

# Knockout of ERK1 MAP Kinase Enhances Synaptic Plasticity in the Striatum and Facilitates Striatal-Mediated Learning and Memory

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## Summary

Extracellular signal-regulated kinases (ERK1 and 2) are synaptic signaling components necessary for several forms of learning. In mice lacking ERK1, we observe a dramatic enhancement of striatum-dependent long-term memory, which correlates with a facilitation of long-term potentiation in the nucleus accumbens. At the cellular level, we find that ablation of ERK1 results in a stimulus-dependent increase of ERK2 signaling, likely due to its enhanced interaction with the upstream kinase MEK. Consistently, such activity change is responsible for the hypersensitivity of ERK1 mutant mice to the rewarding properties of morphine.

Our results reveal an unexpected complexity of ERK-dependent signaling in the brain and a critical regulatory role for ERK1 in the long-term adaptive changes underlying striatum-dependent behavioral plasticity and drug addiction.

## Introduction

Activity-dependent changes in synaptic strength such as long-term potentiation (LTP) have been postulated to be part of the cellular mechanisms of learning and memory formation. In particular, over the last decade, pharmacological and genetic approaches have convincingly demonstrated the importance of the integrity of synaptic plasticity for the formation of hippocampus-dependent memories (Martin et al., 2000b; Mayford and Kandel, 1999). It is highly believed that binding of glutamate and other neurotransmitters to their receptors can activate intracellular signaling cascades that eventually lead to gene transcription and synaptic remodeling (Abel and Lattal, 2001; Sweatt, 1999). However, while several molecular links between synaptic signaling and learning have been found to be essential to the functions of most of the cerebral structures, it is likely that certain mechanisms may be specific to discrete brain areas.

The striatum and its ventral extension, the nucleus accumbens (NA), are key neural elements in the control of a wide variety of behaviors (Graybiel, 1995). It is well established that synaptic integration of glutamatergic and dopaminergic stimuli in the striatum is necessary for normal functions of this structure, from motor planning to reward seeking, and procedural learning (Berke and Hyman, 2000; Nicola et al., 2000). Consistent with a crucial role of dopaminergic signaling in the regulation of striatal activity is the evidence that depletion of dopamine (DA) interferes with both striatum-based motor activity and learning processes, as demonstrated by the cognitive impairment characterizing advanced Parkinson's disease (Olanow and et al., 2000). In addition, striatal nuclei are crucial brain structures implicated in drug addiction (Hyman and Malenka, 2001; Nestler, 2001). The evidence for a direct involvement of dopaminergic signaling in such a variety of functions has also been substantiated by genetic experiments. For instance, ablation in the mouse of D1 and D2 receptors results in opposing effects on basal locomotion and altered behavioral responses to drugs of abuse such as morphine and cocaine (Maldonado et al., 1997; Xu et al., 1994a, 1994b). However, still very little is known of the intracellular mechanisms leading to synaptic plasticity in the striatum. Classical studies on D1- and D2-like receptors have demonstrated that these receptors are differentially coupled to the cAMP/PKA pathway but recent evidence indicates that DA receptor activation in the striatum may also activate other signaling cascades, including the ERK pathway (Nestler, 2001).

The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway is a signaling cascade, controlled by the Ras family of small

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GTPases, which plays a crucial role in a variety of cell regulatory events (Shields et al., 2000; Pearson et al., 2001). The role of the Ras/ERK pathway in cell proliferation, differentiation, and survival is well established, but several lines of evidence have recently pointed to an involvement of Ras/ERK signaling in long-term synaptic changes and behavior (Adams and Sweatt, 2002; Mazzucchelli and Brambilla, 2000; Orban et al., 1999). Prolonged activation of postsynaptic receptors results in translocation of ERK kinases to the nucleus, resulting in phosphorylation and activation of transcription factors such as CREB and Elk-1. These nuclear events initiate cell-specific gene expression programs necessary for synaptic remodeling and long-term changes in synaptic efficacy.

The most abundant ERK kinases in the brain are the products of *ERK1* and *ERK2* genes. Most of the experimental evidence for a role of ERK-dependent signaling in synaptic function has so far relied on the use of inhibitors that do not discriminate between the two kinase isoforms, since they all act on the upstream activators MEK1/2. However, until now, no evidence has been produced for specific functions of ERK1 and ERK2 kinases. Here we show that ERK1 and ERK2 MAP kinases play specific and distinct roles in regulating synaptic function. Mice lacking ERK1 manifest abnormal signaling responses, which are linked to an upregulation of ERK2 activity in the brain. These biochemical changes result in dramatic alterations of synaptic plasticity in the striatum and correlate with altered behavioral responses dependent on this structure, including a striking increase in sensitivity to the rewarding properties of morphine.

## Results

### In Vivo Loss of ERK1 Does Not Affect Brain Development or Alter Expression of ERK2

To address the in vivo roles of ERK1, we have previously generated protein null mutant mice. These mice are viable, fertile, and of normal size, but manifest deficits in thymocyte maturation (Pagès et al., 1999). Before examining possible effects of ERK1 deletion on synaptic function, we first analyzed the brains of mutant mice for anatomical abnormalities. Histochemical analysis with myelin/Nissl staining (Figure 1A) or parvalbumin and calbindin (not shown) did not reveal any anatomical differences between *ERK1* mutant mice and littermate controls, excluding the possibility of an anatomical alteration in brain development.

To assess the expression profile of both ERKs, and to check for possible compensatory changes in ERK2 expression in ERK1-deficient mice, we then dissected selected brain areas and prepared protein extracts. In all structures analyzed by Western blotting, we observed a more pronounced immunoreactivity for p42<sup>ERK2</sup> in comparison to p44<sup>ERK1</sup> in wild-type brains (Figure 1B). This is in agreement with previous reports, indicating that, in some brain areas such as hippocampus, ERK2 is the predominant isoform (English and Sweatt, 1996, 1997). However, no differences in ERK2 expression were detected between *ERK1* mutant and wild-type mouse brains, indicating that loss of one isoform did not result in compensatory increase of the other. This observation

is consistent with results obtained from peripheral tissues, such as embryonic fibroblasts (Pagès et al., 1999). We also probed brain extracts with phospho-specific antibodies recognizing both phospho-ERK1 and phospho-ERK2, to evaluate the in vivo activation state of the two ERK isoforms (Figure 1B). Once again, no differences between mutants and wild-type extracts were observed, confirming that the absence of ERK1 did not alter the basal phosphorylation state of ERK2. Next, we compared the stoichiometry of in vivo phosphorylation of ERK1 with that of ERK2. Scanning and normalization of the signals in wild-type mice revealed that the basal phosphorylation level of p42<sup>ERK2</sup> was much higher than that of p44<sup>ERK1</sup> in all structures examined (Figure 1C). Similar conclusions were also drawn from in vitro experiments with primary cultures (Figure 1D). In wild-type cortical neurons stimulated with glutamate, p42<sup>ERK2</sup> became strongly activated while little phosphorylation was seen in p44<sup>ERK1</sup>, confirming that ERK2 is the major MEK substrate in the brain.

### Altered Behavioral Responses in *ERK1* Mutant Mice

In the absence of evident anatomical abnormalities in *ERK1* mutant mice, we evaluated possible behavioral changes associated with the mutation. We began our behavioral characterization by monitoring basal locomotion in *ERK1* mutant mice and littermates. Mutant and wild-type mice were individually placed in the activity boxes for 10 min on three consecutive days (Martin et al., 2000a). Since the environment is novel, wild-type mice initially show very high levels of locomotor activity, which subsides with time both within and across test sessions. The results showed statistically significant increase in locomotion of *ERK1*<sup>-/-</sup> mice, both in horizontal ( $F_{1,95} = 12.101$ ,  $p < 0.001$ ) and vertical ( $F_{1,95} = 5.688$ ,  $p < 0.05$ ) activity (Figures 2A and 2B), in the first session. Furthermore, whereas wild-type mice showed clear habituation from day 1 to 3 ( $p < 0.001$ ) both in horizontal and in vertical activity, *ERK1* mutants retained a significantly higher activity on day 3, both in horizontal activity ( $F_{1,95} = 17.383$ ,  $p < 0.001$ , genotype effect) and in vertical activity ( $F_{1,95} = 26.441$ ,  $p < 0.001$ , genotype effect). Altogether, these results demonstrate a significant difference between wild-type and *ERK1* mutants in their basal locomotor features.

Next, we subjected the mice to two operant conditioning tests with the aim of assessing possible cognitive dysfunction. We used the active and passive avoidance procedures to test mutant mice since they are reliable and complementary tests for measuring the integrity of the long-term synaptic processes in a number of subcortical structures, including the hippocampus, the amygdala, and the striatum (Schutz and Izquierdo, 1979; Taghzouti et al., 1985; Izquierdo et al., 1997; Roozendaal et al., 1993; Salamone, 1994).

The two-way avoidance ("active avoidance") paradigm is a measure of associative emotional learning and reinforcer-driven control of motor activity (Clincke and Werbruck, 1993). The apparatus consists of two-chambered boxes of the same size and shape with a door in the wall between the two compartments. In the procedure, a warning light (conditioned stimulus, CS) pre-

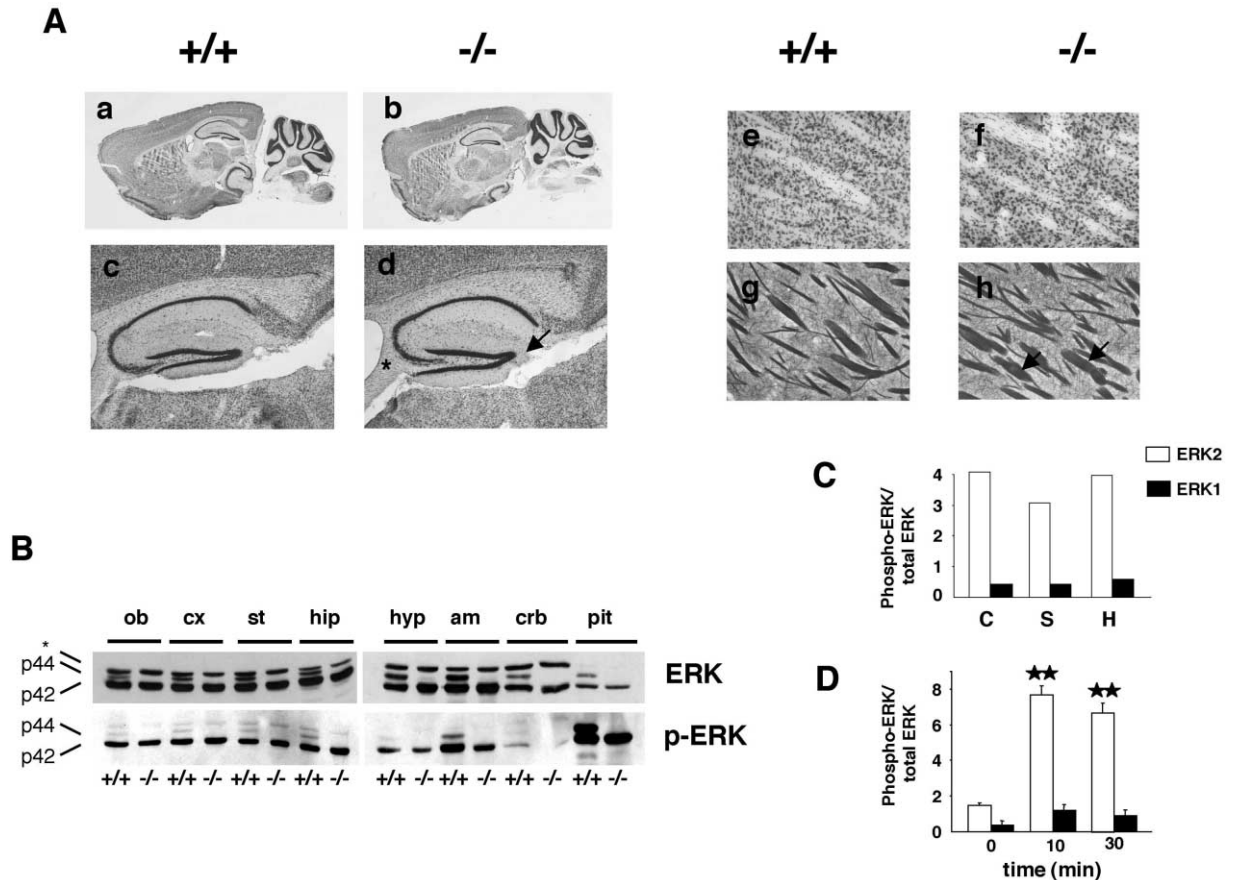


Figure 1. Anatomical and Biochemical Analysis of *ERK1*<sup>-/-</sup> Brains

(A) *ERK1* mutant brain sections (b, d, f, h) as compared to littermate controls (a, c, e, g). All pictures were taken from 40  $\mu$ m parasagittal cryosections. (A) Cyto- and myeloarchitecture visualized by cresyl violet staining (a–f) or gold chloride myelin stain (g and h). (a and b) Overview showing normal brain size and overall cyto- and myeloarchitecture in *ERK1*<sup>-/-</sup> brains. (c and d) Hippocampal formation (magnification 5 $\times$ ). There is no obvious size reduction in *ERK1*<sup>-/-</sup> mice and the cell, and synapse layers of the dentate gyrus and Ammon's horn are normally developed. Perforant path (arrows), fimbria (asterisk), and corpus callosum have normal appearance. (e–h) Details of dorsal striatum showing normal cell density as well as unaffected number and appearance of fiber bundles (arrows) in the internal capsule (magnification 20 $\times$ ). (B) Normal ERK2 expression in *ERK1* mutant brains. Immunoreactivity to both anti-ERK and anti-phospho-ERK antibodies of wild-type and *ERK1* mutant mice brain extracts (ob, olfactory bulbs; cx, neocortex; st, striatum; hip, hippocampus; hyp, hypothalamus; am, amygdala; crb, cerebellum; pit, pituitary gland, 10  $\mu$ g/lane). In addition to p44<sup>ERK1</sup> and p42<sup>ERK2</sup>, a nonspecific band of p46 (asterisk) is recognized by the anti-ERK antibodies. (C) Quantification of normalized p44 and p42 bands intensities from (B) (C, cortex; S, striatum; H, hippocampus). (D) Embryonic cultures (E16) were prepared from cortex of wild-type mice and then stimulated with 100  $\mu$ M glutamate for the indicated times. Protein extracts (10  $\mu$ g) were separated onto SDS-PAGE and probed with either anti-ERK or anti-phospho-ERK antibodies. Band intensities were quantified as mean from three independent experiments and plotted as normalized relative intensity of both p42 and p44 (\*\*  $p < 0.001$ , t test).

cedes a mild electric footshock (unconditioned stimulus, US) by 5 s and overlaps it for 25 s. Over extensive training (5 days, 100 trials per day), mice learned to associate the US and the CS, and avoid the US by running into the opposite compartment before the US onset. As shown in Figure 2C, both wild-type and mutant mice gradually but efficiently learned to avoid the electric shock. However, *ERK1* mutants were significantly faster in learning the task. Two-way ANOVA revealed a significant interaction of genotype and training ( $p < 0.01$ ). Post-hoc analysis indicated significant genotype differences on days 2, 3, and 4. At day 5, statistical differences between the groups were no longer seen, and both groups apparently reached a learning plateau. However, memory retrieval monitored after 5, 10, 20, and 30 days suggested a tendency for superior performance of the KO mice (not

shown). Importantly, to rule out the possibility that the result obtained was not due to associative learning, we measured both footshock sensitivity and basal locomotor activity (in absence of shock) in the shuttle box. No statistical differences were seen in either parameter (see inset in Figure 2C for basal activity). Therefore, these results indicate that *ERK1* mutant mice show an enhanced rate of learning in the active avoidance paradigm.

Multiple-trial learning paradigms, such as active avoidance, do not allow precise discrimination between behavioral changes occurring at the level of short-term (STM) or long-term memory (LTM) formation. This precision is possible, however, with passive avoidance in which short- and long-term memory formation can be probed at 30 min and 24 hr, respectively (Sahgal, 1993).

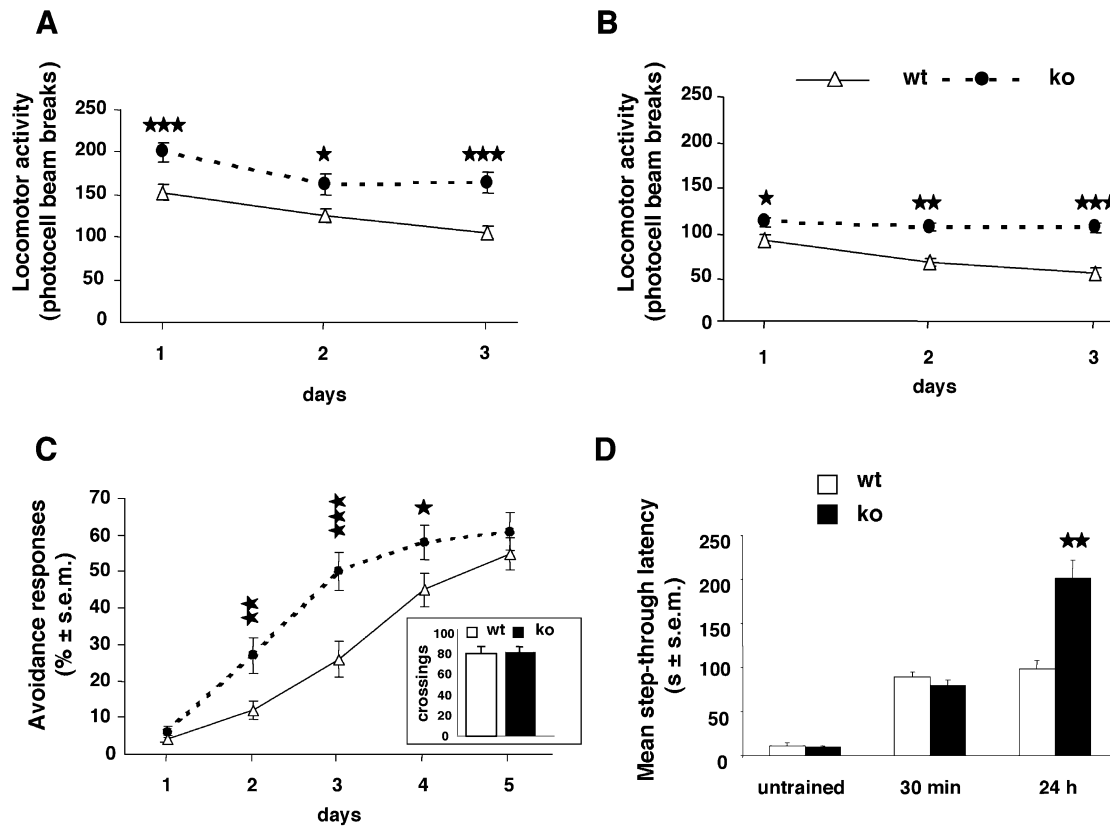


Figure 2. Hyperactivity and Improved Performances in Active and Passive Avoidance Task of *ERK1*<sup>-/-</sup> Mice

Animals (wild-type, wt, n = 53; *ERK1*<sup>-/-</sup>, ko, n = 44) were placed for 3 consecutive days in activity boxes for 10 min. (A) Horizontal and (B) vertical activity was measured and plotted as indicated. (C) Active avoidance behavioral responses are indicated as percentage of shock avoidance, over 5 days (16 mice each genotype). In the inset, number of crossings at the end of the experiment indicates no differences between groups in basal locomotory activity. (D) Passive avoidance learning is indicated as step-through latency, for untrained mice, trained mice probed at 30 min (wild-type, n = 8; *ERK1*<sup>-/-</sup>, n = 10), and 24 hr after training (wild-type, n = 15; *ERK1*<sup>-/-</sup>, n = 17). Values are mean ± SEM. \*\*\* p < 0.001, \*\* p < 0.01, \* p < 0.05, genotype effect.

The apparatus consists of two chambers: a larger dark chamber, separated by a door from a smaller one kept strongly illuminated. A floor grid placed in the dark chamber permits passage of footshocks. This test takes advantage of the natural tendency of mice to prefer dark spaces over lit spaces. The task begins by placing the mouse in the lit box, then opening the door between the chambers. When the mouse enters the dark compartment after a short latency, the door is closed and a single footshock is administered. A single training session is normally sufficient to learn the task, i.e., to learn not to enter the dark compartment. The time spent in the lit side (step-through latency) is the measure of passive avoidance.

Both *ERK1* mutant mice and controls successfully learned to avoid punishment in the dark compartment (main effect of treatment,  $F_{1,16} = 540.664$ ,  $p < 0.001$ ) (Figure 2D). There was no significant difference between *ERK1* mutants and their wild-type littermates in step-through latency at 30 min. In contrast, long-term retention tested 24 hr after training was significantly better in *ERK1*-deficient compared to control mice ( $F_{1,30} = 8.574$ ,  $p < 0.01$ ). These data confirm that *ERK1* mutant mice manifest improved LTM in avoidance tasks.

### Loss of ERK1 Affects Synaptic Plasticity in a Region-Specific Manner

The results obtained from the operant conditioning tests imply that in vivo loss of ERK1 results in a marked enhancement of memory consolidation, while learning and STM appear to be normal. Altogether, the behavioral data suggest that in *ERK1* mutant neuronal networks, changes in cell signaling might have significant effects on synaptic function. We therefore examined synaptic plasticity in hippocampus (CA1), basolateral amygdala, and ventral striatum (nucleus accumbens), all of which are known to be implicated in behavioral plasticity (LeDoux, 2000; Martin et al., 2000b; Nicola et al., 2000). Tetanic stimulation (two trains of 100 pulses at 100 Hz for 1 s) of the neocortical inputs to nucleus accumbens resulted in potentiation that was significantly larger in slices from *ERK1* knockout mice than in wild-type slices (Figures 3A and 3D). Although both groups showed similar initial levels of potentiation, responses in nucleus accumbens of *ERK1*<sup>-/-</sup> mice increased reliably between 15 and 30 min after tetanus, to a final value significantly greater than littermate controls ( $190\% \pm 21\%$  of baseline for *ERK1*<sup>-/-</sup>, n = 8;  $128\% \pm 7\%$  for *ERK1*<sup>+/+</sup>, n = 10,  $p < 0.01$ ). In contrast, the effects of ERK1 deletion

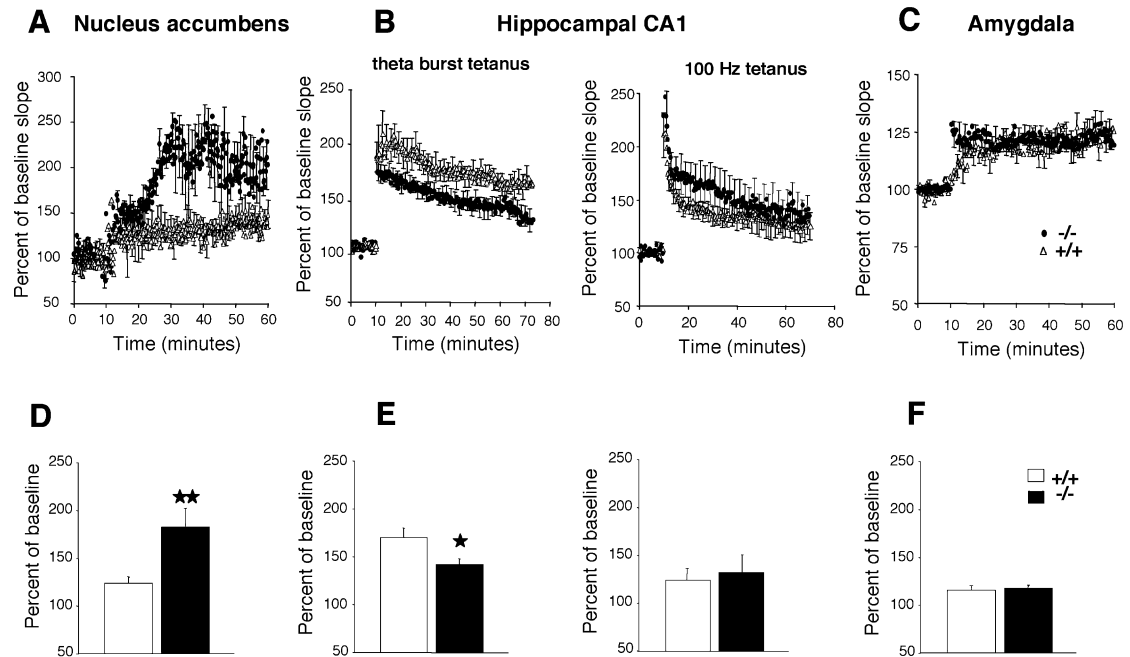


Figure 3. Synaptic Plasticity Is Altered in *ERK1*<sup>-/-</sup> Mice

(A) Tetanic stimulation of neocortical inputs to sagittal slices of nucleus accumbens produced significantly larger LTP in *ERK1* mutant mice (●) than in wild-type littermate controls (△). Two trains of 100 pulses at 100 Hz were delivered after a 15 min baseline period. (D) Comparison of the average percent of LTP shown in (A) across the 60 min after tetanus. (B–E) Theta-burst stimulation delivered to Schaffer collateral/commissural inputs to CA1 produced significantly less LTP in knockouts, compared to wild-type controls, while tetanic stimulation produced equal magnitude LTP in both *ERK1*<sup>-/-</sup> and *ERK1*<sup>+/+</sup> slices. (C–F) Theta-burst tetanus delivered to the lateral amygdala produced equivalent magnitude LTP in the basolateral amygdala of *ERK1*<sup>-/-</sup> and *ERK1*<sup>+/+</sup> slices. A repeated measures ANOVA comparing genotype by post-tetanus time (in 10 min bins) revealed a significant main effect of genotype in nucleus accumbens and in hippocampus (theta-burst stimulation only). Values are mean ± SEM. \*\* p < 0.01, \* p < 0.05, genotype effect.

on synaptic plasticity in the hippocampus depended on the type of stimulation used to induce LTP (Figures 3B and 3E). Three trains of theta-burst stimulation (each train consisting of ten bursts of four pulses at 10 Hz, 5 Hz burst frequency) induced LTP in both knockout and wild-type CA1, but the LTP in *ERK1*<sup>-/-</sup> slices was significantly smaller than in wild-type (171% ± 10% of baseline for *ERK1*<sup>+/+</sup> slices, n = 8; 143% ± 6% for *ERK1*<sup>-/-</sup>, n = 17, p < 0.05). Surprisingly, however, when hippocampal slices were tetanized with two 100 Hz trains of 100 pulses, the magnitude of LTP did not differ between *ERK1*<sup>-/-</sup> and controls (130% ± 13% for *ERK1*<sup>+/+</sup> slices, n = 5; 139% ± 20% for *ERK1*<sup>-/-</sup> slices, n = 3, p = 0.7). Finally, LTP in the basolateral amygdala, elicited with a theta-burst stimulation of the lateral amygdala, did not differ between the two groups (119% ± 5% for *ERK1*<sup>+/+</sup>, n = 3; 121% ± 3% for *ERK1*<sup>-/-</sup>, n = 5, p = 0.60) (Figures 3C and 3F). These results clearly demonstrate that the *ERK1* mutation did not translate in a predictable manner into changes in synaptic physiology: of three brain structures examined, only striatum manifested an increased synaptic plasticity consistent with the behavioral changes observed in the avoidance tasks. Importantly, lack of alterations in amygdalar LTP and very limited changes in hippocampal LTP correlated with lack of significant impairments in either emotional (contextual and cued fear conditioning tests) or spatial (Morris and radial maze tests) LTM formation, respectively (data not shown).

#### Enhancement of ERK2 Signaling in *ERK1* Mutant Neuronal Networks

The experimental evidence presented so far suggests that the loss of one of the two ERK isoforms in the brain results in enhancement of long-term synaptic plasticity in some, but not all, forebrain structures, and that this enhancement might provide the cellular basis for the observed facilitation of LTM. To investigate the molecular mechanisms that may underlie this phenomenon, we analyzed the kinetics of ERK activation in *ERK1* mutant and wild-type mice by culturing primary neurons from neocortex and striatum. Activation of ERK signaling was elicited either by stimulating glutamate receptors or by KCl-mediated membrane depolarization, with similar results. We reproducibly observed that *ERK1* mutant cultures responded with a markedly enhanced increase of ERK2 phosphorylation in comparison to wild-type cells (Figure 4A). Quantification of three independent glutamate stimulation experiments in cortical and striatal cultures is shown in Figure 4B. In wild-type cultures, we observed maximum ERK2 phosphorylation at 10–30 min post-stimulus (corresponding to approximately 10-fold increase over unstimulated levels). In all *ERK1* mutant cultures, the levels of ERK2 phosphorylation at the same time points were significantly higher, ranging from 20- to 40-fold increase over unstimulated levels, in both brain structures. This biochemical phenomenon, however, does not seem to be specific of the analyzed cell types. In fact, we observed a qualitatively similar profile

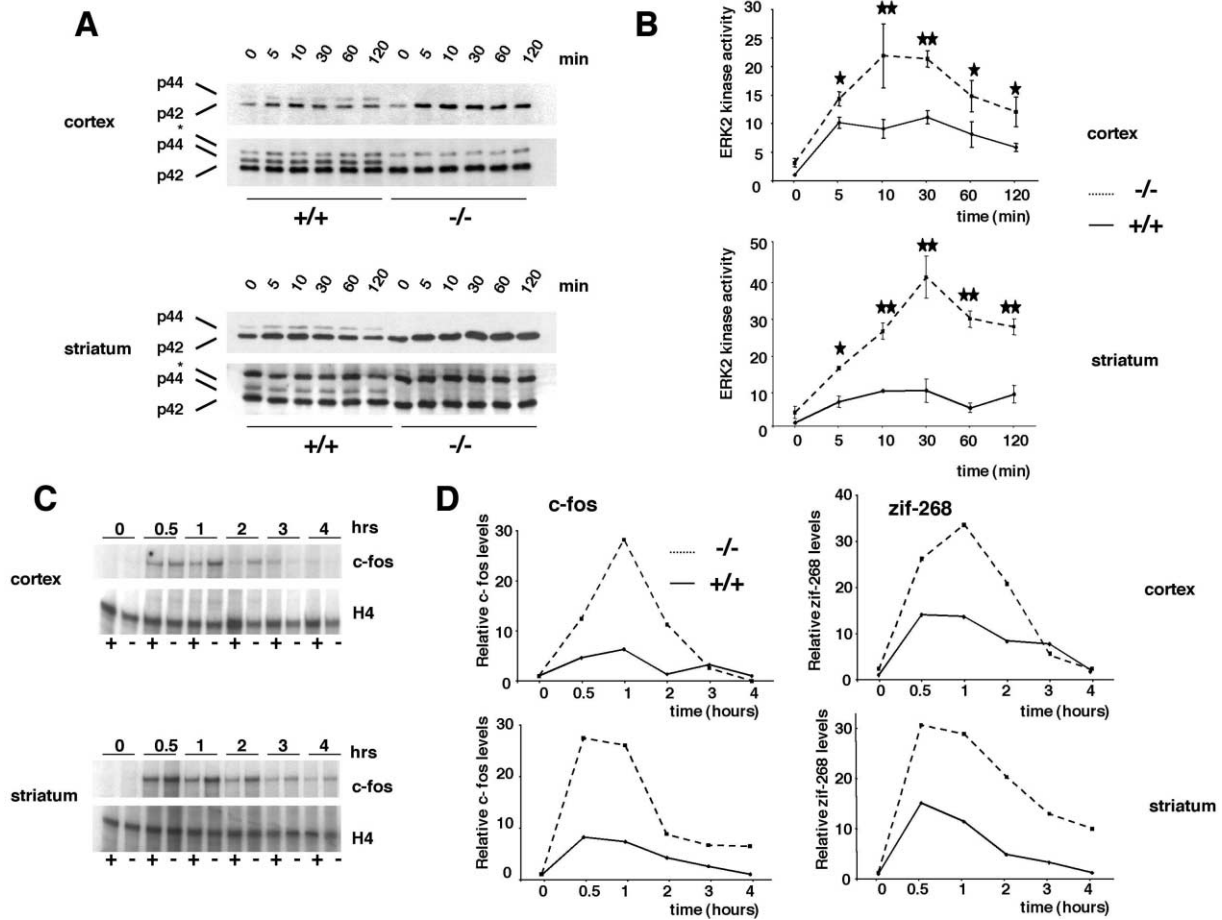


Figure 4. Enhanced ERK2 Signaling in *ERK1* Mutant Brains

(A) Embryonic cultures (E16) were prepared from cortex and striatum, both from wild-type and mutant *ERK1* mice, and then stimulated with 100  $\mu$ M glutamate for the indicated times (min). Protein extracts (10  $\mu$ g) were separated onto SDS-PAGE and probed with either anti-ERK or anti-phospho-ERK antibodies. Asterisk indicates a nonspecific band. (B) Band intensities for p42 ERK2 were quantified from experiments in (A) and normalized phospho-ERK2 levels were calculated as mean from three independent experiments (\*  $p < 0.01$ ; \*\*  $p < 0.001$ , t test). (C) Enhanced ERK2 signaling in *ERK1* mutants results in increased immediate early gene transcription. Cortical and striatal neurons were stimulated with 100  $\mu$ M glutamate for the indicated times and equal amounts of total RNA preparations were subjected to RNase protection assay for *c-fos* gene. H4 histone was used as internal standard for RNA quantitation. (D) Quantification of the relative expression levels for both *c-fos* and *zif-268* are indicated in the graphs, from both cortical and striatal preparations.

of ERK2 hyperactivation in hippocampal cells, embryonic fibroblasts, thymocytes, and astrocytes derived from *ERK1*<sup>-/-</sup> animals (Pagès et al., 1999).

Change of gene expression induced by ERK signaling is known to be dependent on kinase translocation into the nucleus. The activity of ERK kinases on immediate early gene transcription is mediated by two cooperative transcription systems. CRE- and SRE-dependent transcription requires the action of two classes of nuclear factors: CREB-like and TCF family proteins (Elk-1 and Sap-1), respectively (Orban et al., 1999). Immunocytochemical analysis of stimulated *ERK1* mutant neurons with phospho-ERK antibodies confirmed that in mutant cells, ERK2 is rapidly translocated into the nucleus (not shown). However, lack of specific anti-phospho-ERK2 antibodies precluded us to directly determine differences with control cells in either the kinetics or the extent of kinase translocation. Moreover, since commer-

cially available antibodies against both phospho-CREB and phospho-Elk worked poorly on our extracts, we decided to quantitatively assess transcriptional effects of altered ERK signaling by analyzing the activation state of two immediate early genes (IEGs), *c-fos* and *zif-268*, whose transcription rates are strictly dependent on the binding of *trans*-acting factors to the SRE and CRE consensus sequences of their promoters (Mazzucchelli and Brambilla, 2000). As shown in Figure 4C, glutamate-stimulated cortical and striatal cultures from mutant mice transcribed significantly more *c-fos* than wild-type cells. Quantification of the signal for both *c-fos* and *zif-268* is shown in Figure 4D. Interestingly, *c-fos* and *zif-268* activation in *ERK1*-deficient striatum seemed to be more persistent than in cortex: at 4 hr after stimulation, RNA levels for both genes in mutant cells roughly corresponded to 1 hr peak levels of wild-type cells whereas at the same time in cortical mutant neurons signals had

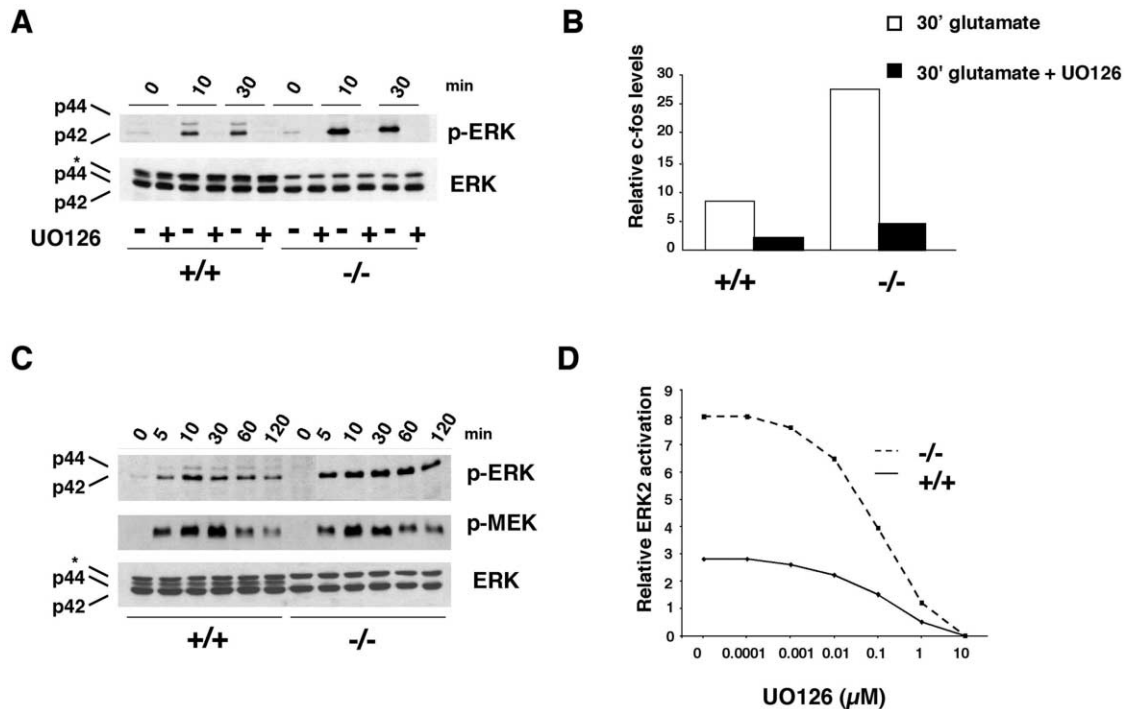


Figure 5. Physiological Responses to ERK2 Signaling Depend on MEK Activity

(A) Cortical cultures were stimulated with 100  $\mu$ M glutamate either in the presence or in the absence of the MEK inhibitor UO126 (10  $\mu$ M), for the indicated times. After SDS-PAGE, protein extracts (10  $\mu$ g/lane) were probed with either anti-ERK or anti-phospho-ERK antibodies. Asterisk indicates a nonspecific band. (B) Cortical neurons were stimulated with 100  $\mu$ M glutamate for 30 min either in the presence or in the absence of UO126 (10  $\mu$ M), and RNase protection assay for *c-fos* gene was performed. (C) Protein extracts prepared from cortical neurons stimulated with 100  $\mu$ M glutamate were run in SDS-PAGE (30  $\mu$ g/lane) and probed with anti-ERK, anti-phospho-MEK, or anti-phospho-ERK antibodies, as indicated. (D) Concentration/response curve for UO126-mediated inhibition of ERK2 phosphorylation. Cortical cultures from either wt and *ERK1* mutant mice were stimulated for 10 min with 100  $\mu$ M glutamate in the presence of increasing concentration of UO126. Normalized phospho-ERK2 levels were plotted as indicated.

returned to basal level. This difference in duration of IEGs transcription might be relevant to the observed physiological and behavioral phenotypes.

In conclusion, it appears that in the absence of ERK1, ERK2-dependent signaling is strongly enhanced in the brain, resulting in a particularly prolonged upregulation of IEGs expression in the striatum.

#### Upregulation of ERK2 Signaling Depends on the Interaction with the Upstream Kinase MEK

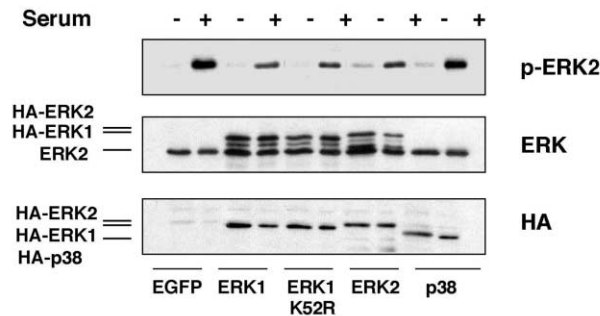
To understand better the potentiation of ERK2 activity in *ERK1*<sup>-/-</sup> mice, we first examined the involvement of MEK. This class of enzymes is believed to be the necessary intermediate linking receptor activation of Ras proteins to ERK stimulation. Therefore, we first confirmed that the enhanced ERK2 phosphorylation was entirely dependent on MEK activity by stimulating neurons in the presence of UO126, a specific inhibitor of MEK (Favata et al., 1998). At a concentration of 10  $\mu$ M, this molecule completely inhibited ERK2 phosphorylation in both wild-type and mutant cells, ruling out the possibility that the enhancement of ERK2 signaling might be caused by a MEK-independent pathway (Figure 5A). Importantly, such MEK dependence was also seen for the super induction

of IEGs since UO126 almost completely blocked expression of *c-fos* in *ERK1* mutant cells (Figure 5B).

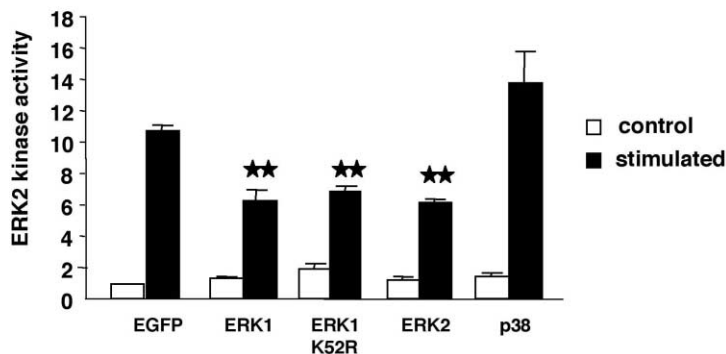
If MEK/ERK2 interaction is necessary for the observed phenotype, a plausible molecular explanation might involve a deregulation of MEK activity in *ERK1* mutant cells. In some situations, ERK-dependent signaling regulates its own activation by several negative feedback mechanisms (Pearson et al., 2001). A possible ERK1-specific negative feedback would be lost in mutant cells and could result in increased phosphorylation and activation of MEK. This does not appear to be the case. As shown in Figure 5C, glutamatergic stimulation of neurons caused equal kinetics of MEK phosphorylation in both wild-type and ERK1-deficient cells, arguing against the possibility of a deregulation of MEK activation in *ERK1* mutants.

As additional confirmation of the integrity of MEK functions, we determined the UO126 inhibition curve of ERK2 phosphorylation, in both control and *ERK1* mutant cells (Figure 5D). Primary neurons were stimulated for 10 min with glutamate, in the presence of increasing UO126 concentration, and ERK2 activation was assessed. UO126 acts as a noncompetitive inhibitor which binds to MEK, MEK-ATP, and MEK-ERK complexes with similar affinity and with Michaelis-Menten kinetics (Fa-

**A**



**B**



**Figure 6. Ectopic Expression of ERKs Attenuates ERK2 Activation in *ERK1*<sup>-/-</sup> Cells**

(A) Fibroblasts prepared from E13 *ERK1*<sup>-/-</sup> embryos were infected with Semliki Forest recombinant viruses at the concentration  $1 \times 10^6$  particles/ml either containing enhanced green fluorescent protein (EGFP), ERK1, ERK1<sup>K52R</sup>, ERK2, or p38 SAPK1 cDNAs, as indicated. After 18 hr of starvation, cells were either immediately extracted or stimulated for 10 min with 20% serum. Protein extract (10  $\mu$ g/lane) were separated in SDS-PAGE and analyzed with anti-phospho-ERK, anti-ERK, or anti-HA antibodies, as indicated. (B) Band intensities for p42 ERK2 were quantified and normalized phospho-ERK2 levels were calculated as mean from three independent experiments (\*\*  $p < 0.01$ , t test).

vata et al., 1998). The two inhibition curves showed no difference in the  $IC_{50}$ , thus demonstrating an overall functional integrity of MEK in ERK1-deficient cells. Importantly, an  $IC_{50}$  of 0.1  $\mu$ M is equal to a previously reported value (Favata et al., 1998). Furthermore, mutant cells displayed higher ERK2 phosphorylation levels at any inhibitor concentration up to 10  $\mu$ M. Since MEK and ERK2 protein levels are unchanged, this observation is consistent with the possibility that, in *ERK1* mutants, ERK2 has a greater access to MEK binding sites, giving rise to an increased accumulation of the activated molecules. MEK kinases are present in the cell at limiting concentrations (Pearson et al., 2001) and a simple explanation for the observed enhancement of ERK2 phosphorylation in *ERK1* mutant neurons might lie in competition between ERK1 and ERK2 for access to MEK. Thus, ERK1 in wild-type cells might compete with ERK2 in binding to MEK; as a consequence, in the absence of ERK1, ERK2 would have greater access to MEK, resulting in higher levels of MEK-mediated ERK2 activation.

We examined this competition model by using Semliki Forest virus (SFV) vectors to express hemagglutinin (HA) epitope-tagged ERK1 and ERK2 proteins in *ERK1* mutant cells (Lundstrom et al., 2001). For these genetic manipulations, we chose embryonic fibroblasts since they can be easily cultured and infected with high efficiency with recombinant SFV-based viral particles. In addition, the use of limited amount of viral preparation allows achieving mild expression levels of the ectopic proteins in these cells. One prediction would be that a

mild excess of either ERK isoform might alter the activation of endogenous ERK2 by displacing it from MEK. As shown in Figure 6A, both ectopic ERK1 and ERK2 significantly attenuated endogenous ERK2 activation as compared to control cells infected with either GFP or HA-p38 SAPK1 MAP kinase. Importantly, ERK1<sup>K52R</sup>, a mutant form lacking kinase activity, was also effective in inhibiting ERK2 activation, demonstrating that no ERK1 enzymatic activity is required for the attenuating effect on endogenous ERK2. Quantitation of three independent experiments is shown in Figure 6B.

#### Modulation of ERK2 Activity Is a Crucial Determinant of LTP Intensity in the Nucleus Accumbens

The experiments on cultured neurons from *ERK1*<sup>-/-</sup> mice described above indicate that the MEK inhibitor U0126 can reduce the stimulus-dependent phosphorylation of ERK2 to wild-type levels. If the mechanism responsible for enhanced expression of LTP in the nucleus accumbens is related to hyperactivation of ERK2, then administration of U0126 in concentrations sufficient to attenuate ERK2 activity should return LTP to wild-type levels. Figures 7A and 7B indicate that accumbens LTP is significantly reduced following administration of both 0.1  $\mu$ M and 1.0  $\mu$ M U0126. At 0.1  $\mu$ M, LTP in *ERK1*<sup>-/-</sup> accumbens was significantly lowered when compared to control medium (t test,  $p < 0.01$ ), and did not differ significantly from LTP in control *ERK1*<sup>+/+</sup> mice (t test,  $p = 0.8$ ), suggesting that when ERK2 activation is brought close to wild-type levels, LTP also returns to wild-type

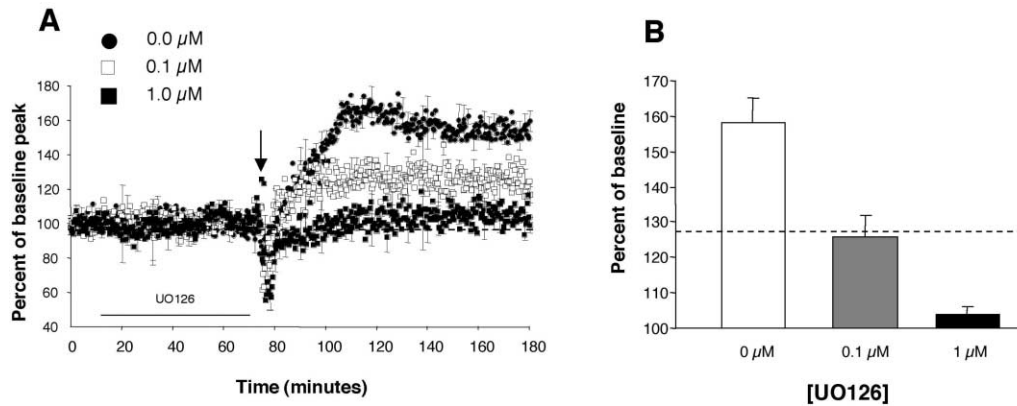


Figure 7. Submaximal Dose of MEK Inhibitor U0126 Rescues Plasticity Enhancement in Nucleus Accumbens of *ERK1* Mutants

(A) U0126 was bath applied at 60 min before tetanic stimulation was delivered to the cortical inputs to the nucleus accumbens and washed out immediately afterwards. U0126 significantly reduced LTP in the nucleus accumbens of *ERK1* knockout mice. (B) Comparison of the average percent of LTP shown in (A) across the 100 min after tetanus indicates that the effect of both concentrations differed significantly from untreated mutant slices ( $p < 0.00001$  for  $1.0 \mu\text{M}$ ;  $p < 0.01$  for  $0.1 \mu\text{M}$ ). The dashed line across the bars indicates the level of LTP in stimulated slices from  $+/+$  animals, which did not significantly differ from the  $-/-$  slices treated with  $0.1 \mu\text{M}$  U0126.

magnitude in nucleus accumbens. At the higher concentration ( $1.0 \mu\text{M}$ ), LTP is completely blocked in both *ERK1*<sup>-/-</sup> and control mice ( $104\% \pm 2\%$  and  $103\% \pm 10\%$  of baseline, respectively).

In conclusion, these results demonstrate a direct quantitative relation between ERK2 activity in nucleus accumbens and LTP induction. In this system, the presence of ERK1 appears to attenuate the effect of ERK2 in promoting long-term plasticity but it is otherwise dispensable.

#### ***ERK1* Mutant Mice Manifest Enhanced Behavioral Responses to the Rewarding Properties of Morphine**

Many of the functions ascribed to the striatum are linked to its nigrostriatal and mesolimbic dopaminergic innervations, which originate in the substantia nigra and in the ventral tegmental area, respectively (Nicola et al., 2000). Aberrant dopaminergic signaling is a critical determinant in the etiology of some major striatal neuropsychiatric disorders. In particular, changes in mesolimbic dopaminergic activity are considered the neural substrate for opiate addiction and increasing dopamine release in the striatum is a property shared by most drugs of abuse, including opiates and psychostimulants (Berke and Hyman, 2000; Hyman and Malenka, 2001; Nestler, 2001). The ERK pathway has recently been shown to have an important role in response to drugs of abuse (Valjent et al., 2000).

To reveal possible alterations in dopaminergic signaling in our mutants, we treated ERK1-deficient mice with the D1-specific agonist SKF 38393 and rapidly dissected striatum. Analysis by Western blot of protein extracts prepared after stimulation at several time points revealed a more sustained ERK2 activation in *ERK1* mutants than in control mice (Figure 8A). These *in vivo* results were also confirmed by treatment of primary striatal cells with the same D1 agonist *in vitro* (not shown).

Importantly, we also examined IEG induction and found that *c-fos* levels were significantly greater in the

striatum of *ERK1*<sup>-/-</sup> mice upon SKF 38393 treatment, thus demonstrating the upregulation of gene expression mediated by dopaminergic signaling in this mouse model (Figure 8B).

To further address the functional significance of increased dopaminergic signaling in the striatum of *ERK1*<sup>-/-</sup> mice, we tested mice in the conditioned place preference (CPP) paradigm, a reliable behavioral test of the addictive and rewarding effects of drugs of abuse (Maldonado et al., 1997). The CPP test reflects a preference for a context which has been repeatedly associated with a drug stimulus. The experimental protocol involves an apparatus with two easily distinguishable chambers connected to each other by a smaller neutral area so that the mice can move freely within the apparatus. During the conditioning phase, the mouse is injected with the drug or saline and confined to one of the chambers for 20 min. For each mouse, drug treatment is on days 1, 3, and 5, in one chamber. Saline administration is on days 2, 4, and 6 when the mouse is confined to the other chamber. Control mice receive saline every day. Twenty-four hours after the final conditioning session, the mice undergo a testing session. The learned association between the context and the appetitive stimulus results in mice spending more time in the drug-associated context.

When we tested the reinforcing effects of morphine on *ERK1* and control mice, significant rewarding responses to the drug were observed in both groups of animals ( $F_{1,49} = 14.010$ ,  $p < 0.001$ ) (Figure 8C). However, *ERK1* knockout mice showed a strikingly stronger drug reinforcement ( $F_{1,22} = 9.446$ ,  $p < 0.01$ ) in spending almost 3-fold more time in the morphine-paired chamber than the control animals.

This last result demonstrates a striking sensitivity of *ERK1* mutants to the rewarding effects of morphine and strongly supports the hypothesis that increased synaptic plasticity in the nucleus accumbens of these mutants has important functional consequences for multiple behaviors that depend on striatal processing.

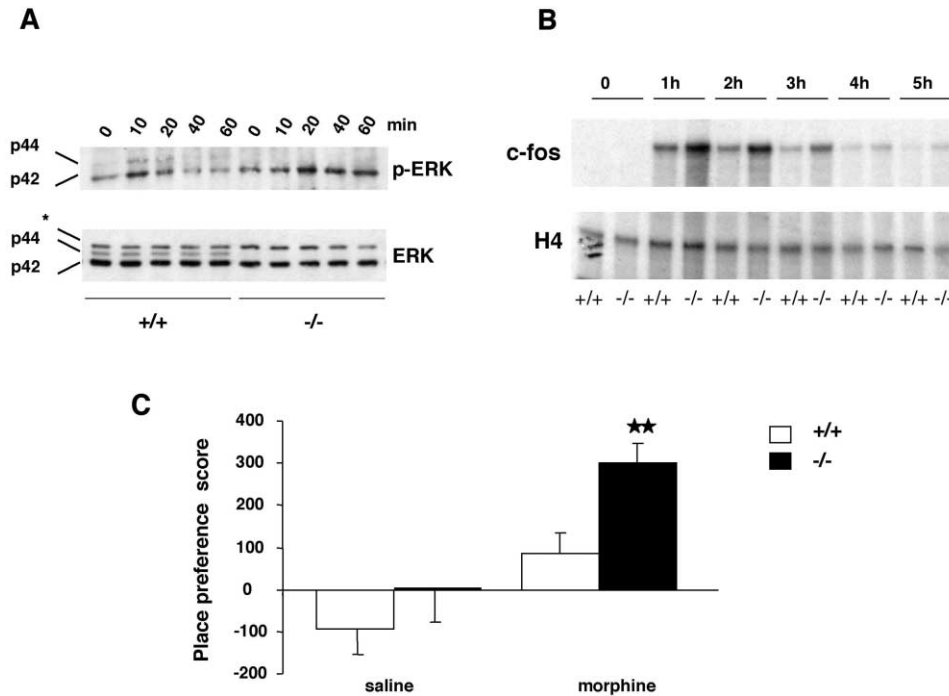


Figure 8. D1 Dopaminergic Signaling Is Upregulated in ERK1-Deficient Mice

The D1-specific agonist SKF 38393 was intraperitoneally injected in mutant and wild-type animals (10 mg/kg). The striata were rapidly dissected at the indicated times after agonist administration. For each time point, the striata of three animals were pooled and proteins or RNA extracted. (A) ERK activation was analyzed by Western blot with an antiserum specifically recognizing the phosphorylated form of ERKs. The treatment rapidly and robustly activated ERK2. In control animals, ERK2 phosphorylation peaked at 10 min and then rapidly decreased to basal level within 1 hr after injection. In contrast, in ERK1-deficient mice, phospho-ERK2 immunostaining reached a higher peak and remained very intense even at later points (up to 60 min). As a control for equal loading, the same protein extracts were probed with an anti-ERK antibody. The asterisk (\*) indicates a nonspecific band. (B) Superinduction of *c-fos* in ERK1-deficient mice. Total RNA preparations (5  $\mu$ g) were subjected to RNase protection assay for *c-fos* and histone H4. The treatment rapidly induced *c-fos*, but its transcription was both more sustained and prolonged in *ERK1*<sup>-/-</sup> mice than in control littermates. (C) Enhanced place preference conditioning for morphine in *ERK1* mutants. Mutant animals were either treated with saline (n = 16) or with 5 mg/kg of morphine (n = 11), as wild-type groups (saline treated n = 13; morphine treated n = 13). Data are expressed in scores calculated as the difference between postconditioning and preconditioning time spent in the compartment associated with the drug. Values are mean  $\pm$  SEM. \*\* p < 0.01, genotype effect.

## Discussion

A large body of evidence indicates that the Ras/ERK cascade is implicated in cognitive processes such as long-term memory formation. To date, the experimental evidence for such involvement has essentially been based on pharmacological manipulations with drugs inhibiting MEK kinases. These proteins are crucial intermediates linking receptor-mediated activation of Ras GTPases and their cytoplasmic effectors, the ERK1 and ERK2 kinases. The results with MEK blockers have formed the basis for a working model in which both ERK1 and ERK2 kinases significantly contribute to LTP and LTM formation. One of the predictions of this model is that loss of either ERK1 or ERK2 might result in a significant inhibition of neural plasticity. The availability of viable and fertile *ERK1* mutant mice has allowed us to test this prediction. Our findings reveal unexpected complexity of the interplay between ERK1 and ERK2 kinases in brain function. Here we propose a model which suggests a general upmodulatory role of ERK1 on ERK2 signaling, and we provide evidence for differential functions of the two isoforms. The absence of ERK1 does not produce predictable changes in synaptic plas-

ticity, as suggested by simple blockade of MEK activity, but rather reveals the existence of area-specific mechanisms that translate cell signaling variations into physiological responses.

### Upregulation of ERK2 Signaling in *ERK1* Mutant Cells Is Due to Altered MEK/ERK Interaction

Very little is known about differential activities of p44<sup>ERK1</sup> and p42<sup>ERK2</sup>. However, early observations reporting a differential phosphorylation of the two MAPKs in vivo and in vitro led to the speculation that the two enzymes might be subjected to different post-translation regulation, especially in the brain (Mazzucchelli and Brambilla, 2000). In particular, our results confirm that ERK1 is poorly phosphorylated in comparison to ERK2 and therefore suggest that p44 may manifest little direct signaling potential in vivo. In addition, our data reported here, together with our earlier evidence on embryonic fibroblasts derived from *ERK1* mutant mice, clearly suggest an upmodulatory role of ERK1 on ERK2 signaling. The recent observation that *ERK2* mutant mice are embryonic lethal in comparison to the viability of *ERK1*-deficient animals is in support of the idea that p44 may play an accessory function to p42 (Adams and Sweatt,

2002). Although rather surprising, our findings are not inconsistent with earlier reports, considering that either inhibition or activation of ERK signaling without discriminating between p42 and p44 would mask any regulatory action of one isoform on the other. In trying to understand the molecular basis underlying possible functional differences between ERK1 and ERK2, several aspects should be taken into account. For instance, it is possible that ERK1 and ERK2 interact with different affinities with the upstream kinase MEK1/2, and this fact may affect their activation state. Particularly interesting is the recent identification of interacting domains on both MEKs and ERKs which mediate their stable associations and are necessary for efficient ERK activation. Moreover, to complicate the matter, the existence of several scaffold proteins for all possible MEK/ERK module combinations may also be relevant for a fine tuning of their signaling properties (Pearson et al., 2001).

While the exact mechanisms of such modulation will require direct *in vitro* binding measurements with purified components, here we propose a molecular model in which at least part of the regulatory effect of ERK1 is exerted at the level of MEK-ERK interaction. Our prediction would be that any change of the stoichiometry of endogenous MEK-ERK2 binding would result in a significant alteration of downstream signaling. The stronger experimental evidence for such a model comes from ectopic expression of recombinant proteins in *ERK1* mutant cells in which either an excess of ERK1 or ERK2 significantly attenuated the activation of endogenous ERK2. Any indirect effect due to activation or inhibition of other components of the pathway by the exogenous ERK kinases could be ruled out by the fact that the same result was obtained with a kinase-deficient ERK1 construct, further supporting the competition model. In addition, although we cannot completely exclude the contribution to the biochemical phenotype of a differential regulation of ERK-specific phosphatases in mutant cells, this possibility is unlikely since we observed neither reproducible differences in the half-life of ERK2 dephosphorylation kinetics between wild-type and ERK1-deficient neurons nor significant changes in the protein levels of MKP-1, 2, and 3, three MAPK phosphatases, within the same time period.

#### Region-Specific Modulation of Synaptic Plasticity in *ERK1* Mutants

We expected that targeted deletion of ERK1 would modify synaptic plasticity since alterations in cell signaling and gene expression frequently affect long-term changes in synaptic strength. Moreover, a significant body of evidence implicates ERK-dependent processes in the induction of LTP. On the other hand, the complexity of the biochemical phenotype suggested several *a priori* possibilities which could be tested in different brain structures. (1) ERK1 does not affect LTP and consequently no changes in LTP of the mutant mice would be evident. (2) ERK1 is essential “*per se*” in promoting synaptic changes, independently of ERK2, and a general reduction of LTP could occur. (3) ERK1 functions to modulate ERK2 activity and the strong enhancement of the latter would cause an increase in LTP in the mutants. (4) The absence of ERK1 produces a compensatory “ex-

cess” of ERK2 activity, which would deregulate synaptic functions and therefore inhibit LTP in the mutant mice.

Our results obtained by analyzing three key structures essential for cognitive functions indicate that none of these four possibilities seems to have general validity, although some may apply in a structure-specific manner. Hypotheses 1 and 2 (ERK1 is either totally dispensable or absolutely essential for synaptic functions and associated behavioral responses) are clearly not consistent with the data that revealed LTP changes in two of the three structures analyzed. LTP in nucleus accumbens is strongly enhanced and we observed corresponding changes in behavior. Similarly, LTP in hippocampus was altered, but we failed to detect changes in LTP of synapses in the amygdala. We cannot formally exclude the possibility that our stimulation protocol for amygdala simply failed to detect changes. However, we believe that our assessments of LTP in the amygdala are reliable for the following two reasons. First, the same theta burst stimulation has been used in the past for other genetically modified mice, showing alteration of LTP in mice with a corresponding alteration of amygdala-dependent behaviors (Brambilla et al., 1997; Krezel et al., 2001). Second, we were unable to detect alterations in either contextual or cued fear conditioning, two forms of emotional learning crucially dependent on the integrity of the amygdala. The situation with respect to the hippocampus is somewhat different. Clearly, the effect of the mutation is dependent on the stimulation protocol. While tetanus at 100 Hz did not show abnormalities, we observed a small but statistically significant reduction of LTP with the theta burst protocol. These results are consistent with a role of ERK kinases in the 5 Hz dependent hippocampal LTP as shown by Winder et al. who observed an effect of MEK inhibitors on this type of LTP while the 100 Hz form was unaffected (Winder et al., 1999). Consistently, Sweatt and collaborators failed to detect LTP alterations at 100 Hz in the hippocampus of an independently generated line of *ERK1* mutants, although results were not reported for any other tetanus protocol (Selcher et al., 2001). In any case, the detected changes in hippocampal LTP are not as dramatic from the behavioral point of view as those observed for the nucleus accumbens, since little effect was seen by applying both water and radial maze procedures to the mutant mice.

Taken as a whole, the electrophysiological results demonstrate an interesting and counterintuitive pattern. The simplest conclusion is that ERK1 is an important modulator of synaptic plasticity, as demonstrated in two of three structures we examined. The discovery that this modulation appears to limit plasticity in the nucleus accumbens but enhance in CA1 was unexpected, but consistent with the idea that the same signaling molecules or pathways may operate differently in different types of neurons or different structures. In this respect, the experiment with a suboptimal concentration of UO126, sufficient to reduce ERK2 activity in *ERK1* mutant slices to wild-type levels and to rescue the LTP phenotype in the nucleus accumbens, is particularly important for several reasons. First, it demonstrates a causal link between enhancement of ERK2 activity and changes in LTP. Second, it confirms that ERK1 is dispensable for normal striatal function, provided that

ERK2 activity is kept to normal levels by other means. Third, it provides a strong evidence for different response thresholds to ERK-dependent signaling in the analyzed neuronal networks.

Future efforts to link the mechanisms of synaptic plasticity to learning and memory will need to consider the possibility that physiological effects observed in a target structure may not apply in the same way to other structures that could also contribute to the tested behaviors.

### Enhanced Behavioral Responses in *ERK1* Mutants Are Linked to Changes in Striatal LTP and Cell Signaling

The enhancement of LTP we observed in the nucleus accumbens of *ERK1*<sup>-/-</sup> mice suggests a rather straightforward interpretation of the behavioral data. The observed phenotypes in *ERK1*-deficient mice are consistent with both the known effects of dopamine signaling in the striatum on locomotion and procedural forms of memory. The consequences of DA receptor activation appear to be potentiated in our mutant mice. This is most likely due to an enhanced and prolonged activation of ERK2, which results in a sustained expression of downstream transcription factors in the striatum. Increased ERK2 signaling in response to DA receptor activation in *ERK1*<sup>-/-</sup> mice could also result in increased locomotion, particularly during exploration of the activity boxes, which might be expected to engage dopaminergic systems. In addition, when the animals are challenged in cognitive tasks such as the two avoidance paradigms, the same underlying molecular alterations might be responsible for the observed memory enhancement. It is, however, important to note that Sweatt and collaborators, while also observing increased locomotion in *ERK1* mutant mice, failed to detect altered memory formation in the passive avoidance paradigm. At the moment, we have no clear explanation for this discrepancy, although it is possible that procedural differences in the performance of the test and/or differences in genetic background contributed to these conflicting results.

To date, little work has been performed on the molecular mechanisms of synaptic plasticity in the nucleus accumbens, and virtually nothing has been published in transgenic mice which correlates changes in LTP with increased behavioral responses depending on this structure. Our data, showing enhanced LTP in the nucleus accumbens and increased LTM in the *ERK1* mutants, provide an example of a genetic alteration of intracellular signaling intermediates that supports this correlation. Moreover, the recent idea that neural adaptations to drugs of abuse may involve the same molecular elements as those essential to behavioral plasticity is strongly confirmed by our results since *ERK1* mutants respond aberrantly to the rewarding properties of morphine. In this respect, the *ERK1* mutants' hypersensitivity to drug treatment seems to correlate with a prolonged *c-fos* induction in striatal neurons in comparison, for instance, to cortical cells prepared from the same animals. This fact leaves open the possibility that enhanced ERK2 activation in the striatum may result in a sustained induction of crucial genes implicated in drug abuse. Interestingly, the behavioral phenotype seen in *ERK1*

mutants strikingly resembles that found in a transgenic line overexpressing  $\Delta$ fosB, a *c-fos*-dependent protein induced in the striatum upon repeated drug injections and believed to be a crucial molecular element for long-term adaptations to pharmacological abuse (Kelz et al., 1999). Future work with genetically modified mice will certainly help to elucidate the still largely obscure signaling mechanisms underlying the synaptic plasticity and pathophysiology of the nucleus accumbens.

### Experimental Procedures

#### Mice

All *ERK1* mutant and wild-type mice used for behavioral analysis were generated as F2 littermates of a strain backcrossed in C57BL/6 background for one generation. Active and passive avoidance results were also replicated with mutant mice kept five generations in C57BL/6 background. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (National Institute of Health and European Community Guidelines on the Care and Use of Laboratory Animals) and approved by the local ethics committee.

#### Histology

Myeloarchitecture was visualized using a gold chloride stain as previously described (Schmued and Heimer, 1990). After mounting on gelatin-coated glass slides, cryosections were incubated for 30 min to 4 hr at 37°C in a phosphate-buffered solution of 0.2% hydrogen-tetrachloroaurate-trihydrate, 0.9% NaCl, and 2.5% sodium-thiosulfate. Development of the stain was monitored microscopically, stopped in distilled water, and fixed in 2.5% sodium-thiosulfate. Before removal of the brains, the mice had been deeply anesthetized with a lethal of Nembutal and perfused transcardially with 4% phosphate-buffered PFA.

#### Biochemistry, Cell Culture, and Molecular Biology

Wild-type and *ERK1* mutant mice (five animals each genotype) were sacrificed and protein extracts were prepared and pooled from different brain areas. Embryonic cultures (E16) were prepared from cortex and striatum, both from wild-type and mutant *ERK1* mice, and then stimulated as previously described (Schwarzschild et al., 1999). After incubation with 100  $\mu$ M glutamate, protein extracts (10  $\mu$ g) were prepared, separated onto SDS-PAGE, and subjected to Western blotting. Antibodies used in this work were purchased from New England Biolabs and Santa Cruz Biotechnologies and used following the manufacturer's protocol. For quantitative comparison of the phosphorylation state of ERK1 and ERK2, we determined the relative recognition efficiency of the anti-ERK antibodies by means of HA-tagged recombinant proteins expressed in embryo fibroblasts. In some experiments, cultures were stimulated with 100  $\mu$ M glutamate either in the presence or in the absence of the MEK inhibitor UO126 (10  $\mu$ M) (Promega). For RNase protection experiments, cortical and striatal neurons were stimulated with 100  $\mu$ M glutamate and total RNA preparations were subjected to RNase protection assay for *c-fos* and *zif-268* genes, as previously described (Foulkes et al., 1991). H4 histone was used as internal standard for RNA quantitation. For the preparation of recombinant SFV particles, the less cytopathic vector pSFV(PD) with the Ser<sup>259</sup>→Pro and Arg<sup>350</sup>→Asp point mutations in nonstructural protein 2 was used to insert the cDNAs for EGFP (Clontech) and mouse *ERK1*<sup>K52R</sup> mutant, ERK2, p38 SAPK-1 (Lundstrom et al., 2001). For in vivo pharmacological treatments, three animals of each genotype were injected intraperitoneally with SKF 38393 (10 mg/kg, Sigma). The striata were rapidly dissected at the indicated times after agonist administration.

#### Electrophysiology

All slices were prepared from adult mice, using standard techniques, as previously described (Brambilla et al., 1997). Briefly, following cervical dislocation, the brains were removed to ice cold artificial cerebrospinal fluid (ACSF) containing additionally 1 mM kynurenic acid to block glutamatergic excitatory transmission. Brains were

then mounted on a Vibratome (TPI) and coronal sections were made for experiments on hippocampus and amygdala, while sagittal slices were prepared for nucleus accumbens experiments. Following at least 1 hr incubation at room temperature, slices were transferred one by one to a submersion bath containing kynurenic acid-free ACSF for recording of extracellular field potentials. For CA1, stimulation was delivered to Schaffer collaterals and recordings made in stratum radiatum; in basolateral amygdala, the stimulation was of lateral amygdala, and the white matter underlying prefrontal cortex was stimulated to evoke responses in the rostral nucleus accumbens. Pre-tetanus response were monitored once per 20 s for at least 10 min; post-tetanus measurements were carried out for at least 60 min.

#### Locomotor Assay

Locomotor activity was measured using activity boxes consisting in individual plastic rectangular area (9 × 20 × 11 cm, Imetronic, France) (Martin et al., 2000a). The boxes contain a line of photocells 2 cm above the floor to measure horizontal movements, and another line located 6 cm above the floor to measure vertical activity. Mice were individually placed in the activity boxes and the ambulatory locomotor activity and total activity (ambulatory movements plus small movements) were recorded during 10 min in a low luminosity environment. In order to habituate the animals to the test environment and to obtain a stable baseline, basal locomotor activity was measured on days 1, 2, and 3.

#### Avoidance Tasks

Active avoidance behavioral training was performed as previously described (Brambilla et al., 1997). The apparatus consisted of two identical compartments. A light was switched on alternately in the two compartments and used as a conditioned stimulus (CS). The CS preceded the onset of the unconditioned stimulus (US) by 5 s, and overlapped it for 25 s. At the end of the 30 s period, both CS and US were automatically terminated, and the cycle begun in the other compartment. The US was an electric shock (0.2 mA) continuously applied to the grid floor. An avoidance response was recorded when the animal avoided the US by running into the dark compartment within 5 s after the onset of the CS. Animals were subjected to 100 cycles/day for 5 days. In passive avoidance mice, were trained on a step-through inhibitory avoidance task, as previously described (Brambilla et al., 1997). The apparatus was divided into two compartments: the smaller was illuminated while the larger one was equipped with a removable cover to allow it to be in darkness. The floor of the larger compartment consisted of two oblique stainless steel plates folded at the bottom through which scrambled constant current could be delivered. On the training day, each mouse was placed in the lit compartment. After few seconds of latency (15 s maximum), when the animal had stepped with all four paws into the dark side, the door was closed, and two footshocks (2 × 0.4 mA, 50 Hz, 2 s) were delivered with an interval of 5 s. The mouse was then removed from the apparatus and returned to its home cage. Retention was tested 0.5 or 24 hr later following a similar procedure, except that no shock was administered. A maximum step-through latency of 300 s was allowed in the test session.

#### Conditioned Place Preference

The rewarding effects of morphine were evaluated by using the conditioned place preference paradigm as previously described (Maldonado et al., 1997). The place preference apparatus consisted of two different Plexiglas compartments separated by a central neutral area. It was located in a sound proof testing room with low luminosity.

The protocol consists of three different phases.

#### Preconditioning Phase

Mice were placed in the middle of the neutral area and their location recorded for the following 18 min.

#### Conditioning Phase

This phase consisted of 6 consecutive days of alternative drug or saline injection. Doors matching the walls of the compartment allowed the confinement of the mice for 20 min immediately after injection. Each mouse received three pairings with morphine in one

chamber and three pairings with saline in the opposite chamber. Saline-treated mice received saline during the 6 days of this phase.

#### Postconditioning Phase

The test was conducted exactly as in the preconditioning phase (free access of each compartment for 18 min, 24 hr after the final conditioning session). Place preference was quantified in terms of time spent in drug-paired side. A score was calculated for each mouse as the difference between postconditioning and preconditioning time spent in drug-paired compartment.

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#### References

- Abel, T., and Lattal, K.M. (2001). Molecular mechanisms of memory acquisition, consolidation and retrieval. *Curr. Opin. Neurobiol.* 11, 180–187.
- Adams, J.P., and Sweatt, J.D. (2002). Molecular psychology: roles for the ERK MAP kinase cascade in memory. *Annu. Rev. Pharmacol. Toxicol.* 42, 135–163.
- Berke, J.D., and Hyman, S.E. (2000). Addiction, dopamine, and the molecular mechanisms of memory. *Neuron* 25, 515–532.
- Brambilla, R., Gnesutta, N., Minichiello, L., White, G., Roylance, A.J., Herron, C.E., Ramsey, M., Wolfer, D.P., Cestari, V., Rossi-Arnaud, C., et al. (1997). A role for the Ras signalling pathway in synaptic transmission and long-term memory. *Nature* 390, 281–286.
- Clincke, G.H.C., and Werbrouck, L. (1993). Two-way active avoidance. In *Behavioural Neuroscience Vol I, A Practical Approach*, Arjun Sahgal, ed. (Oxford: Oxford University Press).
- English, J.D., and Sweatt, J.D. (1996). Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. *J. Biol. Chem.* 271, 24329–24332.
- English, J.D., and Sweatt, J.D. (1997). A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *J. Biol. Chem.* 272, 19103–19106.
- Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273, 18623–18632.
- Foulkes, N.S., Borrelli, E., and Sassone-Corsi, P. (1991). CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* 64, 739–749.
- Graybiel, A.M. (1995). Building action repertoires: memory and learning functions of the basal ganglia. *Curr. Opin. Neurobiol.* 5, 733–741.
- Hyman, S.E., and Malenka, R.C. (2001). Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat. Rev. Neurosci.* 2, 695–703.
- Izquierdo, I., Quillfeldt, J.A., Zanatta, M.S., Quevedo, J., Schaeffer, E., Schmitz, P.K., and Medina, J.H. (1997). Sequential role of hippocampus and amygdala, entorhinal cortex and parietal cortex in formation and retrieval of memory for inhibitory avoidance in rats. *Eur. J. Neurosci.* 9, 786–793.
- Kelz, M.B., Chen, J., Carlezon, W.A., Jr., Whisler, K., Gilden, L., Beckmann, A.M., Steffen, C., Zhang, Y.J., Marotti, L., Self, D.W., et

- al. (1999). Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* **401**, 272–276.
- Krezel, W., Dupont, S., Krust, A., Chambon, P., and Chapman, P.F. (2001). Increased anxiety and synaptic plasticity in estrogen receptor beta-deficient mice. *Proc. Natl. Acad. Sci. USA* **98**, 12278–12282.
- LeDoux, J.E. (2000). Emotion circuits in the brain. *Annu. Rev. Neurosci.* **23**, 155–184.
- Lundstrom, K., Rotmann, D., Hermann, D., Schneider, E.M., and Ehrenguber, M.U. (2001). Novel mutant Semliki Forest virus vectors: gene expression and localization studies in neuronal cells. *Histochem. Cell Biol.* **115**, 83–91.
- Maldonado, R., Saiardi, A., Valverde, O., Samad, T.A., Roques, B.P., and Borrelli, E. (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* **388**, 586–589.
- Martin, M., Ledent, C., Parmentier, M., Maldonado, R., and Valverde, O. (2000a). Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. *Eur. J. Neurosci.* **12**, 4038–4046.
- Martin, S.J., Grimwood, P.D., and Morris, R.G. (2000b). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu. Rev. Neurosci.* **23**, 649–711.
- Mayford, M., and Kandel, E.R. (1999). Genetic approaches to memory storage. *Trends Genet.* **15**, 463–470.
- Mazzucchelli, C., and Brambilla, R. (2000). Ras-related and MAPK signalling in neuronal plasticity and memory formation. *Cell. Mol. Life Sci.* **57**, 604–611.
- Nestler, E.J. (2001). Molecular basis of long-term plasticity underlying addiction. *Nat. Rev. Neurosci.* **2**, 119–128.
- Nicola, S.M., Surmeier, J., and Malenka, R.C. (2000). Dopaminergic modulation of neuronal excitability in the striatum and nucleus accumbens. *Annu. Rev. Neurosci.* **23**, 185–215.
- Olanow, C.W., et al. (2000). Basal Ganglia, Parkinson's Disease and Levodopa Therapy. A supplement to *Trends Neurosci.* **23**.
- Orban, P.C., Chapman, P.F., and Brambilla, R. (1999). Is the Ras-MAPK signalling pathway necessary for long-term memory formation? *Trends Neurosci.* **22**, 38–44.
- Pagès, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P., and Pouyssegur, J. (1999). Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science* **286**, 1374–1377.
- Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22**, 153–183.
- Roozendaal, B., Koolhaas, J.M., and Bohus, B. (1993). The central amygdala is involved in conditioning but not in retention of active and passive shock avoidance in male rats. *Behav. Neural Biol.* **59**, 143–149.
- Sahgal, A. (1993). Passive avoidance procedures. In *Behavioural Neuroscience Vol I, A Practical Approach*, Arjun Sahgal, ed. (Oxford: Oxford University Press).
- Salamone, J.D. (1994). The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. *Behav. Brain Res.* **61**, 117–133.
- Schmued, L.C., and Heimer, L. (1990). Iontophoretic injection of fluoro-gold and other fluorescent tracers. *J. Histochem. Cytochem.* **38**, 721–723.
- Schutz, R.A., and Izquierdo, I. (1979). Effect of brain lesions on rat shuttle behavior in four different tests. *Physiol. Behav.* **23**, 97–105.
- Schwarzschild, M.A., Cole, R.L., Meyers, M.A., and Hyman, S.E. (1999). Contrasting calcium dependencies of SAPK and ERK activations by glutamate in cultured striatal neurons. *J. Neurochem.* **72**, 2248–2255.
- Selcher, J.C., Nekrasova, T., Paylor, R., Landreth, G.E., and Sweatt, J.D. (2001). Mice lacking the ERK1 isoform of MAP kinase are unimpaired in emotional learning. *Learn. Mem.* **8**, 11–19.
- Shields, J.M., Pruitt, K., McFall, A., Shaub, A., and Der, C.J. (2000). Understanding Ras: 'it ain't over 'til it's over.' *Trends Cell Biol.* **10**, 147–154.
- Sweatt, J.D. (1999). Toward a molecular explanation for long-term potentiation. *Learn. Mem.* **6**, 399–416.
- Taghzouti, K., Simon, H., Louilot, A., Herman, J.P., and Le Moal, M. (1985). Behavioral study after local injection of 6-hydroxydopamine into the nucleus accumbens in the rat. *Brain Res.* **344**, 9–20.
- Valjent, E., Corvol, J.C., Pages, C., Besson, M.J., Maldonado, R., and Caboche, J. (2000). Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *J. Neurosci.* **20**, 8701–8709.
- Winder, D.G., Martin, K.C., Muzzio, I.A., Rohrer, D., Chruscinski, A., Kobilka, B., and Kandel, E.R. (1999). ERK plays a regulatory role in induction of LTP by theta frequency stimulation and its modulation by beta-adrenergic receptors. *Neuron* **24**, 715–726.
- Xu, M., Hu, X.T., Cooper, D.C., Moratalla, R., Graybiel, A.M., White, F.J., and Tonegawa, S. (1994a). Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. *Cell* **79**, 945–955.
- Xu, M., Moratalla, R., Gold, L.H., Hiroi, N., Koob, G.F., Graybiel, A.M., and Tonegawa, S. (1994b). Dopamine D1 receptor mutant mice are deficient in striatal expression of dynorphin and in dopamine-mediated behavioral responses. *Cell* **79**, 729–742.