MAPK CASCADE SIGNALLING AND SYNAPTIC PLASTICITY

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The mitogen-activated protein kinase (MAPK) cascade that leads to the activation of extracellular signal-regulated kinases-1 and -2 (ERK1 and ERK2) has a key role in the differentiation of some cell types and the proliferation of others. However, several recent reports implicate this cascade in the control of synaptic plasticity in the adult brain. ERK signalling seems to be essential for characterized neuronal transcriptional events, and might also regulate synaptic targets to control plasticity. Another recently emerging story is the involvement of a 'parallel' but distinct kinase cascade leading to the activation of p38 MAPK, which might control distinct forms of synaptic plasticity.

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Several MAPK cascades have been characterized in mammalian cells (reviewed extensively by Cobb and colleagues[1]) of which the best-studied involves the activation of ERK1 and ERK2 in response to growth factors and other stimuli. To activate ERK1 and ERK2, extracellular stimuli cause (through mechanisms that have been reviewed extensively elsewhere[2,3]) an increase in the active, GTP-bound form of the small G protein Ras (FIG. 1). Elevated Ras–GTP levels result from increased activity of guanyl nucleotide exchange factors (GEFs, which catalyze the exchange of GDP for GTP on Ras), decreased activity of GTPase-activating proteins (GAPs, which promote the slow intrinsic hydrolysis of GTP to GDP catalysed by Ras itself), or a combination of these two processes. Ras–GTP then triggers activation of the protein kinase Raf, which in turn phosphorylates and activates the enzyme MAPK/ERK kinase (MEK). MEK's role is to phosphorylate and activate ERK1 and ERK2 (also known as p44 and p42 MAPK, respectively)[4]. ERKs are serine/threonine kinases, whose targets include transcription factors, cytoskeletal proteins, regulatory enzymes and, importantly, other kinases. These downstream kinases include ribosomal protein S6 kinases (RSKs, a group of kinases with previously identified cytoplasmic and nuclear targets[5]) and the RSK-related mitogen- and stress-activated kinases (MSKs) that are restricted to the nucleus. Evidence indicates that MSKs probably mediate important MAPK-dependent transcriptional events[6–8].

In the years following the identification of ERKs, signalling that is mediated by these kinases was found to have a vital role in the responses of many cell types to growth factors and other mitogens (see REFS 1,9 for review), controlling both proliferation and differentiation. However, a puzzling observation was that ERKs, their upstream regulators and many downstream targets, are highly expressed in mature neurons, which are terminally differentiated and do not divide[10]. This raised a simple question: what are ERKs doing in these cells, when their roles in proliferation and differentiation are clearly no longer required? This question became even more intriguing when it was discovered that ERKs are activated in neurons in response to excitatory glutamatergic signalling[11–13], which controls many forms of synaptic plasticity that are thought to underlie higher brain processes such as learning and memory.

Routes to neuronal ERK activation

As described above, ERK1 and ERK2 are activated when extracellular stimuli increase Ras–GTP levels by altering the balance between the activities of Ras–GEF and Ras–GAP. The 'classical' route to Ras–ERK activation through receptor tyrosine kinases, adaptor proteins (such as Grb2) and GEFs (such as Sos; for review, see REFS 2,3) can operate in neurons in response to neurotrophins. However, neuronal ERK activation in response to direct membrane depolarization[14] or

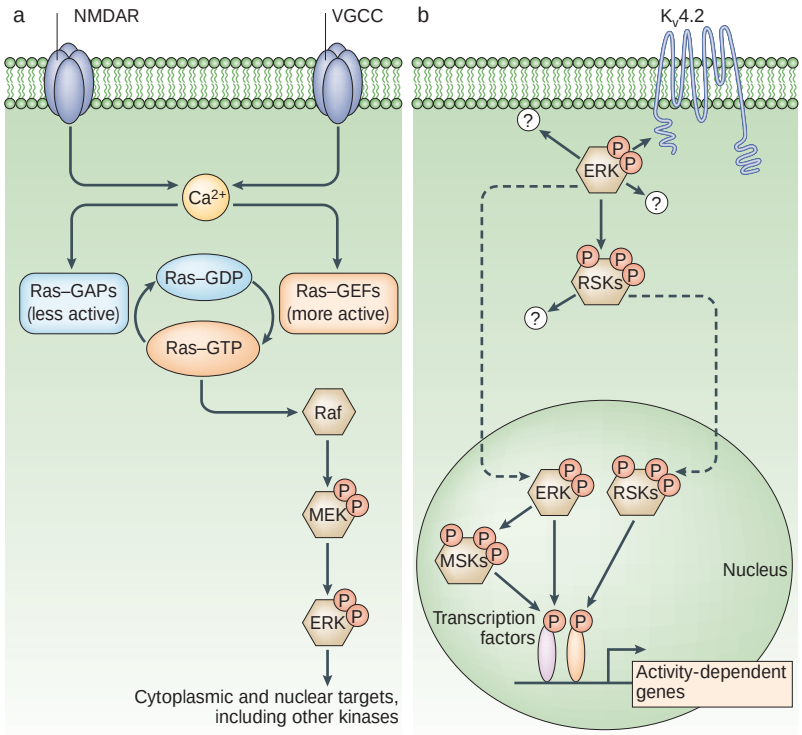

Figure 1 | **Activation of extracellular signal-regulated kinase (ERK) by synaptic signalling, and downstream targets. a** | Calcium influx, either through NMDA (*N*-methyl-D-aspartate)-type glutamate receptors (NMDARs) or voltage-gated calcium channels (VGCCs) triggers an increase in the levels of Ras–GTP. This leads to the activation of Raf, mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) and ERK, allowing phosphorylation of both nuclear and cytoplasmic ERK substrates. The precise route by which calcium activates Ras signalling is unknown, but could involve activation of Ras–guanyl nucleotide exchange factors (GEFs), inhibition of Ras–GTPase-activating proteins (GAPs), a change in the localization of these enzymes (which would alter their likelihood of 'seeing' Ras), or a combination of all of these factors. The precise route to Ras activation might differ depending on the neuronal cell type and/or the extracellular stimulus. **b** | Following its activation, ERK phosphorylates extranuclear targets such as the voltage-dependent K^+ channel $K_V4.2$ and downstream kinases such as ribosomal protein S6 kinases (RSKs). A pool of activated ERK and RSK translocates to the nucleus, where ERK phosphorylates and activates the constitutively nuclear mitogen- and stress-activated kinases (MSKs). In the nucleus, ERKs, RSKs and MSKs phosphorylate transcription factor substrates. The best-characterized of these substrates is CREB (cyclic-AMP-responsive element (CRE)-binding protein), which might be phosphorylated by MSKs, RSKs or both. It is highly unlikely that this figure represents the entire range of neuronal ERK/RSK/MSK targets, and the identification of further substrates will be of great interest.

glutamatergic signalling[15,16] proceeds by a different route. ERK activation that is induced by these stimuli is Ras-dependent, but activation of Ras in response to these signals requires calcium influx[14] (see REF. 17). This calcium influx can be facilitated by NMDA (*N*-methyl-D-aspartate)-type glutamate receptors or voltage-gated calcium channels (FIG. 1a). Precisely how elevated calcium levels trigger Ras activation is yet to be elucidated. Although calcium-dependent GEFs such as Ras–guanyl nucleotide releasing factors (Ras–GRFs[18]) and GAPs (for review, see REF. 17) have been identified, the precise GAPs and GEFs that are involved in neuronal Ras regulation might differ depending on the stimulus and/or cell type. In neurons, signal transduction 'downstream' of Ras seems to occur through the classical kinase cascade, though subtle differences are evident when compared with non-neuronal cells.

LONG-TERM POTENTIATION (LTP). A possible mechanism for information storage, LTP is a long-lasting strengthening of synaptic responses following specific patterns of 'firing'.

LONG-TERM DEPRESSION (LTD). An enduring weakening of synaptic strength that is thought to interact with LTP in the cellular mechanisms of learning and memory.

Functions of neuronal ERK signalling

The presence of all of the key elements of the Ras–ERK signalling cascade in neurons, and the activation of this pathway in response to glutamate receptor stimulation, implicate this cascade in neuronal function. However, elucidating the specific roles of ERK and its downstream kinases in the adult brain has been difficult. A major advance was the development of specific inhibitors of MAPK cascades. These inhibitors have been extremely useful to the study of these kinases in many tissues and cell types, including those of the nervous system. The inhibitors can be applied to primary neuronal cultures, acute or organotypic slices, and can even be infused into a behaving animal. They act on endogenous proteins, and can reach and inhibit their target relatively rapidly so that indirect or prolonged effects (for example, those requiring protein synthesis, or autocrine signalling loops) are minimized. Several years ago, a new cell-permeant inhibitor of ERK activation was described[19,20]. Both this compound, PD 98059, and a more potent, structurally unrelated inhibitor, U0126 (REF. 21), target the protein kinase MEK. PD 98059 and U0126 have now been used in over 3000 studies to implicate signalling through MEK (and therefore MEK's only known substrate, ERK) in various cellular processes.

An important caveat to the use of kinase inhibitors is their specificity — inhibitors that act as ATP competitors are seldom specific, as all kinases use ATP (see REFS 22,23 for an enlightening analysis of this point). Importantly, neither PD 98059 nor U0126 inhibit MEK by competing with ATP. Instead, both compounds seem to act by preventing the activation of MEK by its 'upstream' kinase, Raf[22]. PD 98059 and U0126 are therefore more likely to be specific than are other kinase inhibitors.

The use of MEK inhibitors to block specific forms of synaptic plasticity provided some of the first convincing evidence that ERK signalling is crucial to brain function. Synaptic plasticity is thought to be crucial for information processing in the brain and to underlie many complex behaviours such as learning and memory. The best-studied forms of synaptic plasticity in the central nervous system are LONG-TERM POTENTIATION (LTP) and LONG-TERM DEPRESSION (LTD) of excitatory synaptic transmission[24]. The molecular mechanisms underlying LTP and LTD have been extensively characterized, and the regulation of protein phosphorylation has a central role in induction of LTP and LTD. In 1997, English and Sweatt provided the first evidence that ERK signalling is involved in plasticity, using the MEK inhibitor PD 98059 to block LTP induction in the hippocampus[25]. The same authors had previously shown that ERK was activated in hippocampal slices by high-frequency stimulation patterns that induce LTP[26]. Interestingly, PD 98059 prevented both increases in ERK activity and induction of LTP. These findings have now been replicated and extended by many other groups[16,27–32] (see FIG. 2a, for example).

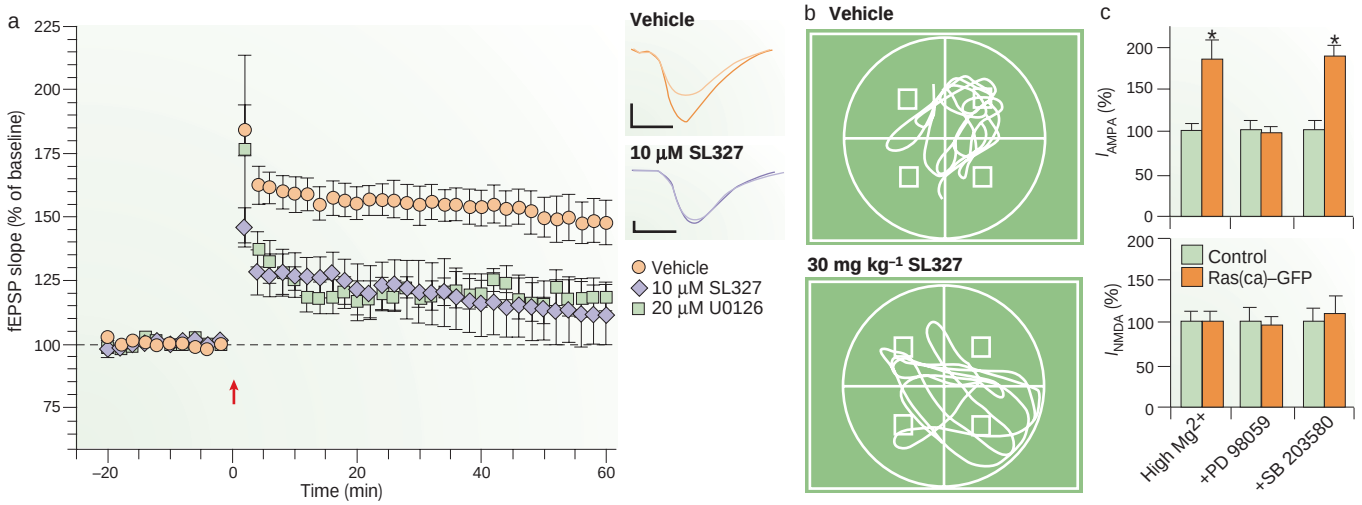

Figure 2 | **Extracellular signal-regulated kinase (ERK) activation is required for synaptic plasticity, learning and memory, and increased α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) transmission. a** | Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitors prevent certain forms of long-term potentiation (LTP). Pre-incubation of rat acute hippocampal slices with the MEK inhibitors SL327 or U0126 prior to high-frequency stimulation (red arrow) prevented subsequent LTP. Modified, with permission, from REF. 32 © (2003) Cold Spring Harbor Laboratory Press. Interestingly, the authors demonstrated that only certain forms of LTP are ERK-dependent. The insets show representative traces from vehicle- and SL327-treated mouse slices before (faint) and after (bold) tetanization. Scale bars are 1 mV by 8 ms. fEPSP, field excitatory postsynaptic potential. **b** | Impaired performance of MEK inhibitor-treated animals in a water maze task. Mice given an intraperitoneal injection of the MEK inhibitor SL327 or vehicle control were placed in a water maze containing a hidden escape platform. The mice could learn the location of the platform by reference to distal visual cues. Both SL327- and vehicle-treated animals learned to find the platform following training. The platform was then removed and the mice were subsequently tested for their ability to remember the previous position of the platform. The figure shows a trace of the swim path of a vehicle-treated mouse and an SL327-treated animal. The vehicle-treated animal searched intensely in the area of the maze where the platform was previously positioned (the top right quadrant, viewed from above). However, the SL327-treated mouse searched aimlessly around the pool and seemed unable to remember the location of the platform. Modified, with permission, from REF. 42 © (1999) Cold Spring Harbor Laboratory Press. **c** | Ras-dependent increases in AMPAR transmission are blocked by MEK inhibitors. Infection of organotypic hippocampal slices with Sindbis virus expressing Ras(ca)–GFP (a GFP-tagged constitutively active Ras) caused an increase in AMPAR-mediated synaptic transmission in infected cells. This increase was prevented by the MEK inhibitor PD 98059, but was unaffected by the p38 MAPK inhibitor SB 203580 (upper panel). The active Ras construct did not alter NMDAR (*N*-methyl-D-aspartate receptor)-mediated transmission (lower panel). Importantly, the authors also demonstrated that Ras(ca)–GFP expression occludes subsequent pairing-induced LTP in infected cells. Modified, with permission, from REF. 16 © (2002) Cell Press.

Many forms of synaptic plasticity require ERK

The findings described above implicate ERK signalling in the best-studied form of synaptic plasticity, 'classical' LTP, which occurs at hippocampal CA3–CA1 synapses and requires activation of the NMDA subtype of glutamate receptor. Over the past few years, a number of studies have demonstrated a requirement for ERK activity in several other forms of synaptic plasticity. These include NMDA receptor (NMDAR)-independent forms of LTP in hippocampal area CA1 (REF. 33), several forms of LTP in the dentate gyrus[34], and LTP in the amygdala, which is associated with fear-dependent learning[35,36] (for review, see REF. 37).

Another important study demonstrated a requirement for ERK activation in cortical LTP[38]. This form of synaptic plasticity is thought to underlie the 'rewiring' of synaptic connections that is observed during MONOCULAR DEPRIVATION. Consistent with this supposition, MEK inhibitors prevented the shift in ocular dominance that is normally observed during monocular deprivation. Taken together, these findings indicate that ERK activation is a requirement that is common to many forms of synaptic plasticity in the forebrain. However, the precise targets for ERK in each of these forms of plasticity might differ.

Interestingly, inhibition of ERK signalling also prevents cerebellar LTD in cultured Purkinje cells[39]. However, the role of ERK in this case seems to be to maintain metabotropic glutamate receptors (mGluR) — activation of which is required for LTD to occur — at the Purkinje cell surface, rather than to participate in 'downstream' signalling.

Involvement of ERK in learning and memory

LTP is generally considered to be the most promising candidate for a cellular mechanism that underlies learning and memory, but its precise relationship to these processes is still far from clear. There are numerous forms of LTP and, although many of these are ERK-dependent, there are some that are not (for example, REF. 40). For LTP researchers and those interested in ERK signalling, it would therefore be reassuring if the MEK inhibitors that prevent LTP also affect learning and memory in behaving animals. Experiments of this nature have been performed and clearly support a role for ERK activation in memory processes.

MONOCULAR DEPRIVATION
In mammals, depriving one eye of visual input when neuronal connections between the eyes and visual cortex are forming weakens synaptic connections between these areas. Connections from the 'open' eye populate the area of visual cortex that is normally reserved for the deprived eye. The deprived eye becomes functionally disconnected from the visual cortex, leaving the animal behaviourally blind in this eye, even when visual input is restored.

The forms of long-term memory in mammals in which the involvement of ERK has been best-characterized are spatial learning and fear conditioning. The most common spatial learning experiments generally assess the ability of an animal to learn and remember the location of a hidden platform in a water maze. Training in the maze leads to activation of ERK in the hippocampus[41]. Intrahippocampal infusion of PD 98059 (REF. 41) or systemic administration of a more potent inhibitor of ERK activation, SL327 (a derivative of U0126 that crosses the blood–brain barrier)[42] impairs the ability of animals to subsequently remember the location of the hidden platform (FIG. 2b). In these studies, the MEK inhibitors greatly impaired memory retention, but had lesser effects on memory acquisition. In an interesting extension of their earlier work, Dash and colleagues found that infusion of PD 98059 into the entorhinal cortex — a brain area with projections to the hippocampus — also impaired performance in a maze task[43].

Another form of long-term memory, fear conditioning, is generally studied by placing an animal in a particular environment (or 'context'), delivering an audible cue and then administering a foot-shock. Subsequently, the extent to which animals have typical fear responses (such as freezing) when re-presented with either the cue or the context is determined. As for the water-maze task, training in this experiment leads to ERK activation[27,36]. When re-presented with either the cue or the context, animals that were pretreated with MEK inhibitors have decreased levels of activated ERK and freeze far less frequently, indicating that they have not learned to associate the cue or context with the foot-shock[27,36].

Despite being extremely important to survival, the ability to remember taste is a less well-studied form of memory. Exposure of rats to a new taste results in ERK activation in the insular cortex (which contains the taste cortex). Conditioned taste aversion, a model of learning in which animals learn to associate a new taste with an aversive reaction, is also prevented by MEK inhibitors[44]. Taken together, these results strengthen the view that ERK activation is necessary not only for LTP, but for several forms of learning and memory. The involvement of ERK in memory processes has been discussed in greater detail in a recent review[37].

ERK and the modulation of synaptic transmission

The studies described above show that ERK is required for various forms of synaptic plasticity, and for learning and memory. However, these studies did not address the role(s) of this kinase at a cellular level. More recently, two cellular processes that probably underlie changes in synaptic transmission have been reported to be ERK-dependent.

Hippocampal LTP has been used as a cellular model of learning and memory for many years, but there has been intense controversy regarding its underlying mechanisms. Several recent publications indicate that an increase in the number and/or activity of postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors (AMPARs) is a key step in LTP[45–47]. A second area of intense study is the activity-dependent remodelling of dendritic spines and filopodia. This cytoskeletal remodelling, often termed structural plasticity, is also induced by stimuli that can lead to LTP[48,49].

Cytoskeletal changes and AMPAR insertion could be linked. The formation of new spines and increases in spine size are probably accompanied by the insertion of greater numbers of glutamate receptors into the postsynaptic membrane, leading to increased synaptic transmission. However, further studies are needed to precisely define the interplay between spine size, spine number and changes in synaptic transmission.

Direct control of AMPAR transmission by ERK

Malinow and colleagues recently established a clear link between ERK activity and AMPAR transmission. Employing an elegant system that has yielded several other important findings, they used recombinant AMPARs whose activity could be detected using an 'electrophysiological tag' to investigate the effects of Ras–ERK signalling on AMPAR responses and synaptic plasticity in hippocampal slices[16]. The authors found that expression of a constitutively active form of Ras led to a tonic increase in synaptic AMPAR responses. Crucially, this effect was blocked by PD 98059, indicating that at least one essential function of Ras in this system is to activate ERK (FIG. 2c).

Furthermore, increased AMPAR transmission induced by an active form of calcium/calmodulin-dependent protein kinase II (CaMKII) was also PD 98059-sensitive, indicating that ERK activity is required for CaMKII to drive the insertion of AMPARs into synapses. This finding was striking, as many studies have demonstrated a key role for CaMKII in synaptic plasticity and learning (for review, see REFS 50,51) but without fully defining all of the molecular targets for this kinase. Now it seems that at least one crucial role of CaMKII in LTP is to trigger ERK activation, possibly by acting on Ras regulators such as synaptic Ras–GAP (SynGAP; see below).

The authors also reported that pairing-induced, NMDAR-dependent LTP was prevented by dominant-negative Ras and by PD 98059. This mirrored the results that had been obtained by other researchers using acute hippocampal slices, by demonstrating that: (i) ERK activity affects the long-term changes in AMPAR response that are induced following overexpression of activated Ras or CaMKII; (ii) ERK is essential for the rapid change in AMPAR transmission that occurs during this particular type of synaptic plasticity. These findings are exciting, but the precise route from NMDAR activation to Ras–ERK activation is still unknown and, more interestingly, the key substrate(s) for ERK that lead to increased AMPAR transmission have yet to be identified.

It should perhaps be noted at this point that ERK activation, while necessary for many forms of synaptic plasticity, is not sufficient to induce or mimic LTP. Several electrophysiological stimulation protocols or agonist treatments activate ERK without altering long-term synaptic plasticity. By contrast, overexpression of activated Ras occludes LTP[16], indicating that Ras

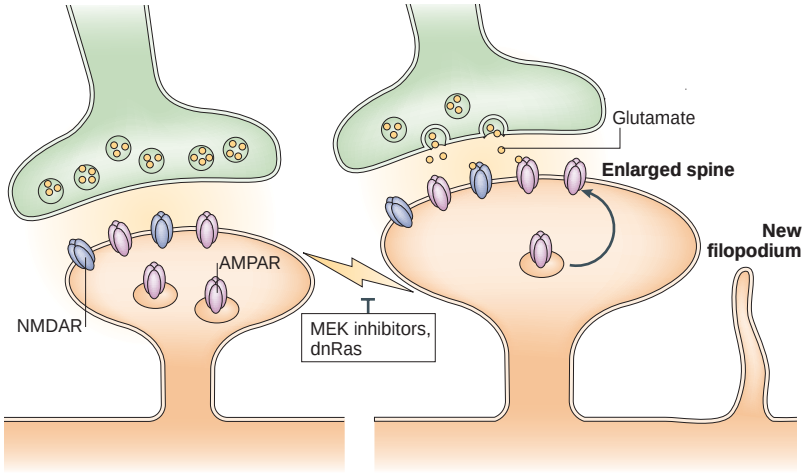

Figure 3 | **Extracellular signal-regulated kinase (ERK) signalling is required for processes that underlie synaptic plasticity.** Following synaptic glutamate release in organotypic slices to activate NMDARs (*N*-methyl-D-aspartate receptors)[16], or repeated depolarization or NMDAR activation of primary neuronal cultures[54,55], the postsynaptic neuron inserts new AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) into the synapse, dendritic spines enlarge, and new filopodia (which might be precursors for additional synaptic contacts) and spines appear. Each of these changes could contribute to increased synaptic transmission. Importantly, these changes are prevented by mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitors, or by dominant-negative Ras (dnRas) expression, indicating a requirement for ERK activation in each of these processes.

activation is sufficient for LTP. These results indicate that other Ras targets (possibly phosphatidylinositol 3-kinase signalling, which is also required for certain steps in LTP[52,53]) are also important to synaptic plasticity.

ERK activation is required for structural plasticity

A requirement for ERK activation in structural plasticity has also been demonstrated. Studies using time-lapse imaging to examine the formation of new dendritic spines and filopodia in cultured hippocampal neurons found that new spines were only formed following repeated depolarization, rather than following a single depolarization[54]. Interestingly, only repeated depolarization — perhaps a simple biochemical correlate of repeated learning — induced sustained activation of ERK. As with LTP experiments in slices, this sustained ERK activation was not merely correlated with spine formation but was essential for it, as the MEK inhibitor U0126 prevented both ERK activation and the formation of new spines and filopodia. A complementary study, also using cultured hippocampal neurons, reported increases in ERK activity and dendritic spine number following NMDAR activation. This increase in spine number was prevented by MEK inhibitors[55] and was dependent on protein synthesis. These studies did not determine the precise role(s) of ERK in spine morphogenesis, which could be to directly regulate synaptic structure by phosphorylating synaptic proteins, to control translation of dendritic mRNAs, to induce transcription of genes that are necessary for synaptic remodelling, or a combination of these factors. However, further dissection of the role of ERK in this or related systems might shed light on the interplay between structural plasticity and changes in synaptic transmission. A schematic summary of the involvement of ERK in structural plasticity and AMPAR transmission is presented in FIG. 3.

Substrates for ERK in synaptic plasticity

ERK activity is evidently essential for several forms of synaptic plasticity, for certain types of learning, and to cellular changes that are thought to underlie these events. However, as hinted at in the previous section, the identity of the substrates that are phosphorylated by ERK in these situations is far from being resolved. Following LTP-inducing stimuli, phospho-ERK immunoreactivity develops rapidly in both dendritic and somatic regions of hippocampal neurons[56,57], indicating that ERK substrates might be present throughout the neuron.

$K_V4.2$ and cytoskeletal proteins are ERK targets

The requirement for ERK activation in the control of gene expression is well documented and is probably an essential function of this cascade in the regulation of synaptic plasticity (see below). Indeed, certain forms of U0126-sensitive potentiation are prevented if nuclear translocation of ERK does not occur[31]. However, active ERK, while clearly present in nuclei of stimulated cells, is also present at high levels in the cell cytoplasm and in dendrites[31,58,59]. Furthermore, MEK inhibitors exert their effects on LTP relatively rapidly (within 20–30 min) following high-frequency stimulation[25,27,30,32,59] (see FIG. 2a). This short period is unlikely to be sufficient for translocation of ERK from distal dendrites to the nucleus, followed by activation of transcription and subsequent translation and trafficking of the new gene product to potentiated synapses.

It seems more likely that ERK and its downstream effector kinases also control the phosphorylation of cytoplasmic proteins much closer to synapses. Extranuclear targets that are phosphorylated by ERK during learning have already been reported in invertebrates[60,61], and evidence that ERK regulates synaptic and/or cytoplasmic proteins in mammals is now accumulating. One possible extranuclear target for ERK is the voltage-dependent K^+ channel $K_V4.2$ — an excellent substrate for ERK[62] that is phosphorylated in an ERK-dependent manner in the hippocampus following various stimuli[58]. Increased phosphorylation of this channel at ERK sites during LTP has recently been reported[63]. The possibility that other extranuclear proteins are targets for ERK (and/or ERK's downstream effector kinases) presents an interesting area for future research.

Direct transcription-factor targets for ERK

Translocation of activated ERK from the cytoplasm to the nuclei of non-neuronal cells is a well-documented phenomenon[64,65]. This process also occurs in neurons[31,56], indicating that nuclear proteins such as transcription factors are important targets of ERK signalling in the regulation of synaptic plasticity. Several of these transcription factors are probably the same as those that have been identified as ERK substrates in other systems. The best-characterized involvement of such proteins in a

Box 1 | RSK2, an ERK effector genetically implicated in neuronal function

Many experiments that are discussed in the main text relied on MEK (mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase) inhibitors to implicate ERK signalling in the process under investigation. However, in any field, results can be viewed with greater confidence if complementary approaches draw a similar conclusion. In this regard, genetic studies have also provided compelling evidence for the importance of ERK signalling in neuronal function and plasticity. These studies have included both analyses of the phenotypes of knockout and transgenic mice, and identification of mutations in MAPK cascade enzymes as causative factors in human neurological conditions.

A well-known example of the latter situation is Coffin-Lowry syndrome (CLS), an inherited condition that is characterized clinically by 'severe psycho-motor retardation'; that is, poor coordination and low intelligence quotient (IQ)[115]. Genetic linkage studies showed that this condition is caused by point mutations in the ribosomal protein S6 kinase *RSK2* gene[77], which codes for a protein kinase that is directly phosphorylated and activated by ERK both *in vitro*[116] and *in vivo*[20] (see also the section on cyclic-AMP-responsive element (CRE)-binding protein (CREB) phosphorylation). Analysis of cells taken from CLS sufferers shows that *RSK2* mutations are heterogeneous, but invariably cause a reduction or loss of RSK2 kinase activity[117]. More recently, the phenotype of an *RSK2*-knockout mouse was reported[76]. These mice seem to be a good model for CLS, in that they have poor coordination and poor learning in a maze task (but it is unclear whether the coordination deficit contributes to the poor water-maze performance). Taken together, these findings indicate an important role for RSK2 in correct neuronal development and/or function.

Interestingly, the gene coding for another RSK isoform, RSK4, lies in a region of the X-chromosome that is commonly deleted in non-specific X-linked mental retardation[118], and several RSK isoforms are located in brain regions that are important for learning and memory[78]. At a subcellular level, RSK proteins are present in postsynaptic density fractions[87] and NMDAR (*N*-methyl-D-aspartate receptor)-containing protein complexes[85]. These findings indicate that all RSKs might have important roles in neuronal development and/or function.

An interesting question that arises from these findings is why other isoforms (RSK1, 3 and 4) cannot compensate for the loss of RSK2 function in CLS, as these kinases are closely related and probably possess similar substrate specificity *in vitro*. A possible reason comes to light when the respective RSK carboxy (C) termini are examined. All four RSK isoforms terminate in a S-T-X-L-COO- sequence that is known to bind to PSD95/Discs large/ZO1 (PDZ)-domain containing proteins. Indeed, the C tails of RSK1 and RSK2 (and presumably those of RSK3 and RSK4, although this has not been tested directly) bind PDZ domain proteins *in vitro* and *in vivo*[119]. PDZ-domain containing proteins have important roles in the differential subcellular targeting of interacting proteins, and organize proteins into signalling complexes[120]. Interestingly, the PDZ-domain binding specificities of RSK1 and RSK2 differ, and they interact with different PDZ domain proteins *in vivo*[119]. This indicates that, although the kinase activities of different RSKs *in vitro* might be similar, they 'see' different substrates *in vivo* and therefore might be unable to compensate for one another.

learning model is that of the transcription factor Elk1, which is phosphorylated in an ERK-dependent manner in the insular cortex of animals exposed to new taste stimuli[44]. Elk1 is also phosphorylated in a MEK-inhibitor-sensitive manner following LTP induction in the dentate gyrus[56], and in the hippocampi of animals subjected to context-dependent fear conditioning[66]. However, no MEK inhibitors were used in the latter study to confirm that this learning model was ERK-dependent. Transcription-factor targets of ERK that have been identified in other tissues, such as Myc and C/EBPβ (CCAAT/Enhancer-binding protein β) (for review, see REF. 67), have not yet been definitively shown to be targets in the brain.

CREs
Elements in the promoter region of many signal-regulated genes, recognized by CREB family transcription factors.

CREB as a target for ERK during plasticity

The transcription factor CREB (cyclic-AMP-responsive element (CRE)-binding protein) is perhaps the most intensively studied kinase substrate in the field of learning and memory (recently reviewed in REFS 68,69). Studies in invertebrates and rodents indicate that CREB-dependent transcription is essential for many forms of learning and memory (for review, see REF 69), although recent data indicate that the requirement for CREB might differ depending on the learning model[69,70].

Phosphorylation of CREB at Ser133 leads to recruitment of other transcription machinery to CREs to regulate gene transcription (reviewed in REFS 68,69,71). As such, it is probable that CREB phosphorylation at Ser133 is an important step in the induction of gene expression that is essential to learning. Much attention has therefore been focused on the identity of the kinase(s) that phosphorylate this residue. It now seems likely that the rapid initial increase in CREB Ser133 phosphorylation in response to neuronal activity is mediated by the nuclear protein kinase CaMKIV[72]. However, the prolonged phosphorylation of CREB that is essential for CRE-dependent transcription is prevented by MEK inhibitors, indicating a requirement for ERK signalling in the maintenance of Ser133 phosphorylation[72,73].

Although ERK cannot phosphorylate Ser133 directly, this residue can be phosphorylated *in vitro* by two sets of kinases that are downstream of ERK — RSKs and MSKs. *In vitro* experiments showed that CREB Ser133 is a reasonable substrate for RSK1, RSK2 and RSK3 (REFS 6,74), and an excellent substrate for MSKs[6]. It would therefore seem likely that one or more of these downstream kinases mediates prolonged CREB phosphorylation. Early studies implicated RSK2 as the ERK-dependent CREB kinase in response to neurotrophin signalling[75]. It is tempting to propose that RSK2 mediates ERK-dependent CREB phosphorylation in response to neuronal activity because mutations in the *RSK2* gene cause learning deficiencies in mice[76] and humans[77] (see BOX 1), and RSK2 protein is prominently expressed in brain regions that are important for cognitive function[78]. However, no mention of impaired CREB phosphorylation was made in publications from three separate groups who deleted the *RSK2* gene by homologous recombination[76,79,80], despite obvious phenotypes that are caused by the absence of this kinase. It is probable that the learning deficits observed in the absence of RSK2 function[76,77] are due, at least in part, to impaired phosphorylation of neuronal RSK2 substrates other than CREB.

In contrast to *RSK2* knockouts, cells derived from mice lacking MSKs exhibit greatly impaired CREB phosphorylation in response to a number of stimuli[7,8]. However, the neuronal signalling pathways of these mice are yet to be elucidated. Therefore, the ERK-dependent CREB kinase(s) that phosphorylate Ser133 in response to neuronal activity have not been unequivocally identified, and might differ depending on the cell type.

Box 2 | GAPs and GEFs genetically link ERK signalling in plasticity

The neurofibromatosis-1 (NF1) protein is another link between Ras–extracellular signal-regulated kinase (ERK) signalling and learning and memory. Mutations in the *NF1* gene, which codes for a Ras–GTPase-activating protein (GAP), cause learning deficits in humans[121], and similar mutations in mice provide a good model for this condition[122]. Analysis of *NF1*$^{+/-}$ mice indicates that Ras is excessively activated in these animals, and their learning deficits can be rescued by manipulations that decrease Ras functionality[123]. So, it seems likely that learning and memory require a 'happy medium' for the dynamic regulation of Ras–ERK signalling — when levels are artificially low (as in the presence of MEK inhibitors) or too high (as in *NF1*$^{+/-}$ mice) then learning ability is impaired.

Another Ras regulator that is implicated in learning and memory is Ras–guanyl nucleotide releasing factor (Ras–GRF), a calcium-sensitive guanyl nucleotide exchange factor (GEF) for Ras[18]. Ras–GRF expression is highly brain-specific, and it is perhaps no surprise that this protein should regulate neuronal Ras function. Indeed, recent findings indicate that Ras–GRF1 can directly bind the NMDAR (*N*-methyl-D-aspartate receptor) NR2B subunit, and that disrupting this interaction prevents NMDAR-dependent ERK activation[124]. However, the exact processes that are controlled by Ras–GRF are unclear as different lines of Ras–GRF-knockout mice exhibit distinct deficits in different learning models[125,126]. As yet, no satisfactory explanation for these differences has been proposed but, taken together with data from the *NF1*$^{+/-}$ mice, it seems clear that aberrant regulation of neuronal Ras (and so, presumably, ERK) signalling leads to cognitive impairments.

'Memory genes': ERK-induced gene targets

Transcription factors such as CREB and Elk — the phosphorylation of which is ERK-dependent — probably regulate the transcription of many different genes during plasticity. Identification of these genes and the mechanism(s) by which their newly transcribed products might control long-term changes in plasticity, and even learning and memory, is an intriguing future endeavour. As might be expected from the results on CREB phosphorylation, MEK inhibitors prevent transcription of CRE-reporter genes following both LTP in hippocampal slices[28] and a fear-dependent learning model in behaving rats[81]. However, precisely which CRE-containing genes are essential for LTP and/or learning remains unclear.

Genes containing other promoter elements such as serum response elements (SREs) are probably also targets of ERK signalling, as SREs are recognized by transcription factors such as Elk1. Knowledge of specific gene targets for ERK signalling during plasticity, however, is limited to a few reports. For example, transcription of the immediate early gene *Zif268* in the dentate gyrus is prevented[56] or attenuated[59] by infusion of MEK inhibitors into this region prior to LTP. Upregulation of the immediate early gene *Arc*, which has an essential but undefined role in LTP and memory[82], is also MEK-dependent during brain-derived neurotrophic factor (BDNF)-induced LTP in the dentate gyrus[83] and following treatment of primary hippocampal cultures with forskolin[84]. However, the involvement of ERK signalling in the induction of these or other genes during more widely studied forms of LTP, or during learning models, has not been reported.

Key players in neuronal ERK signalling

Combined pharmacological approaches (discussed above) and genetic studies (BOXES 1,2) have provided strong evidence for the involvement of ERK in neuronal development and plasticity. There are other findings that are less 'cut-and-dried' in their conclusions, but which identify several other factors that are probably important in the regulation of neuronal ERK signalling.

Traditional biochemical approaches, recently combined with proteomic techniques, have demonstrated the presence of several upstream regulators and downstream effectors of ERK in complexes, isolated from mouse forebrain, that contain NMDARs[85]. Less comprehensive, earlier studies had demonstrated the presence of ERK, RSK1 and the Ras–GEF Sos, in isolated POSTSYNAPTIC DENSITY (PSD) fractions[86,87]. Many components that are necessary for Ras–ERK signalling might therefore be present at NMDAR-containing PSDs. However, studies indicate that little ERK is synaptically localized. So, ERK might only loosely be associated with NMDAR complexes or complexed with NMDARs only at intracellular locations.

It is of interest that SynGAP[88,89], which might act as a 'brake' on ERK activity *in vivo*, is present at such high levels in the PSD as to allow its identification biochemically[89,90]. SynGAP was identified in the yeast two-hybrid system by its interaction with the third PSD95/Discs large/ZO1 (PDZ) domain of PSD95, a core component of the PSD[88], and by classical biochemical methods that purified, sequenced and identified proteins from rat brain PSD preparations[89]. Both of these studies identified a 135-kDa protein that contained a C-terminal PDZ ligand (through which SynGAP binds PSD95), a Ras–GAP domain and other interaction motifs. The selective localization of SynGAP at excitatory synapses indicates that it is important for the regulation of synaptic Ras signalling by NMDAR activation or other synaptic signals. However, the mechanisms of SynGAP regulation are still unclear and are under investigation. Mice with targeted deletions of the *SynGAP* gene die shortly after birth[91,92]. Interestingly, the heterozygote (*SynGAP*$^{-/+}$) mice have increased levels of phospho-ERK[91] and increased synaptic targeting of AMPARs[92], as well as impaired hippocampal synaptic plasticity and spatial learning[91,92]. These results indicate that SynGAP is crucial to synaptic plasticity and learning and memory.

Another cascade, another function?

A kinase cascade involving sequential phosphorylation and activation of kinases that are homologous to, but distinct from, their counterparts in the classical Ras–ERK cascade leads to activation of the p38 MAPK[93–95]. p38 MAPK was initially characterized owing to its role in the response of cells to various adverse stimuli such as heatshock and bacterial endotoxin. 'Upstream' kinases that are homologous to, but distinct from, MEK and Raf have been characterized as p38 activators in non-neuronal cells (reviewed in REF. 96). However, like ERK1 and ERK2, p38 is also highly expressed in brain regions that are important for learning and memory[97], and is now emerging as a key player in the regulation of synaptic plasticity.

As for ERKs, studies investigating the role of p38 have been aided tremendously by the use of cell-permeant kinase inhibitors. p38 activity can be blocked

POSTSYNAPTIC DENSITY (PSD). In electron micrographs of glutamatergic synapses, an ultrastructural thickening is visible on the postsynaptic side. This PSD can be purified biochemically owing to its resistance to detergents, and is enriched in NMDA-type glutamate receptors, as well as in many scaffold/adaptor proteins and signalling enzymes.

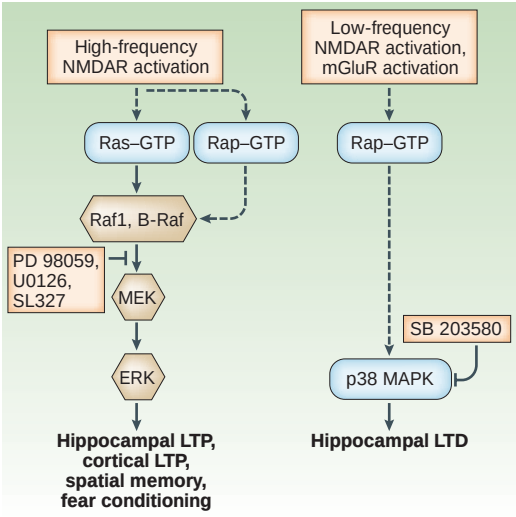

Figure 4 | **Parallel mitogen-activated protein kinase (MAPK) cascades might regulate distinct forms of synaptic plasticity.** High-frequency NMDAR (*N*-methyl-D-aspartate receptor) stimulation preferentially activates Ras–extracellular signal-regulated kinase (ERK) signalling and leads to long-term potentiation (LTP). ERK-dependent plasticity is prevented by the MAPK/ERK kinase (MEK) inhibitors PD 98059, U0126 and SL327. By contrast, low-frequency NMDAR stimulation or activation of metabotropic glutamate receptors (mGluRs) activates p38 MAPK and leads to long-term depression (LTD). In this case, LTD has been reported to be prevented by the p38 inhibitor SB 203580. The precise role of Rap (which has been implicated in both LTP and LTD) is yet to be established. ERK signalling is required for hippocampal and cortical LTP, and for certain forms of spatial memory and fear-dependent learning. p38 MAPK has been implicated in two forms of hippocampal LTD, although its roles in behaviour are yet to be established.

by the compound SB 203580 (REF. 98) or by closely related inhibitors. SB 203580 inserts into the ATP-binding pocket of p38 (that is, it is an ATP competitor) but binds a residue in the pocket that is conserved in only a small number of kinases, and is therefore reasonably specific[99]. This specificity is enhanced by the fact that p38 has a relatively high K_m for ATP (that is, it binds ATP weakly), which allows SB 203580 to 'compete' more effectively with ATP on p38 than it might on other kinases[100] (but see REF. 101). Interestingly, the two isoforms of p38 (p38α and p38β) are localized in different subcellular regions of neurons[97]. However, no attempt has been made to assign specific roles to each isoform in the brain because the SB 203580 compound inhibits them both[102].

p38 MAPK signalling in neuronal plasticity

Given the prominent expression of p38 in the adult brain, and bearing in mind the neuronal roles of ERK, it is perhaps not surprising that p38 signalling should also regulate synaptic plasticity. A form of hippocampal LTD that depends on mGluR activation was reported to be inhibited by SB 203580 but not by PD 98059, indicating a requirement for p38 MAPK but not for ERKs in this form of plasticity[29]. Perfusion of an active form of p38 could mimic and occlude this mGluR LTD.

Although this study was enlightening, the authors did not dissect either the route by which p38 was activated during LTD or what its downstream targets might be. Indeed, the role(s) of this kinase might be complex, because the authors also demonstrated effects of postsynaptically infused p38 on presynaptic function, indicating that this kinase regulates a retrograde messenger. Provocative new results indicate a requirement for p38 activation in a different form of hippocampal LTD that depends on NMDAR activation[16]. Behaviourally, little has been attributed to p38, although classical eye-blink conditioning — a form of associative learning that is controlled by the cerebellum — is prevented by infusion of SB 203580 into the cerebellar vermis[103]. The precise targets for p38 in these processes are not clear.

Upstream of p38 — what's the Rap?

The p38 MAPK studies described above did not fully elucidate the mechanism by which this kinase might be activated during LTD. However, in one study, the Ras-related small G protein Rap was reported to lie upstream of p38 (REF. 16), because both p38 activation and NMDAR-dependent LTD were prevented by the expression of dominant-negative Rap in hippocampal organotypic slices. This is consistent with the finding that Rap2 (but not, apparently, Rap1) is enriched in NMDAR-containing protein complexes[85]. Furthermore, the identification of two RapGAPs that are enriched in PSDs indicates that Rap signalling is probably important at synapses[104,105]. Overexpression of one of these RapGAPs (spine-associated RapGAP), which should inhibit Rap and so also p38 signalling, leads to increased size of dendritic spines[104]. This result could be explained by a switch in the balance between 'potentiative' signalling that is controlled by Ras–MAPK and 'depressive' signalling that is mediated through Rap–p38.

This simple model, whereby Ras–ERK signalling controls potentiation of synaptic transmission and Rap–p38 signalling controls depression (FIG. 4), is unlikely to be the whole story. This is because other studies have provided compelling evidence that, in certain neuronal cell types, Rap signalling can control the activation of ERK1 and ERK2 owing to its ability to activate the B-Raf isoform[106,107] (for review, see REF 108). B-Raf is an isoform of Raf that is highly expressed in the brain and in dendrites. Conversely, the expression of another Raf isoform, Raf1, is restricted to neuronal cell bodies and does not extend to dendrites[109]. So, B-Raf is the best candidate for the Raf isoform that participates in synaptic signalling, although the relative contributions of Ras and Rap to B-Raf activation in response to neuronal activity have not been examined (and might differ depending on the cell type).

Bearing this in mind, a recent study reported reduced B-Raf activity and a concomitant decrease in the activity of a membrane-associated pool of ERK[63] in a transgenic mouse overexpressing a dominant-negative *Rap1B* transgene under the control of an α-CamKII promoter. These mice also exhibited decreased ERK-dependent LTP and deficits in hippocampus-dependent learning. It is tempting to conclude that Rap→B-Raf signalling (which would be blocked in these mice) is normally essential for

Box 3 | Central roles and modulators: the long-term potentiation (LTP) debate

As discussed in this journal[127] and elsewhere[128], claims that a molecule 'is essential for' LTP and/or learning and memory are sometimes made without strong supportive evidence. Although most researchers agree that certain proteins (for example, NMDARs (*N*-methyl-D-aspartate receptors) and calcium/calmodulin-dependent protein kinase II (CaMKII)) have pivotal roles in LTP, there are other proteins that, when manipulated, impair LTP, but that might only modulate this process. For example, proteins that are mainly involved in the regulation of, for instance, inhibitory transmission, might still alter LTP when knocked out or inhibited. Whether extracellular signal-regulated kinase (ERK) signalling is a central player or a modulator, or lies somewhere in between, is presently unclear, as most experiments using mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitors cannot effectively discriminate between these two possibilities.

The strongest evidence for a direct role of ERK in LTP is that constitutively active Ras constructs mimic and occlude LTP, and that MEK inhibitors prevent this effect[16]. However, as discussed in the main text, Ras probably signals to other effectors in addition to ERK, so even in this case ERK's role might be essential but still modulatory. If prolonged CREB (cyclic-AMP-responsive element (CRE)-binding protein) phosphorylation is indeed ERK/ribosomal protein S6 kinase (RSK)/mitogen and stress-activated kinase (MSK)-dependent[72,73], and CREB phosphorylation is essential for the induction of genes necessary for LTP/learning, then an active role for ERK is indicated.

However, these two findings alone are probably insufficient to build a convincing case. As stressed in our main conclusions, the identification of *in vivo* kinase substrates is crucial to our understanding of the relative contributions of different signalling pathways to synaptic plasticity. So, if the main ERK/RSK/MSK substrates in the hippocampus and/or cortex are found to regulate constitutive membrane trafficking, inhibitory transmission and so on, a modulatory role for this cascade would be strongly supported. However, if ERK and/or its downstream kinases are found, for instance, to regulate glutamate receptors by direct phosphorylation, to phosphorylate postsynaptic proteins that control synapse-specific receptor insertion/recycling or presynaptic proteins that regulate neurotransmitter release, or to control activity-dependent mRNA translation (all of which are probably more central to plasticity processes), then a pivotal role for ERK signalling in LTP is much more likely. Further experiments with non-phosphorylatable versions of identified substrates could then clarify the necessity and/or sufficiency of these proteins for LTP. Until such substrates are identified, however, conclusions regarding the centrality of ERK signalling (and other 'candidate' pathways such as phosphatidylinositol 3-kinase/Akt signalling) should be drawn cautiously, and the frustrations of those outside the field, although understandable, should be kept in check.

ERK-dependent plasticity and learning. However, the level of expression of the inhibitory Rap1B construct was extremely high in these mice, and could have interfered with endogenous Ras→B-Raf→MEK→ERK signalling. Whether endogenous Rap1B→B-Raf signalling is important during synaptic plasticity and/or learning is not yet clear. The lack of effect on LTP of overexpression of Rap, reported by Zhu and co-workers[16], is apparently at odds with these findings, and as yet there is no satisfactory explanation for these differences.

Exciting possibilities and general caveats

Studies using cell culture systems and acute and organotypic slices, and investigations using behaving animals, have all indicated a requirement for the activation of ERK or p38 MAPK in the control of several forms of synaptic plasticity, and in learning and memory (FIGS 2,4 and BOX 3). So, what are the main questions that should be addressed in the future?

Perhaps the most important piece of the puzzle that is still missing is the identification of the direct substrates of the kinases in question. This is a problem that is not limited to neuroscience, and more information will probably be obtained by combining inhibitor studies with proteomic/bioinformatic approaches. These encompass the recently developed phospho-substrate motif antibodies[110] and 'phospho-proteomics', in which high-sensitivity mass spectrometric methods are adapted to specifically identify phosphorylated proteins[111–114]. Together, these different approaches have the potential to identify precise *in vivo* substrates for individual kinases, delivering greater insight into the processes that they regulate.

Given the roles of many MAPK cascade enzymes in non-neuronal processes, conventional knockout approaches might generate severe phenotypes in which subtle neuronal changes are obscured. However, the use of temporally or spatially restricted knockouts of MAPK cascade enzymes might yet yield important information on their roles in the brain.

A more general problem that is yet to be tackled comprehensively is that of synapse-to-nucleus signalling. Are activated ERK molecules translocated all the way from activated synapses to the cell body and/or the nucleus (a considerable distance in most neurons), or are different 'pools' of MAPK cascade enzymes localized at each site? Each of these possibilities raises further questions. For example, if ERK is translocated, does it 'tag' (for example, by phosphorylation of specific substrates) the activated synapses from which it originated? Conversely, if there are spatially separate pools of ERK, are they activated by the same stimuli, and what are the precise targets of each? Further examination of this question might reveal precisely how ERK signalling can regulate phosphorylation of both synaptic proteins and transcriptional regulators, and which of these substrates are necessary for different forms of plasticity. The interplay between ERK signalling and other transduction pathways during plasticity is an area of daunting complexity, but one that will also need to be dissected.

All in all, current studies into neuronal MAPK signalling have provided new insight into the mechanisms of synaptic plasticity, learning and memory. The prospects for enhancing our understanding of brain function are excellent as work to elucidate these mechanisms, and the precise functions of particular kinases, continues.

Note added in proof

Tonegawa and colleagues recently reported deficits in neuronal activity-induced protein translation caused by MEK inhibitors or by a dominant-negative *MEK1* transgene, supporting a role for ERK in the control of protein synthesis during plasticity[129].

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Competing interests statement

The authors declare that they have no competing financial interests.

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