Engagement of the PFC in consolidation and recall of recent spatial memory

Wanda C. Leon,1 Martin A. Bruno,1,5 Simon Allard,1 Karim Nader,2 and A. Claudio Cuello1,3,4,6

1Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3G 1Y6, Canada; 2Department of Psychology, McGill University, Montreal, Quebec H3G 1Y6, Canada; 3Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec H3G 1Y6, Canada; 4Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec H3G 1Y6, Canada; 5Facultad de Ciencias Médicas, UCCuyo-CONICET, San Juan J5400, Argentina

The standard model of system consolidation proposes that memories are initially hippocampus dependent and become hippocampus independent over time. Previous studies have demonstrated the involvement of the medial prefrontal cortex (mPFC) in the retrieval of remote memories. The transformations required to make a memory undergo system’s consolidation are thought to require synaptic plasticity. In this study, we investigated the participation of the mitogen-activated protein kinase (MAPK)/ERK pathway in acquisition, memory consolidation, and recent memory recall of the Morris water maze (MWM). However, MEK inhibitor resulted in impairments on recent memory retrieval either when applied at the end of the learning phase (memory consolidation) or prior to the retention test. The results strongly support the concept that recently acquired and consolidated spatial memories require the mPFC, and that local activation of the MAPK/ERK pathway in the mPFC is necessary for the consolidation and recall of recent memories.

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The standard model of memory system consolidation postulates that memories are initially hippocampus dependent and are stored in the neocortex over time (Marr 1971; McClelland et al. 1995; Squire and Alvarez 1995; Wiltgen et al. 2004; Frankland and Bontempi 2005; Smith and Squire 2009). In humans, damage to the hippocampus results in a disruption of recently acquired memories, while sparing remote memories (Scoville and Milner 1957). In support of such an idea, functional imaging studies from rodents and humans have indicated that hippocampal activity is associated with the retrieval of recent but not remote memories. Moreover, dorsal hippocampus inactivation impairs performance of recent, but not old memories (Maviel et al. 2004). It should be noted, however, that the temporal gradient identifying system consolidation has not usually been found (Ryan et al. 2001; Lehmann et al. 2007).

It has been proposed that the mPFC, specifically the anterior cingulate cortex (ACC), controls the retrieval of old memories (Frankland and Bontempi 2005; Blum et al. 2006; Frankland et al. 2006). Furthermore, it has been shown that inactivation or lesion of the ACC blocks the expression of old contextual fear (Frankland et al. 2004) and spatial memories (Teixeira et al. 2006). At the synaptic level, synaptic plasticity mechanisms are thought to be required for these time-dependent changes to occur (Frankland et al. 2001; Frankland and Bontempi 2005). Prevalent theories of how hippocampus-dependent memories change over time posit that the mPFC should not undergo synaptic plasticity or be functionally necessary for the expression of recent memories (Frankland and Bontempi 2005).

Recent reports have revealed inconsistencies regarding the role of the mPFC in system’s consolidation, as they have indicated that the mPFC is also necessary for the formation of recent memories (Zhao et al. 2005; Blum et al. 2006). Specifically, it has been demonstrated that inhibition of the NR2B subunit of the mPFC disrupts the expression of newly acquired trace-conditioned memories (Zhao et al. 2005). These findings suggest that the mPFC plays an early role in memory formation.

In this study we used a modified version of the MWM to allow the monitoring of the mitogen-activated protein kinase (MAPK)/ERK signaling pathway by blocking and determining the degree of ERK1/2 phosphorylation as learning and memory tasks were assessed. The hidden platform version of the MWM has been used extensively to measure cognitive deficits in rodents (Morris 1984; Brandeis et al. 1989; Nieto-Escamez et al. 2004). Most of the previous work was done using a typical 5-d protocol, where the synaptic consolidation process may occur repeatedly over the training period. In our study, we used a modified single-day learning MWM protocol to better differentiate between acquisition, consolidation, and retrieval processes and for a more accurate determination of MAPK/ERK activity or its blockade. Given that extracellular signal regulated kinase-1 and 2 (ERK1/2) signaling is involved in long-term potentiation (English and Sweatt 1996, 1997; Thomas and Huganir 2004) and in learning and memory formation (Atkins et al. 1998; Blum et al. 1999; Sweatt 2001; Adams and Sweatt 2002; Silva 2003; Peng et al. 2010), we examined the expression of ERK1/2 in the hippocampus and mPFC.

To clarify whether the mPFC plays a role during recent spatial memory formation, we measured the levels of ERK activation and we transiently and selectively inhibited the ERK signaling pathway using the upstream MEK inhibitor U0126. The inhibitor was used either prior to acquisition of the MWM, right after acquisition, or after 24 h, but 30–40 min prior to the probe test.
We found that ERK activation was not induced in the mPFC during acquisition, but did increase during the subsequent synaptic consolidation period and during the retrieval of a new memory. However, inhibition of the ERK pathway in the mPFC resulted in an impairment of consolidation and recall of recent spatial memories without affecting the acquisition of the MWM task. These findings indicate that the acquired information is consolidated in the mPFC and that this brain region is necessary for the retrieval of recent spatial memories.

Results

Acquisition of a spatial memory induces ERK activation in the hippocampus, but not in the prefrontal cortex (PFC)

Consistent with previous studies demonstrating a role of the hippocampus in the acquisition and consolidation of recent spatial memories, ERK-phosphorylation was significantly increased in the hippocampus, but not in the PFC of rats during acquisition. Figure 1A illustrates the acquisition phase (left) and retention test (right) of rats trained on the hidden platform version of the single-day learning of the MWM. Animals required progressively less time to find the submerged platform from trials 1 to 6, as they learned the spatial task (***, P < 0.001, one-way ANOVA). Retention trials (probe tests) were performed 24 h after finishing the acquisition phase in the presence of the hidden platform. The latency to find the submerged platform was considered as an index of memory retention. Since our initial investigation, we have observed no significant changes in ERK phosphorylation between naive and control rats exposed to swimming (see Supplemental Fig. S2). We performed all experiments with animals exposed to the swimming for equivalent times. Trained animals spent significantly less time searching for the platform 7.33 ± 0.87 (mean ± SEM, n = 9) than control animals 48.4 ± 5.3 (mean ± SEM, n = 6) (**, P < 0.01, unpaired t-test), indicating that trained animals were able to retrieve the newly acquired spatial information 24 h later. The levels of hippocampal and mPFC ERK phosphorylation over acquisition trials are illustrated in Figure 1, B and C, respectively. The hippocampal ERK phosphorylation reached the maximum during the third trial (**, P < 0.01, one-way ANOVA), while in the mPFC no significant change was observed during the diverse stages of the training period (P > 0.05, one-way ANOVA).

ERK activation occurs in the mPFC and hippocampus during recent memory recall

The hypothesis of systems consolidation proposes that the hippocampus, but not mPFC, should be activated and required for the expression of a hippocampus-dependent memory (Bontempi et al. 1999; Frankland et al. 2004). To investigate this issue, trained and control animals were sacrificed after the probe test, and the possible engagement of the mPFC was evaluated by measuring ERK phosphorylation levels using neurochemical and immunohistochemical methods. By quantitative Western blotting and confocal microscopy, we found that ERK was phosphorylated in both the mPFC and hippocampus after the retrieval of a 1-d-old memory. Figure 2, A and B, illustrate representative Western blots for phospho and total ERK levels during recent memory recall in both the hippocampus and mPFC of trained animals. In trained animals, we observed a significant post-training increase in ERK phosphorylation in both hippocampus (**, P < 0.01, unpaired t-test) and mPFC (*, P < 0.05, unpaired t-test) immediately after the retrieval of recently acquired spatial information. As shown in Figure 2, A and B, the increase in ERK phosphorylation was not a result of an increase in total ERK protein expression. To
gather more direct information on the neuroanatomical sites of the increased ERK phosphorylation in the hippocampus and mPFC, we performed immunofluorescence and imaged by confocal microscopy (see Fig. 2C,D,F,G). Consistent with our Western blot results, there was a significant increase in the number of pERK-immunoreactive neurons observed in the mPFC of trained animals after recent memory recall (*, $P < 0.05$, unpaired t-test; Fig. 2E). We also quantified the number of immunoreactive neurons in the FR2 region of the frontal cortex, where we did not detect significant changes after the probe test.

Furthermore, the application of the inhibitor U0126 in mPFC had no effect in basal levels of neurons displaying ERK phosphorylation in the FR2, indicating that inhibition was restricted to mPFC without affecting neighboring cortical regions (see Supplemental Fig. 3). This suggests that the phosphorylation is region specific and does not occur throughout the neocortex. In the hippocampus, we found that the average intensity of the pERK immunoreactivity was increased in the CA1 region of trained animals when compared with controls (**, $P < 0.01$, unpaired t-test; Fig. 2H).

**Figure 2.** Stimulation of ERK pathway in both the PFC and hippocampus after 24-h retrieval of a recent spatial memory. (A,B) Representative Western blot analysis revealing phosphorylated extracellular regulated kinase (pERK) and total ERK in the hippocampus and PFC. Note that ERK activation (phosphorylation) occurs during recent memory recall in both hippocampus and PFC of trained animals ($n = 9$), as revealed by the relative optical density of immunoreactions as compared with controls ($n = 6$). Statistical difference of ERK activation in the hippocampus and PFC were of (*) $P < 0.05$ and (**) $P < 0.01$, respectively, in relation to control groups. (C,D) Phospho-ERK immunoreactivity as revealed by confocal microscopy of the PFC of the control ($n = 5$) and trained ($n = 5$) animals. The pERK immunoreactive cells were mainly present in laminae II and laminae III of the PFC. (E) Trained animals showed a significant increase in the number of immunoreactive cells when compared with control animals (*, $P < 0.05$). (F,G) Representative confocal images of the CA1 region of the dorsal hippocampus of controls and trained animals, respectively, after a 24-h probe test. (H) Quantification of the signal intensity in the CA1 region of control ($n = 5$) and trained animals ($n = 5$) applying an image-analysis system. The area representing the pyramidal cells was isolated using a minimum intensity threshold mask. The average brightness of the signal was then measured using the “brightness” option of the MCID software (see Materials and Methods section). The signaling intensity was significantly increased in trained animals when compared with control animals (**, $P < 0.01$).
Inhibition of ERK pathway in the mPFC did not disrupt the acquisition phase of Morris water maze task

We further investigated whether the mPFC is engaged during the acquisition phase of the single-day learning of the Morris water maze task. For this we performed a bilateral intra-mPFC infusion (Fig. 3A) of the specific MEK kinase inhibitor U0126 prior to the training. U0126 had no effect on the acquisition of the MWM task. A two-way ANOVA revealed a significant training effect (P < 0.001) and a nonsignificant (P > 0.05) training × treatment interaction (Fig. 3B). To verify independently whether U0126 injections inhibited the ERK phosphorylation pathway in the PFC area, we measured the levels of phospho ERK and total ERK by Western blotting at the end of the acquisition phase. We observed that U0126 infusion into the mPFC resulted in a significant inhibition of ERK phosphorylation (***, P < 0.001 unpaired t-test) as compared with the vehicle-infused group (Fig. 3C). No significant ERK phosphorylation changes (P > 0.05 unpaired t-test) were found in the hippocampus of rats receiving either U0126 or saline (control group) in the mPFC area (Fig. 3D).

Inhibition of the ERK pathway in the mPFC area impairs consolidation of a newly acquired spatial memory

To examine whether ERK phosphorylation in the mPFC is engaged in spatial memory during the consolidation phase, U0126 or saline was bilaterally infused after the end of the last trial of acquisition phase in single-day MWM protocol. Animals were then tested 24 h later for retention of memory. The mPFC-U0126 injected group spent a longer time to find the submerged platform 48.30 ± 7.351 n = 5 (mean ± SEM) compared with the vehicle-treated controls 5.800 ± 0.5017 n = 5 (**, P < 0.01, unpaired t-test; Fig. 4B). This data suggests that the mPFC plays a significant role in the synaptic stabilization of a newly acquired spatial memory.

The U0126-treated animals revealed a significant decrease in ERK phosphorylation when compared with the vehicle-treated controls. No change in the total ERK protein expression was observed in any experimental group (Fig. 4C,D). The effect observed 24 h after the U0126 injection in Figure 4, B–D is not due to the remaining inhibitory activity of the compound. The inhibitory effects of U0126 at the injected concentration (1.0 ug/ul) in the mPFC area were transient, as no reduction in ERK phosphorylation was observed after the probe test when the animals were treated the previous day (data not shown).

Inhibition of the ERK signaling pathway in the PFC prevents the expression of recent spatial memory

We further examined the role of the ERK activation in the mPFC in the retrieval of recent spatial memory. For this purpose, we infused U0126 in the mPFC 24 h after the end of the acquisition/consolidation phase and 30–40 min before the probe test (Fig. 5A). Animals injected with the ERK inhibitor U0126 were impaired in the expression of the acquired spatial memory. The latency to find the hidden platform was significantly higher when the animals were treated the previous day (data not shown).
involvement of the mPFC in the consolidation and retrieval of recent spatial memories. Neurochemical aspects underlying these processes were also investigated. It is difficult to separate acquisition and consolidation in the standard version of the water maze task that requires training over multiple days, where consolidation may occur repeatedly at the end of each training period. Additionally, a previous lesion study has shown that mPFC was not required for the acquisition of the classical MWM (de Bruin et al. 1994). However, when the task demand is changed by altering the configuration of the environment, the use of multiple distal cues, or by varying the number of trials, an engagement of mPFC was revealed (Compton et al. 1997). In the present study, by applying 1-d-six trials protocol for the acquisition of the MWM task, thus increasing the task demands of the MWM, we found that such neocortical involvement could occur in earlier stages of memory formation. Thus, during recent spatial memory recall, there was an activation of the ERK pathway not only in the hippocampus, but also in the mPFC after applying the novel single-day learning of the MWM. These results would be in line with a recent finding reported by Blum et al. (2006). By using a different behavioral task, they demonstrated that mPFC activation was shown to be necessary not only for remote but also recent trace fear conditioning memory. Our observations indicate that retrieval of recent memory is mPFC dependent and that its activation may depend on the executed behavioral spatial task.

Since hippocampal ERK phosphorylation has been shown to be required during recent spatial learning and memory (Blum et al. 1999; Selcher et al. 1999; Clark et al. 2005) we also investigated whether or not the bilateral administration of U0126 in the mPFC after the acquisition phase and retesting for “recent memory” (B) After 24 h, the rats injected with the MEK inhibitor U0126 in the PFC fail to remember the previously learned location of the hidden platform, as revealed by the markedly increased escape latency time (***, P < 0.01). (C) The effective inhibition of the activation (phosphorylation) of ERK in the PFC of treated rats, without change in total ERK protein expression, as shown in the representative Western blot and demonstrated quantitatively by analysis of the relative (pERK normalized against total ERK) optical density of immunoreactive bands (D) (**, P < 0.01).

There is a body of evidence supporting the idea that aging memories become more dependent on neocortical sites (Bontempi et al. 1999; Frankland et al. 2001, 2004; Maviel et al. 2004). Our data shows that the involvement of the neocortex in consolidation is an earlier event than is proposed in the standard model of system memory consolidation (Takehara et al. 2003; Maviel et al. 2004; Frankland and Bontempi 2005; Teixeira et al. 2006). Some scientists have failed to find these gradients in rodents or human imaging (Ryan et al. 2001; Lehmann et al. 2007). Interestingly, we found that such neocortical involvement could occur in earlier stages of memory formation. This study also demonstrated that the bilateral inhibition of the ERK pathway in the mPFC before training had no effect on the acquisition of spatial information. These results would be in agreement with a previous report in which, using a different behavioral task, Runyan and colleagues showed that mPFC does not directly participate in the encoding of fear conditioning, but impaired recent memory retention (Runyan et al. 2004). Our results show a similar involvement of the same brain region in spatial memory. Taken together, the evidence suggests that mPFC is involved in many types of early memory formation.

Finally, we investigated whether or not the bilateral administration of U0126 in the mPFC after the acquisition phase and before the probe test was able to disrupt recent spatial memory consolidation and retrieval. Our results revealed that mPFC ERK phosphorylation during spatial memory consolidation takes place 2 h after the acquisition of single-day learning of the MWM (Supplemental Fig. S1). In addition, we found that U0126 infusion into the mPFC immediately after the acquisition phase.
Figure 5. Disruption of the ERK pathway in the PFC after recent memory consolidation prevents memory recall. (A) Performance of rats \( n = 10 \) in learning the MWM task and time of injection of the MEK inhibitor in the PFC during the memory consolidation period (\( * \), \( P < 0.01 \)). (B) Note the failure of PFC U0126-treated rats \( n = 5 \) in retaining the acquired memory of the location of the submerged platform when the ERK pathway is disturbed after consolidation of recent memory. (C) The effective drug-induced inhibition of ERK phosphorylation in the PFC by Western blot analysis. (D) The effective MEK inhibitor blockade of the ERK activation as consequence of recent memory recall.

of single-day learning of the MWM blocks the synaptic consolidation of recent spatial information (Fig. 4B,C). This indicates that in our experimental paradigm the infusion of MEK inhibitor in the mPFC should have disrupted spatial memory consolidation and retrieval within the half-life of the U0126, which has been shown to be \( \sim 2 \) h. Therefore, behavioral testing was performed within the effective period of the compound half-life (London and Clayton 2008). These previous findings reinforce the role of mPFC in the consolidation and retrieval of recently acquired spatial memories. These observations are consistent with the observations of Blum et al. (2006), who demonstrated that temporary inactivation of the medial mPFC impaired recent trace fear memory recall. Other investigators have also shown that transient disconnection of the hippocampal–prefrontal circuit disrupts the retrieval of spatial memory (Floresco et al. 1997; Blum et al. 2006; Wang and Cai 2006). The above, along with the present study, support the notion that the hippocampal–prefrontal circuit plays an important role in the consolidation and retrieval of recent spatial memory.

In summary, we have shown that by using a modified version of the classical MWM (single-day learning), it is possible to manipulate the ERK cell-signaling pathway by infusing the upstream MEK inhibitor (U0126) directly and restricted to the mPFC. This approach permitted us to demonstrate that mPFC participation is necessary for the consolidation and retrieval of recent spatial memories.

Materials and Methods

Animals

Four month-old male Fischer-344 rats were used in this study, housed two to a cage with food and water ad libitum, and maintained on a 12-h light–dark cycle. All procedures were performed according to guidelines approved by the McGill University Animal Care Committee and the Canadian Council on Animal Care.

Surgery

Rats were anesthetized with equithesin and placed in a stereotaxic apparatus (David Kopf Instruments). The skin covering the skull was cut in the midline and retracted sideways. Bilateral cannulae (22-gauge stainless steel, HK Scientific) were implanted in the mPFC (bregma +3.2 mm, lateral +0.75 mm and depth –1.5 mm) (Paxinos and Watson 1986). A solid cylindrical dummy cannula was inserted to occlude the cannula guide at the end of the surgeries. Animals were given a 7-d rest period following surgery, before behavioral training.

Pharmacological infusion

Animals were quickly anesthetized with isoflurane (AErrane, Baxter Healthcare) in an induction chamber. The anesthesia was maintained during the U0126 infusion procedure with a table-top anesthesia system (Harvard Apparatus) equipped with a single O2 flowmeter set at 50 mL O2/minute mixed 2.5% isoflurane via a face mask.

Prior to the drug infusion each dummy guide was removed and a 28-gauge internal cannula was inserted at the depth –1.5 mm prefrontal area, below the level of bregma. The cannula was connected by a polypropylene tube to a 1.0-ul Hamilton syringe that delivered the solution using an automated pump (Bioanalytical System, Inc). The selective MEK inhibitor U0126 (Promega Corp.) was dissolved in 25% dimethyl sulfoxide (DMSO) in a phosphate-buffered saline (PBS) solution (Davies et al. 2000). A solution of 1.0 ug/ul of U0126 was infused bilaterally into PFC 30–40 min before behavioral training (pretraining), immediately after training (post-training), or 24 h later and before the retention probe, at a rate of 0.2 ul/min for 5 min. The cannula was left in place for an additional 2 min to allow diffusion and to minimize backflow. At the end of infusions, the cannulae were removed and the cannula guides were occluded with the dummy cannulae. All vehicle control infusions consisted of the same volume of solution used for the pharmacological inhibitor (25% DMSO in PBS).

Behavioral procedure

Training phase

Prior to the initiation of the behavioral procedure rats were handled during 5 d for a period of 5 min to minimize stress. Rats were trained in a modified version of the MWM task (single-day learning) (Morris et al. 1982; Morris 1984) to locate a hidden escape platform in a circular 1.4-m pool. The pool was filled with opaque water and the testing room contained extra-maze visual cues. Each rat received six trials, with an intertrial interval of 15 min in a single-day learning. Subjects were released into the pool from one of four starting positions, and the location of the platform remained constant throughout training (northeast quadrant of the pool). The time to find the escape platform (escape latency) was measured. If an animal failed to find the platform, it was briefly placed on the platform by the experimenter. Animals were allowed to remain on the platform for a period of 10 sec. Control animals also received six trials, allowing them to swim freely without the presence of the platform. They were allowed to swim for an average of time similar to trained animals. Visual and locomotor impairments were ruled out by raising the platform above the surface of the water. No difference was found among the experimental groups.
For determining whether swimming in the MWM pool affected basal ERK phosphorylation levels in the mPFC, naïve (not exposed to swimming pool) (n = 5) and control (exposed to swimming pool) (n = 5) rats were sacrificed and the tissue was stored at −80°C for further analysis. The Western blot analysis of ERK phosphorylation revealed no changes between naïve and control animals, and therefore consecutive studies were carried out only with control rats (see Supplemental Fig. S2).

For the recent spatial memory recall experiments, control (n = 6) and trained rats (n = 9) were trained in a single day of the MWM.

In the pretraining experiment (before acquisition of MWM) rats were given a recovery period of 30–40 min after the infusion with either 1.0 ug/ul of U0126 or saline (n = 10), respectively. In the post-training experiment (immediately after acquisition of MWM) rats were infused with either U0126 (n = 5) or saline (n = 5) and retested after 24 h. For the mPFC consolidation phase experiment (n = 5 per time point) rats per time point were sacrificed at 0, 60, 120, and 240 min after the acquisition of MWM task. Finally, 24 h after the acquisition phase of MWM and 30–40 min before the retention probe, rats were injected with either U0126 (n = 5) or saline (n = 5).

For the time course ERK phosphorylation experiments in the mPFC and hippocampus during acquisition of 1-d protocol, rats were sacrificed right after each trial (n = 4).

For the immunocytochemical analysis of ERK phosphorylation, (n = 5) trained and (n = 5) control animals were used. All animals were sacrificed shortly after the retention probe, 24 h after acquisition.

Retention probe
After completing the single-day learning of the MWM, memory recall was determined by a probe test. Recent memory formation was evaluated by the latency for each animal to find the submerged platform in one trial of 60 sec. This probe test was performed 24 h after the acquisition, and measured the ability to consolidate and retrieve recent spatial memories.

Preparation of protein extracts
At the appropriate time points, immediately after acquisition, consolidation and recent spatial memory recall, animals were sacrificed and the brain tissue (hippocampal and medial prefrontal area) was quickly removed. Both mPFC tissue and whole hippocampus were homogenized in 10× cell lysis buffer (Cell Signaling Technology, Inc.) and protease inhibitor cocktail (Roche Diagnostics).

Western blot analysis
Samples of whole hippocampus and mPFC homogenate from individual rats were normalized by total protein content through dilution with cell lysis buffer. The amount of protein individual rats were normalized by total protein content. Samples of whole hippocampus and mPFC homogenate from hippocampus were homogenized in 10× polyacrylamide gels and transferred (Bio-Rad Laboratories, Inc.) using Bovine Serum Albumin (BSA) was measured using a conventional protein assay protocol through dilution with cell lysis buffer. The amount of protein was bigger than 10 μg/ul. The Western blot of ERK phosphorylation in the mPFC, naïve (Roche Diagnostics). Signaling Technology, Inc.) and protease inhibitor cocktail (www.learnmem.org 303 Learning & Memory) was resolved in 12% polyacrylamide gels and transferred (Bio-Rad Laboratories, Inc.) using Bovine Serum Albumin (BSA) was measured using a conventional protein assay protocol through dilution with cell lysis buffer. The amount of protein samples of whole hippocampus and mPFC homogenate from whole hippocampus were homogenized in 10× cell lysis buffer (Cell Signaling Technology, Inc.) and protease inhibitor cocktail (Roche Diagnostics) and using Kodak Biomax XAR imaging film. The immunoreactive bands were determined by densitometry of the films using MCID image analysis system. The phosphorylated forms of ERK were normalized to total ERK.

Immunocytochemical analysis
All animals were deeply anesthetized to areflexia with 0.4 mL/kg of Equithesin, 6.5 mg of chloral hydrate, and 3 mg of sodium pentobarbital in a volume of 0.3 mL, i.p. per 100 g body weight. Anesthetized animals were perfused transcardially with cold saline, followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), (pH 7.4). Rat brains were then removed, post-fixed for 4 h in the same fixative solution, and then cryoprotected in 30% sucrose in 0.1 M PB.

Tissues were embedded in an optimal cutting temperature (OCT) compound (Tissue-Tek) and 35-μm-thick sections were cut on a cryostat (Leica CM-3050-S) and collected in PBS containing 0.2% Triton-X 100 (PBS + T). Brain sections were washed for 30 min with PBS + T and incubated in 10% normal goat serum (NGS) for 60 min.

The tissue was then incubated at 4°C for 24 h in 5% NGS and a polyclonal antibody raised in rabbit against the phosphorylated ERK kinase (Cell Signaling Technology, Inc.) at a concentration of 1 in 50 in PBS + T. Sections were then washed for 30 min in PBS + T, following which they were incubated for 2 h at room temperature in a goat anti-rabbit biotinylated IgG (1:200; Vector). The sections were then washed again with PBS + T and incubated for 2 h at room temperature in Alexa Fluor 488 conjugated to streptavidin (1:200; Molecular Probes). Sections were then washed for 30 min in PBS and mounted on gelatin-subbed slides, dried overnight, and coverslipped with Aqua Polymount (Polysciences, Inc.). Changes in pERK immunoreactivity were determined by capturing images of immunoreactive cells using a Zeiss LSM 510 confocal microscope equipped with an argon laser. All settings regarding lasers and digital gain were kept constant for all images from all animals. Confocal images were exported as TIFF files and analyzed with an image analysis system (MCID Elite version 7, Imaging Research, Inc.). For both the PFC and the hippocampus, three pictures, corresponding to three different fields, were taken per section, and four sections per animal were used. A total of 12 pictures for the PFC and 12 pictures for the hippocampus were quantified per animal. More precisely, we quantified in the Cg1 and Cg2 areas of the anterior cingulate cortex and the CA1 region of the hippocampus since they were the regions with the most apparent phosphorylation.

Quantification of pERK immunoreactive neurons in the mPFC
Immunoreactive neurons were counted using the MCID image analysis software. In short, a brightness threshold was set to separate immunoreactive cell bodies from the background. The software considered continuous areas above threshold that were bigger than 10 μm² as cell bodies. All of the cell bodies were added for each confocal image and these counts were averaged for each animal. To control for proper quantification, two animals were manually counted and the numbers were compared with the ones generated by the MCID software. The neuronal counts were highly comparable using both techniques. The images were always taken in the Cg1 and Cg2 areas of the mPFC.

Quantification of the hippocampus
A threshold for confocal images was obtained using a brightness criterion to isolate immunoreactive neurons. The minimal intensity threshold was kept consistent throughout all confocal images and reliably isolated immunoreactive neurons. Following thresholds, the brightness index immunoactivity were determined by LUC image analysis software that represented the average brightness level of all immunoreactive material that was above the minimal

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brightness criterion. A relative brightness of 1 means that all the selected pixels were saturated and a relative brightness index of 0 means that all of the pixels were black. Since all of the tissue was incubated with the same antibody mixtures and imaged with consistent confocal settings, we interpreted the relative brightness as an index of protein levels. Due to a different cellular organization and a different distribution of the immunoreactivity, we could not properly isolate a specific immunoreactive neuronal population in the CA1 region of the hippocampus and quantify the number of neurons as we did in the mPFC. Therefore, the relative brightness of the overall neuronal population per confocal image was quantified in an attempt to find a cytological equivalent of the data obtained by Western blotting.

**Statistics**

For the behavioral studies, the data was analyzed for statistical significance using a one-way ANOVA and two-way ANOVA. Comparisons between groups were done using a two-tailed unpaired t-test. A value of $P < 0.05$ was used as a criterion for statistical significance.

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