perhaps from the nucleus. In either scenario, the dendritic pMAPK signaling described here displays spatiotemporal characteristics suitable for storing a local signal at active synapses, which would allow site-specific interpretation of a globally distributed signal[49].

Methods

Transfection and pharmacology. Calcium phosphate transfections, immunocytochemistry and culture of dissociated hippocampal CA3/CA1 pyramidal neurons were done as previously described[24,46]. Before stimulation, neurons at 8–9 days *in vitro* (d.i.v.) were preincubated in 1 μM TTX for 1–2 h to block spontaneous neuronal activity; TTX was also present during the inter-stimulus periods and the post-stimulus phase. The stimulation (90 mM KCl in isotonic Tyrode) clamps the membrane potential of neurons close to 0 mV, and considering known current–voltage relationships, provides maximal activation of voltage-gated sources of calcium influx. Submaximal stimulation (with either greater or lesser depolarization) would allow for recruitment of intrinsic depolarization mechanisms, and effects of the MEK inhibitors would then be complicated by their known effects on neuronal excitability[8]. TTX was typically not present during the stimuli; as would be expected, inclusion of TTX during this type of stimulus had no effect (data not shown). Stimuli were applied by rapidly changing the perfusion solution over the coverslip substrate bearing the cultured neurons from control to high-K^+ Tyrode's solution. Pharmacological agents were added 15–20 min before stimulation unless otherwise indicated, and were also present in the recovery solutions. Drugs were obtained from RBI (Alexandria, Virginia; nimodipine, APV, CNQX, forskolin), Elan Pharmaceuticals (Dublin, Ireland; ω-CTx-MVIIC), Calbiochem (La Jolla, California; TTX, KN-93, KN-92, W-7, chelerythrine, calphostin C, KT5720, RpcAMPS, AFC, AGC) and Promega (Madison, Wisconsin; BDNF, U0126, PD98059). The EGFP expression plasmid was obtained from Clontech (Palo Alto, California); expression plasmids for RasN17 and empty vector were purchased from Upstate Biotechnology (Lake Placid, New York). Rap1N17 was a gift from P. Stork (Vollum Institute). Antibody to dually phosphorylated ERK1/2 was obtained from Promega or Upstate Biotechnology and was used at 1:1000. Anti-MAP2 monoclonal antibody (Roche/Boehringer Mannheim, Mannheim, Germany) was used at 1:500. Secondary antibodies were from Jackson Immunoresearch (West Grove, Pennsylvania).

Explant culture. Isolated dentate gyrus/CA3 dissected from 2–4-day-old rats was placed on Matrigel-coated coverslips and cultured for up to 3–4 weeks. We found that the calcium phosphate EGFP transfection procedure[24] could be efficiently done in the explants at 5–7 d.i.v. and that EGFP expression remained stable for two weeks or more, allowing for high-resolution, real-time, long-term tracking of dendritic morphology. Dendritic spine maturation proceeded as *in vivo*, with early filopodia consolidating into mature head-neck spines associated with presynaptic terminals.

Imaging. To image and quantify immunofluorescence, confocal microscopy was done as previously described[24,46]. For purposes of quantification, apical dendrites from all clearly identifiable individual neurons within randomly selected fields were included. In analysis of filopodium formation, standard confocal microscopy techniques using stacked Z-series data ruled out artifacts from shifting focus or changes in dendrite positioning. For tracking morphological changes, individual EGFP-transfected dentate neurons were maintained in a continuous perfusion chamber at room temperature and were visualized with a 100× Nikon oil immersion objective (NA 1.3). Dendritic spine length and shape changes were analyzed with NIH Image software[50]. Intracellular calcium imaging with fura-2 was done as previously described[24].

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