Research

Role of the phosphoinositide 3-kinase-Akt-mammalian target of the rapamycin signaling pathway in long-term potentiation and trace fear conditioning memory in rat medial prefrontal cortex

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Phosphatidylinositol 3-kinase (PI3K) and its downstream targets, including Akt (also known as protein kinase B, PKB), mammalian target of rapamycin (mTOR), the 70-kDa ribosomal S6 kinase (p70S6k), and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), may play important roles in long-term synaptic plasticity and memory in many brain regions, such as the hippocampus and the amygdala. The present study investigated the role of the PI3K/Akt-mTOR signaling pathway in the medial prefrontal cortex (mPFC), also a crucial neural locus for the control of cognition and emotion. Western blot analysis of mPFC tissues showed an activation of phosphorylation of Akt at the Ser473 residues, mTOR, p70S6k, and 4E-BP1 in response to long-term potentiation (LTP)-inducing high-frequency stimulation (HFS). Infusion of PI3K inhibitors (wortmannin and LY294002) and an mTOR inhibitor (rapamycin) into the mPFC in vivo suppressed HFS-induced LTP as well as the phosphorylation of PI3K/Akt-mTOR signaling pathway. In parallel, these inhibitors interfered with the long-term retention of trace fear memory examined 3 d and 6 d after the trace fear conditioning training, whereas short-term trace fear memory and object recognition memory were kept intact. These results provide evidence of involvement of activation of the PI3K/Akt-mTOR signaling pathway in the mPFC for LTP and long-term retention of trace fear memory.

The phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates important cellular functions, such as gene expression, cell cycle progression, cell growth, and differentiation (Franke et al. 1997; Katso et al. 2001). PI3K has many downstream targets that include a serine/threonine protein kinase, Akt, also known as protein kinase B (PKB) (Franke et al. 1997; Cantley 2002). An important effector of Akt is the serine/threonine protein kinase termed mammalian target of rapamycin (mTOR) (Sarbasov et al. 2005). In turn, activation of mTOR leads to the phosphorylation and activation of the 70-kDa ribosomal S6 kinase (p70S6k) (Pullen and Thomas 1997; Lawrence et al. 2004) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (Gingras et al. 1998), which are important regulators of protein translation (Hay and Sonenberg 2004).

A role for PI3K/Akt in the brain has been investigated, as PI3K/Akt has been implicated in the induction and maintenance of long-term potentiation (LTP) in the hippocampal CA1 region (Opazo et al. 2003), the leading experimental model for the synaptic changes that may underlie learning and memory. PI3K/Akt has also been suggested for LTP at the perforant path-granule cell synapses (Horwood et al. 2006). In addition, PI3K/Akt has been shown to be required for amygdala fear conditioning (Lin et al. 2001). mTOR, an effector of Akt, was also implicated in synaptic plasticity. The first evidence was provided by showing that rapamycin, a selective inhibitor of mTOR activity (Takei et al. 2001, 2004; Hay and Sonenberg 2004), blocks long-term facilitation in

Aplysia (Casadio et al. 1999). Subsequent studies suggested that mTOR has been implicated in the regulation of LTP in the hippocampus (Tang et al. 2002; Cammalleri et al. 2003). Furthermore, neurobehavioral testing had revealed that mTOR signaling in the hippocampus plays an important role in long-term memory consolidation of an inhibitory avoidance learning task (Bekinschtein et al. 2007). Two downstream targets of mTOR, p70S6k and 4E-BP1, are important regulators of protein translation, and regulation at the translational level might play an important role in long-lasting forms of synaptic plasticity (Casadio et al. 1999; Tang et al. 2002; Kelleher Jr. et al. 2004). Previous studies have shown that the dendritic mTOR-p70S6k cascade is required for the protein synthesis-dependent late phase of LTP in the hippocampus (Tang et al. 2002; Cammalleri et al. 2003). Collectively, these data suggest that the PI3K/Akt-mTOR-p70S6k cascade may play an important role in LTP and long-term memory in the hippocampus. However, few studies have investigated the role of this signaling pathway in synaptic plasticity and long-term memory in rat medial prefrontal cortex (mPFC), a brain region critically involved in the control of cognition and emotion (Goldman-Rakic 1995).

Recognition memory tasks have recently become widely applied and are especially suited to test the effects of pharmacological interventions on learning and memory. Furthermore, different forms of recognition memory, such as the standard one-trial novel object preference task, recognition of a familiar object (Ennaceur and Delacour 1988), one-trial object-place recognition (Mumby et al. 2002), temporal order memory (Mitchell and Laiacina 1998; Mumby et al. 2002), and, recently, episodic-like memory (Dere et al. 2005a,b; Kart-Teke et al. 2006) can be mea-

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sured in rats and mice. Much evidence has indicated a critical role that the mPFC may contribute to recognition memory; damage to this region has been shown to impair recognition memory tasks (Kolb et al. 1994; Chiba et al. 1997; Mitchell and Laiacina 1998; Kesner and Ragazzino 2003; Hannesson et al. 2004; Browning et al. 2005; Barker et al. 2007). These findings suggest that the mPFC plays an important role in recency discriminations for objects or spatial locations, and, furthermore, that the mPFC may be concerned with the integration of object and place information necessary for object-in-place discriminations.

Trace conditioning is a form of learning that requires the association of a conditioned stimulus (CS) and an unconditioned stimulus (US) separated by time (Pavlov 1927). Human and animal studies have designated that the hippocampus (Clark and Squire 1998) and the mPFC (Runyan and Dash 2004; Runyan et al. 2004) are involved in the trace fear conditioning memory. In this study, we investigated the role of the PI3K/Akt-mTOR signaling cascade in the mPFC-dependent object recognition memory and trace fear conditioning memory tasks.

Results

Characteristics of basal synaptic transmission, PPF, and LTP in the mPFC

In vivo, stimulation of the mPFC in the contralateral hemisphere evokes a characteristic field excitatory postsynaptic potential (fEPSP), an initial positive deflection followed by a negative deflection, followed by a second positive deflection in its contralateral counterpart (Gemmell and O'Mara 2000; Roder et al. 2003). Basal synaptic transmission, paired-pulse facilitation (PPF), and LTP induction were tested in this study. Basal synaptic transmission, represented by the slope and the amplitude of fEPSPs in response to electrical stimulation of varying current intensities, was measured. The input–output functions (Fig. 1A) shows that both normalized fEPSPs slope and normalized fEPSPs amplitude were increased with electrical stimulation of increasing current intensities. PPF represents an increase of the second postsynaptic response (pulse 2) relative to the first (pulse 1). At the interpulse interval of <30 msec (i.e., 20 msec) or at the interpulse interval of >100 msec (i.e., 150–700 msec) (Fig. 1B), pulse 2 failed to facilitate significantly relative to the pulse 1 (~100%). However, significant facilitation occurred at the interpulse interval of 30–100 msec (repeated-measures ANOVA and Tukey's *t*-test, $F_{(9,230)} = 8.52$, $P < 0.001$, $n = 12$). At the interpulse interval of 50 msec, the mean magnitudes of facilitation in fEPSPs slope and

Figure 1. Basal synaptic transmission, paired-pulse facilitation (PPF), and long-term potentiation (LTP) in the mPFC. (A) Normalized input–output functions. Mean ($\pm$ SEM) fEPSPs slope (upper panel) and fEPSPs amplitude (lower panel) as a function of stimulus intensity. (Inset) Representative waveforms indicating where the slope and amplitude of the fEPSPs measurement was taken. (B) Mean ($\pm$ SEM) paired-pulse ratios of fEPSPs slope (upper panel) and fEPSPs amplitude (lower panel) as a function of interpulse intervals. (*) Significant increase relative to the pulse 1 (~100%) ($P < 0.05$). (C) The percent of baseline ($\pm$ SEM) of fEPSPs slope after high-frequency stimulation (HFS) to induce LTP in the mPFC. (Inset) Representative fEPSPs waveforms recorded before HFS (baseline), 1 min, 1 h, and 3 h after HFS.

fEPSPs amplitude were 155%–165% relative to the first pulse (~100%). Furthermore, an interpulse interval of 50 msec was the most sensitive measure in the phenomenon of PPF, reflected by the facilitation ratio of pulse 2/pulse 1 as the highest among the tested interpulse intervals (Tukey's *t*-test, $F_{(1,22)} = 15.83$, $P < 0.001$). In the mPFC, LTP of fEPSPs slope and fEPSPs amplitude can be induced by high-frequency stimulation (HFS) in vivo. Upon HFS, the magnitude of fEPSPs slope increased to ~160% relative to the baseline and has no obvious decay as a function of post-HFS time (Fig. 1C). The magnitude of fEPSPs

amplitude after the application of HFS was 155%–165% relative to the baseline, similar to that of fEPSPs slope (data not shown). These results indicated that the cross-commissural pathway between the mPFC is capable of expressing PPF and LTP as measured by either fEPSPs slope or fEPSPs amplitude. Since the patterns of changes in fEPSPs slope and fEPSPs amplitude were similar, only fEPSPs slope data were presented in the subsequent experiments.

Induction of LTP increased the phosphorylation of Akt, mTOR, p70S6k, and 4E-BP1 in the mPFC

To examine whether induction of LTP by HFS in the mPFC could trigger the activation of the PI3K/Akt-mTOR signaling pathway, the mPFC tissues were harvested at a different post-HFS time, and the total and phosphorylated protein levels of Akt, mTOR, p70S6k, and 4E-BP1 were examined by Western blot. As expected, applying HFS to the mPFC did not significantly alter the total protein levels of Akt, mTOR, p70S6k, and 4E-BP1 relative to the test stimulation (all $F_{(4,55)} \leq 1.98$, all P s > 0.05 , data not shown). However, the levels of phosphorylation of these proteins were altered at the different post-HFS time. For Akt at Ser473 residues, significant increased phosphorylation can be detected as early as 5 min, and this increase persisted to 3 h after the HFS (one-way nonparametric ANOVA and the Mann-Whitney U -test, $F_{(4,55)} = 4.58$, $P < 0.01$; Fig. 2A), the deadline that we recorded in the present study. Though the combination of Akt at Ser473 and Thr308 residues activates Akt, for Akt at Thr308 residues, no significant increase was observed at each time point after the HFS delivery ($F_{(4,55)} = 1.32$, $P > 0.05$, data not shown). For mTOR, a significant increase in phosphorylation can be detected from the time point of 30 min to 3 h post-HFS (Fig. 2B). For both p70S6k (Fig. 2C) and 4E-BP1 (Fig. 2D), a significant increase of phosphorylation was observed at the time points of 1 h and 3 h after the HFS delivery ($F_{(4,55)} \geq 2.68$, all P s < 0.05).

PI3K/Akt-mTOR signaling pathway involved in the HFS-induced LTP and the activation of phosphorylation in the mPFC

To investigate whether the PI3K/Akt-mTOR signaling pathway is required in the induction of LTP and in the HFS-induced phosphorylation of Akt, mTOR, p70S6k, and 4E-BP1, two structurally distinct PI3K inhibitors (wortmannin and LY294002), and an mTOR inhibitor (rapamycin) were infused into the mPFC 30 min prior to the application of HFS, respectively. Recordings of evoked fEPSPs in the presence of these inhibitors were monitored continuously for 3 h following the application of HFS, and mPFC tissues were harvested at the end of the electrophysiological recordings and subjected to Western blot analysis. Insertion of the injection needle into the infusion-recording combined electrode and infusion of vehicle (0.05% DMSO in the aCSF) alone or any one of the inhibitors into the mPFC did not significantly change the input-output functions ($F_{(8,117)} \leq 1.99$, all P s > 0.05) and PPF measures ($F_{(9,130)} \leq 1.77$, all P s > 0.05), which were tested 30 min after the initiation of infusion and before the application of HFS (data not shown). However, infusion of these inhibitors significantly reduced the magnitude of LTP and the protein levels of phosphorylation of PI3K/Akt-mTOR and its downstream signaling molecules (Fig. 3). As shown in Figure 3A, LTP of fEPSP slope with a magnitude of ~160% relative to the baseline was observed in the vehicle infusion group, whereas infusion of 4 ng wortmannin of 1 μ L into the mPFC significantly inhibited the magnitude of HFS-induced LTP. During the first 30 min post-HFS, the magnitude of fEPSPs slope in the presence of wortmannin decreased with time and then became stable. Three hours after the HFS, the magnitude of LTP was only ~110% relative to the baseline. Like-

Figure 2. Time course of high-frequency stimulation (HFS)-induced increases in the phosphorylation of Akt at Ser473 (A), mTOR (B), p70S6k (C), and 4E-BP1 (D) in the mPFC. No significant changes in the total and the phosphorylation of these protein kinases as a function of stimulation time were observed in the test stimulation group; thus, the ratios of phosphorylated/total protein levels of each protein kinase in the test stimulation groups were valued as 100%. The densitometry for proteins showing the normalization of phosphorylated/total protein ratios in the HFS-induced LTP group to those protein ratios in the test stimulation group were presented as a function of post-HFS time. (Inserts in the upper portion of each panel) Representative Western blots in the test stimulation and the HFS-induced LTP groups. (*) Represents $P < 0.05$ compared with the test stimulation group (100%) at each time point.

Figure 3. Effects of wortmannin (PI3K inhibitor) and rapamycin (mTOR inhibitor) on LTP induction and phosphorylation of PI3K/Akt-mTOR signaling molecules in rat mPFC. (A) The percent of baseline ($\pm$ SEM) of fEPSPs slope after high-frequency stimulation (HFS) to induce LTP in the vehicle and the wortmannin-infused group. (Inset) Representative fEPSPs waveforms recorded before HFS (pre-HFS, dashed line) and 3 h after HFS (post-HFS, solid line) in the vehicle and wortmannin infusion groups. (B) The densitometry for phosphorylated mTOR (p-mTOR), phosphorylated p70S6k (p-p70S6k), and phosphorylated 4E-BP1 (p-4E-BP1) proteins showing the normalization of phosphorylated/total protein ratios in the vehicle HFS group, the wortmannin-infused test stimulation group, and the wortmannin-infused HFS group to those protein ratios in the vehicle test stimulation group. (*) Represents $P < 0.05$ compared with the vehicle test stimulation group (100); (#) represents $P < 0.05$ compared with the vehicle HFS group; (&) represents $P < 0.05$ compared with the wortmannin-treated test stimulation group. (C) The percent of baseline ($\pm$ SEM) of fEPSPs slope after HFS to induce LTP in the vehicle and the rapamycin-infused group. (Inset) Representative fEPSPs waveforms recorded before HFS (pre-HFS, dashed line) and 3 h after HFS (post-HFS, solid line) in the vehicle and rapamycin infusion groups. (D) The densitometry for phosphorylated p70S6k (p-p70S6k) and phosphorylated 4E-BP1 (p-4E-BP1) proteins showing the normalization of phosphorylated/total protein ratios in the vehicle HFS group, the rapamycin-infused test stimulation group, and the rapamycin-infused HFS group to those protein ratios in the vehicle test stimulation group (100%); (#) represents $P < 0.05$ compared with the vehicle HFS group.

wise, infusion of 1.5 μ g LY294002 of 1 μ L into the mPFC, revealed a similar pattern of decrease in the magnitude of LTP (data not shown). These results indicated that PI3K/Akt is involved in the induction of LTP in the mPFC.

Levels of total and phosphorylated protein levels of mTOR, p70S6k, and 4E-BP1, all substrates of PI3K/Akt, were measured using Western blot analysis. Infusion of wortmannin or LY294002 into the mPFC and application of HFS to the mPFC did not significantly alter the total protein levels of PI3K/Akt-mTOR signaling molecules ($F_{(3,20)} \leq 2.01$, all P s > 0.05 , data not shown). However, the results revealed that infusion of wortmannin (Fig. 3B) or LY294002 (data not shown) into the mPFC reduced the phosphorylation of the PI3K/Akt-mTOR signaling pathway. As shown in Figure 3B, in the vehicle-infused group, application of HFS resulted in significant increases of phosphorylated mTOR, p70S6k, and 4E-BP1 relative to the test stimulation ($F_{(1,9)} \geq 6.02$, all P s < 0.05). Infusion of 4 ng wortmannin of 1 μ L into the mPFC significantly reduced the levels of phosphorylated mTOR, p70S6k, and 4E-BP1 under the test stimulation ($F_{(1,8)} \geq 11.58$, all P s < 0.01), and the degree of reduction was up to 60%–75%, in-

dicating that infusion of 4 ng of wortmannin significantly reduced the basal phosphorylation levels of PI3K/Akt signaling molecules in the mPFC. In the wortmannin-infused group, application of HFS was able to significantly increase the protein levels of phosphorylated mTOR, p70S6k, and 4E-BP1 relative to the test stimulation ($F_{(1,11)} \geq 4.99$, all P s < 0.05), but these phosphorylated protein levels were still significantly depressed compared with those in the vehicle test stimulation group ($F_{(1,11)} \geq 5.05$, all P s < 0.05 , Fig. 3B).

Similarly, infusion of rapamycin, an inhibitor of mTOR, into the mPFC 30 min prior to the application of HFS significantly reduced the induction of LTP (Fig. 3C). Immediately after the HFS, the evoked fEPSPs slope was able to reach 150%–160% relative to the baseline, but the magnitude of the fEPSPs slope decayed with time and became stable ~ 1 h after the HFS. Three hours after the HFS, the magnitude of LTP of fEPSPs slope was only $\sim 115\%$ relative to the baseline (Fig. 3C). mPFC tissues were harvested 3 h after the HFS, and analysis of p70S6k and 4E-BP1, substrates of mTOR, revealed that total protein levels were not significantly changed by the drug infusion or the application of

HFS ($F_{(3,19)} \leq 2.52$, $P_s > 0.05$, data not shown), but phosphorylated protein levels were significantly reduced under the test stimulation ($F_{(1,8)} \geq 6.38$, both $P_s < 0.05$) and under the application of HFS ($F_{(1,10)} \geq 5.19$, both $P_s < 0.05$) in the rapamycin-infused group compared with those in the vehicle-infused group (Fig. 3D). All these results revealed that the magnitude of LTP and the basal and HFS-induced phosphorylation of mTOR, p70S6k, and 4E-BP1 were inhibited by the PI3K/Akt inhibitors or mTOR inhibitor, demonstrating that the PI3K/Akt-mTOR signaling pathway may be implicated in these processes.

PI3K/Akt-mTOR signaling pathway in the mPFC failed to involve object recognition memory

To investigate the role of the PI3K/Akt-mTOR signaling pathway in object recognition memory, wortmannin or LY294002 (distinct PI3K inhibitors) or rapamycin (an mTOR inhibitor) were infused into the mPFC immediately after the sample session of object recognition tasks, respectively. After a delay of 2 h and 24 h, short-term and long-term object recognition memory was tested. Four object recognition tasks, including novel object preference (Fig. 4A), object location (Fig. 4B), object in place (Fig. 4C), and temporal order (Fig. 4D), were used. During the sample sessions, the total time spent in the exploration of objects and the total amounts of time exploring either one of the objects were not significantly different among the vehicle-infused and

the inhibitor-infused groups ($P_s > 0.05$, data not shown). These results indicated that there was no biased exploratory preference in either infusion group, and the motivation, curiosity, and motor functions were balanced among the groups. During the test sessions of the four object recognition tasks, the total exploration time tested 2 h and 24 h after the sample sessions did not differ among the groups (data not shown), suggesting that infusion of PI3K/Akt inhibitor or mTOR inhibitor has no significant effects on the motor or sensory systems. Further analysis of exploration preference tested 2 h or 24 h after the sample sessions found no significant differences among the infusion groups in the four object recognition tasks (one-way ANOVA, all $P_s > 0.05$, Fig. 5A–D).

PI3K/Akt-mTOR signaling pathway in the mPFC is involved in long-term trace fear memory

Trace conditioning is a form of classical conditioning in which a time gap separates the CS from the US, the association of which has been shown to depend on mPFC activity (Runyan et al. 2004). Rats receiving 10 CS–US pairings with a 20-sec trace period and a pseudorandom intertrial interval of 1–4 min (Fig. 6A) were able to exhibit a gradual acquisition curve (Fig. 6B). In addition, previous studies have already shown that these conditioning training trials maximized CS–US association and minimized context–US association, and were allowed for long-term retention

testing at 72 h (Runyan and Dash 2004; Runyan et al. 2004). To determine whether the PI3K/Akt-mTOR signaling pathway in the mPFC is involved in the trace fear conditioning memory, rats were given an infusion of vehicle or PI3K inhibitors (wortmannin and LY294002) or mTOR inhibitor (rapamycin) into the mPFC immediately after trace fear conditioning training. Short-term and long-term trace fear memory was examined at 2 h, and 24 h, 3 d, and 6 d after the infusions, respectively. Retention of fear memory was assessed by monitoring freezing behavior during the trace period following tone presentation in the retention fear memory testing, and the percentage of time the animals remained frozen at each testing time point was averaged for four tone presentations. Contextual fear memory was assessed by the percentage of time the animals remained frozen during a 60-sec period without receiving the CS and the US in the contextual fear memory testing. Bilateral infusion of 4 ng wortmannin, 1.5 μ g of LY294002, or 7 ng of rapamycin of 1 μ L into the mPFC immediately after trace fear conditioning training failed to induce any significant alterations in the 2 h (Fig. 6C), 24 h (Fig. 6D), 3 d (Fig. 6E), and 6 d (Fig. 6F) contextual fear memory (all $F_{(3,33)} \leq 2.53$, all $P_s > 0.05$). No significant difference in 2 h ($F_{(3,33)} = 1.62$, Fig. 6C) or in 24 h ($F_{(3,33)} = 1.78$, Fig. 6D) retention fear memory was observed by infusion of these inhibitors (all $P_s > 0.05$). However, infusion of these inhibitors into the mPFC impaired 3 d ($F_{(3,33)} = 3.59$, $P < 0.05$, Fig. 6E) and 6 d

Figure 4. Diagram of the four object recognition memory tasks. (A) Novel object preference task; (B) object location task; (C) object in place task; (D) temporal order task.

Figure 5. Infusion of PI3K inhibitors (wortmannin and LY294002) or mTOR inhibitor (rapamycin) into rat mPFC did not effect short-term and long-term memories: (A) novel object preference (2 h testing, $F_{(3,28)} = 1.72$; 24 h testing, $F_{(3,28)} = 1.85$); (B) object location (2 h testing, $F_{(3,27)} = 1.65$; 24 h testing, $F_{(3,27)} = 1.69$); (C) object in place (2 h testing, $F_{(3,31)} = 1.71$; 24 h testing, $F_{(3,31)} = 1.73$); (D) temporal order tasks (2 h testing, $F_{(3,29)} = 1.95$; 24 h testing, $F_{(3,29)} = 2.01$). All P s > 0.05.

($F_{(3,33)} = 3.92$, $P < 0.05$, Fig. 6F) retention of fear memory, as indicated by decreased freezing behavior.

To ensure that the differences in retention of fear memory observed 3 d and 6 d after infusion of inhibitors were not caused by a general mPFC dysfunction, we examined the ability of the animals to acquire and remember the trace fear conditioning task 24 h after the 6-d retention and contextual fear memory testing. Animals were trained and tested for their ability to acquire the trace conditioning task; rats were able to acquire the task, as

indicated by increased freezing behavior during the trace periods (data not shown). These results indicated that the impairment in retention of fear memory was attributable to a disruption of memory storage rather than a generalized mPFC dysfunction.

To further determine whether the PI3K/Akt-mTOR signaling pathway is involved only in the mPFC-dependent trace fear conditioning memory, rats were given an infusion of vehicle or PI3K inhibitors (wortmannin and LY294002) or mTOR inhibitor (rapamycin) into the mPFC immediately after delay fear conditioning, pseudorandom (unpaired), US-alone, and CS-alone fear conditioning training, respectively. Short-term and long-term fear memory was also examined. In these four fear conditioning training groups, contextual fear memory was also assessed by the percentage of time the rats remained frozen during a 60-sec period in the contextual fear memory testing. Retention of fear memory was assessed by monitoring freezing behavior during the period of 20 sec (the same time period as that in the trace fear conditioning testing) following the tone presentation and averaging the results. In the delay fear conditioning training experiments, four instead of 10 CS-US pairings were used to reduce the level of conditioned freezing, keeping the retention of delay fear memory identical to that of trace fear memory. As shown in Figure 7A, infusion of PI3K inhibitors or mTOR inhibitor did not induce any significant alterations relative to the vehicle-infused group either in the contextual fear memory ($F_{(3,26)} \leq 1.65$) or in the retention of fear memory ($F_{(3,26)} \leq 1.52$, all P s > 0.10), indicating that the lack of an effect of these inhibitors on delay fear memory was not due to a ceiling effect. Likewise, infusion of inhibitors did not result in any significant alteration in the pseudorandom (Fig. 7B), US-alone (Fig. 7C), and CS-alone (Fig. 7D) contextual fear memory and retention of fear memory ($F_{(3,24)} \leq 2.15$, all P s > 0.05). These results revealed that although four control fear conditioning training paradigms elicited different freezing levels in the contextual and retention of fear memory testing, a null effect of inhibitors was found. These results, together with those from the trace fear conditioning paradigm, suggested that the role of the PI3K/Akt-mTOR signaling pathway in the mPFC was restricted to the long-term retention of trace fear memory.

Discussion

To identify that a signaling pathway is important for the induction of LTP, at least two criteria have to be fulfilled. First, electrophysiological stimulation that induces LTP should be able to activate a biochemical cascade. Second, this biochemical cascade has to be necessary for the induction of LTP, and blockade within this cascade should interrupt the LTP induction. In this study, we provided evidence that the PI3K/Akt-mTOR signaling pathway meets these two criteria of a signaling pathway needed for the induction of LTP in the mPFC. On one hand, LTP induction by HFS in the mPFC was accompanied with an activation of phosphorylation of Akt at Ser473, mTOR, p70S6k, and 4E-BP1. The control group that received the test stimulation did not reveal any significant increase in the phosphorylation of these protein kinases, indicating that the activation of the PI3K/Akt-mTOR signaling pathway is specific to the induction of LTP. On the other hand, infusion of inhibitors of the PI3K/Akt-mTOR signaling pathway, such as wortmannin, LY294002, and rapamycin, into the mPFC significantly reduced the magnitude of LTP. The inhibition of LTP induction was in parallel to that of PI3K/Akt signaling downstream targets, mTOR, p70S6k, and 4E-BP1. In addition, long-term retention of trace fear memory was impaired by infusion of PI3K or mTOR inhibitors into the mPFC, suggesting that the PI3K/Akt-mTOR signaling pathway is also involved in the trace fear conditioning memory tasks.

Figure 6. Experimental protocol for trace fear conditioning training and testing and effects of infusion of PI3K inhibitors (wortmannin and LY294002) or mTOR inhibitor (rapamycin) into the mPFC on the short-term and long-term retention of trace fear memory and contextual fear memory. (A) The exact sequences for (a) trace fear conditioning training, (b) context testing, (c) retention testing, and (d) the whole experimental design. (CS) Conditioned auditory stimulus, (US) unconditioned stimulus. (B) Freezing behavior expressed as a percentage during acquisition. (C) Freezing behavior calculated 2 h after the post-training infusion of vehicle or inhibitors into the mPFC during the retention and context testing. (D) Freezing behavior calculated 24 h after the post-training infusion during the retention and context testing. (E) Freezing behavior calculated 3 d after the post-training infusion during the retention and context testing. (F) Freezing behavior calculated 6 d after the post-training infusion during the retention and context testing. (*) Significant difference compared with the vehicle group ($P < 0.05$).

In this study, the cross-commisural pathway between the mPFC was capable of expressing PPF and LTP of synaptic transmission as measured by either fEPSPs slope or fEPSPs amplitude. The clear and stable fEPSPs recorded in our experiments provided a reliable basis for the long-lasting analysis of synaptic plasticity for periods of up to 3 h. Our results were comparable to those of previous reports (Gemmell and O'Mara 2000; Roder et al. 2003), but a difference in the protocol for the induction of LTP resulted in a slight difference in the magnitude of LTP. In our study, a persistent increase of fEPSPs slope or fEPSPs amplitude (~160% relative to the baseline) was induced in the mPFC in vivo.

PI3K/Akt is known to be involved in multiple aspects of neuronal development and physiology, including differentiation, polarization, growth, survival, cytoskeletal reorganization, regulated secretion, and receptor trafficking (Rodgers and Theibert 2002). For example, in cultured hippocampal neurons, PI3K/Akt was reported to promote neurite initiation, outgrowth, and stability of growth cones (Atwal et al. 2000, 2003; Sanchez et al. 2001; Dijkhuizen and Ghosh 2005), as well as the growth and branching of dendrites (Jaworski et al. 2005). How might PI3K/Akt be involved in the induction of LTP? Opazo et al. (2003) have summarized three possible functions for PI3K/Akt in LTP induction in the hippocampus. First, PI3K/Akt can activate an atypical isoform of protein kinase C (PKC), PKC ζ (Chou et al. 1998; Le Good et al. 1998), which plays an important role in the induction of LTP in the hippocampus (Sacktor et al. 1993; Ling et al. 2002). Second, PI3K/Akt is involved in the trafficking and insertion of AMPA-type glutamate receptors (Passafaro et al. 2001), which are thought to be one of the mechanisms underlying LTP (Malinow and Malenka 2002). Third, PI3K/Akt has an important role in changes in dendritic spine structure induced by activation of synaptic NMDA receptors (Yuste and Bonhoeffer 2001). PI3K/Akt activity provides an essential link between NMDA receptors and extracellular signal-regulated protein kinase (ERK) (Opazo et al. 2003), which is required for LTP induction. These same possible mechanisms might explain the involvement of PI3K/Akt in the induction of LTP in the mPFC of the present study. However, recent studies have demonstrated that PI3K/Akt is a key signaling intermediate downstream of the dopamine receptor (Beaulieu et al. 2005), which is thought to be involved in LTP induction in the mPFC (Sun et al.

2005; Chen et al. 2007). Thus, the important functions of PI3K/Akt in normal dopaminergic synaptic transmission (Emamian et al. 2004; Beaulieu et al. 2005) may also contribute to the role of PI3K/Akt in the LTP of mPFC.

Previous studies of the hippocampus have shown that PI3K activity contributes to the induction of LTP rather than its maintenance or expression because PI3K inhibitors applied after LTP induction do not block LTP (Opazo et al. 2003). However, other studies have suggested that the expression of LTP is also dependent on persistent PI3K activity (Kelly and Lynch 2000; Sanna et al. 2002). In the present study, the role of PI3K/Akt in the induction of LTP or in the expression and maintenance of LTP cannot be distinguished because the PI3K inhibitors, wortmannin and LY294002, were both infused 30 min before the application of LTP-inducing HFS *in vivo*, and the processes of induction and maintenance of LTP occurred in the presence of their actions.

It is known that mTOR regulates the translation initiation complex in a rapamycin-sensitive manner. p70S6k, one downstream target of mTOR (Burnett et al. 1998), increases translational capacity by promoting the expression of several members of the translational machinery whose mRNAs display oligopyrimidine tracts at their 5' ends (Jefferies et al. 1997). 4E-BP1, another downstream target of mTOR (Burnett et al. 1998), is an inhibitor of the cap binding protein eukaryotic initiation factor 4E (eIF4E). Phosphorylation of 4E-BP1 inhibits the repression of eIF4E and thus increases translation of capped mRNAs (Beretta et al. 1996). In response to synaptic activity, phosphorylated mTOR, p70S6k, and 4E-BP1 accumulated in dendrites (Tang et al. 2002; Cammalleri et al. 2003), thus regulating protein synthesis. Protein synthesis in dendrites has been recognized as an important process for the induction of the late phase of LTP (Bhalla and Iyengar 1999), which might contribute to rapamycin, a specific inhibitor of mTOR, reducing LTP induction in the present study. Infusion of rapamycin into the mPFC *in vivo* was performed during the process of the LTP induction, and whether mTOR also plays an important role in the establishment of LTP in the mPFC was not tested in the present study. Previous study on the hippocampus has shown that establishment of the late phase of LTP was not sensitive to mTOR because disruption of this signaling pathway with rapamycin was unable to inhibit the expression of enduring LTP (Cammalleri et al. 2003).

In the present study, infusion of wortmannin, LY294002 (data not shown), or rapamycin inhibited the magnitude of LTP in the mPFC, which was consistent with the previous results

obtained from the hippocampus (Tang et al. 2002; Cammalleri et al. 2003; Opazo et al. 2003). Infusion of 4 ng of wortmannin, 1.5 μ g of LY294002, or 7 ng of rapamycin into the mPFC with an estimated volume of 100 μ L results in an equilibrium concentration of $\sim$ 100 nM, $\sim$ 50 μ M, and $\sim$ 80 nM, respectively, which was comparable to previous studies performed in the hippocampus *in vivo* (Barros et al. 2001; Dash et al. 2002; Bekinschtein et al. 2007). Although the concentration proximal to the infusion site is likely to be higher, 4 ng of wortmannin, 1.5 μ g of LY294002, or 7 ng of rapamycin infusing into the mPFC was able to significantly reduce the phosphorylation of PI3K/Akt downstream tar-

Figure 7. Null effects of infusion of PI3K inhibitors (wortmannin and LY294002) or mTOR inhibitor (rapamycin) into the mPFC on the short-term and long-term fear memory in (A) delay, (B) pseudo-random, (C) US-alone, (D) or CS-alone fear conditioning retention and context testing. (CS) Conditioned auditory stimulus, (US) unconditioned stimulus.

gets (mTOR, p70S6k, and 4E-BP1). Thus, infusion of these inhibitors into the mPFC used in the present study can be accepted as a protocol to elucidate the biological functions of PI3K/Akt in the LTP induction in the mPFC *in vivo*. The partial inhibition of LTP in the mPFC by PI3K inhibitors or mTOR inhibitor most likely indicates that PI3K/Akt-mTOR may be part of important modulatory pathways involved in LTP induction. Some mechanisms underlying LTP induction may arise from an PI3K/Akt-mTOR-independent signaling pathway and cannot be ruled out.

It should be addressed that our data may not be totally consistent with those of other studies. For example, in our study, during the process of LTP induction in the mPFC, phosphorylation of Akt 473 increased as early as 5 min and was sustained 3 h after the LTP-inducing HFS, while phosphorylation of Akt 308 was kept intact, which was consistent with the recent study also showing that the PI3K substrate Akt at Ser473 but not at Thr308 was increased after LTP-inducing tetanus stimulation (Horwood et al. 2006). However, in Lin's study, phosphorylation of Akt was observed at 10 min and 30 min, but not 60 min after LTP-inducing tetanic stimulation in the amygdala (Lin et al. 2001); and in Tsokas's study, immunoreactivity to Akt 308 increased more than twofold in response to HFS in homogenates of excised area CA1 (Tsokas et al. 2007). In our study, significant increases in phosphorylation of 4E-BP1 were observed at 1 h and 3 h after LTP-inducing HFS, whereas a rapid (5 min) and sustained (60 min) phosphorylation of 4E-BP1 was produced by an LTP-inducing acute theta burst stimulation in the mouse area CA1 region (Schmitt et al. 2005). The reason for the differences in the time course of activation, which substrates were activated, and the levels of phosphorylation were unclear, but might be attributable to species differences (rat vs. mouse), brain regions (mPFC vs. amygdala, hippocampus), and LTP-inducing stimulation patterns (HFS vs. theta burst stimulation).

The role of the PI3K-mTOR signaling pathway in memory formation has been explored in the hippocampus. Although dissociations between the mechanisms underlying LTP and long-term memory have been observed (Jun et al. 1998), most studies suggest that PI3K (Dash et al. 2002, 2004) and mTOR (Myskiw et al. 2008) activity may facilitate long-term hippocampus-dependent memory. Intrahippocampal administration of the PI3K inhibitor LY294002 impaired acquisition, short-term memory, long-term memory, and the retrieval of long-term memory in a step-down avoidance task (Barros et al. 2001). LY294002 infused *i.c.v.* *in vivo* impaired long-term consolidation of recognition memory but not short-term recognition memory or spatial learning (Horwood et al. 2006). Bilateral intra-CA1 infusion of rapamycin completely hindered one-trial inhibitory avoidance long-term memory without affecting short-term memory retention (Bekinschtein et al. 2007). Likewise, in the present study, the long-term trace fear memory, which is dependent on the functions of mPFC (Baeg et al. 2001; Runyan and Dash 2004; Runyan et al. 2004), was impaired by infusion of PI3K inhibitors (wortmannin and LY294002) or mTOR inhibitors (rapamycin) into the mPFC immediately after trace fear conditioning training. However, the deficits in retention of long-term fear memory were observed only at 3 d and 6 d, but not 24 h, after the training in this study. The reason for these delayed deficits was unclear, but ceiling-level performance with the robust levels of freezing (80%–100%) might contribute to the null effects of inhibitors on 24-h retention of fear memory. The trace training procedure of 10 CS–trace–US association with 1 mA foot shock (US) used in this study might maximize the levels of conditioned freezing; thus, this fear conditioning paradigm may not be sensitive enough to detect modest alterations in 24-h fear memory retention. In our previous study (Sui et al. 2006), only three CS–trace–US associations were adopted in the trace fear

conditioning paradigms, and the control level of 24-h conditioned freezing was only 40%–60%, comparable to the reported levels in rats. Another interpretation of the delayed retention deficits in the present study could be that infusion of these inhibitors affected extinction-related processes that may have been initiated during the 2-h retention tests. In this study, the same rats that received trace fear conditioning training were examined at each retention test; thus, extinction learning likely is occurring with each nonreinforced CS exposure.

In this study, infusion of these inhibitors into rat mPFC did not affect long-term object recognition memory. These results were unexpected because the object in place and the temporal order tasks depended on the functions of mPFC (Hannesson et al. 2004; Dere et al. 2007), and if PI3K-mTOR signaling is involved in the mPFC memory formation, infusion of these inhibitors should interfere with the mPFC-dependent object recognition memory tasks. The reasons why inhibition of the PI3K/Akt-mTOR signaling pathway did not impair the mPFC-dependent long-term object recognition memory in this study were unknown. One possibility is that in the vehicle-infused group, exploration preference 24 h after training, representing long-term object recognition memory, was only ~60%, approaching the level of the random exploration ratio (50%). Under this situation, exploration preference may not be a sensitive measurement for the long-term object recognition memory, and it was hard to obtain any significant differences if the number of subjects was limited. Repeated training may overcome this decline of memory consolidation in the recognition memory tasks (Horwood et al. 2006), but further experiments are needed. Another possibility is that the PI3K/Akt-mTOR signaling pathway may not be important for mPFC-dependent object recognition memory, even though a recent study has demonstrated that mTOR activity is required for consolidation of object recognition memory in the dorsal hippocampus (Myskiw et al. 2008). Some mechanisms, other than the PI3K/Akt-mTOR signaling pathway, may be necessary for mPFC-dependent object recognition memory, such as protein synthesis and ERK signaling (Nagai et al. 2007).

In this study, our electrophysiological and neurobehavioral data have a certain degree of similarity to those performed on the hippocampus, which was not surprising because the molecular pathways regulating LTP are largely shared between the prefrontal cortex and the hippocampus (Huang et al. 2004). Although our data demonstrated that the PI3K/Akt-mTOR signaling pathway is implicated in mPFC LTP and mPFC-dependent long-term trace fear memory, multiple regulatory processes at the transcriptional, translational, and posttranslational levels may be simultaneously active in LTP, and in learning and memory tasks. For instance, an LTP-inducing stimulus is able to induce activation of ERK, and changes in ERK activity affect mTOR directly; thus, phosphorylation of p70S6k was then followed without any increase in PI3K/Akt activity (Ma et al. 2005; Tsokas et al. 2007). Therefore, a future challenge is to understand how multiple regulatory mechanisms are coordinated by upstream signaling molecules.

In summary, we have used an *in vivo* animal model to characterize certain features of synaptic plasticity in the mPFC. Although *in vitro* preparations offer high experimental tractability, particularly for the study of the underlying mechanisms of LTP, *in vivo* studies address the impact of LTP at the systems level (*i.e.*, its effect on the activity of a large number of neurons). Systems-level analyses are also very important because cognitive functions, including learning and memory, are known to be associated with the activation of large populations of neurons. In this study, we provide evidence showing that the PI3K/Akt-mTOR signaling pathway is involved in the mPFC LTP and mPFC-dependent long-term trace fear memory.

Materials and Methods

Subjects, reagents, and drug preparation

Adult male Sprague-Dawley rats weighting 180–220 g were supplied by the Experimental Animal Center, Shanghai Medical School, Fudan University, China. All animal procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved and monitored by the Ethical Committee of Animal Experiments at the Institute of Neurobiology and the Institutes of Brain Science, Fudan University, China. Animals were maintained on a 12-h light–dark cycle and in a temperature-controlled ($24^{\circ}\text{C} \pm 1^{\circ}\text{C}$) environment with access to food and water *ad libitum*.

All primary antibodies: total Akt antibody (catalog no. 9272), phospho-serine (Ser)473 Akt antibody (catalog no. 9271), phospho-threonine (Thr)308 Akt antibody (catalog no. 9275), total mTOR antibody (catalog no. 2972), phospho-Ser2448 mTOR antibody (catalog no. 2971), p70S6 kinase antibody (catalog no. 9202), phospho-p70S6 kinase antibody (catalog no. 9206), 4E-BP1 antibody (catalog no. 9452), phospho-4E-BP1 antibody (catalog no. 9451), the PI3K inhibitors (wortmannin and LY294002), and a specific inhibitor of mTOR (rapamycin) were purchased from Cell Signaling Technology, Inc. The horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG, enhanced chemiluminescence (ECL) Western Blotting detection reagents and Restore Western Blotting stripping buffer were obtained from Pierce Biotechnology. Protease inhibitor cocktail and polyvinylidene difluoride (PVDF) membrane were obtained from Roche Products. All other reagents were of analytical grade and from Sigma Chemical.

All drugs for intramedial prefrontal cortex (mPFC) infusion were applied by dissolving them to the desired final concentrations in artificial cerebrospinal fluid (aCSF, the composition of the aCSF solution was 117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 25 mM NaHCO_3 , 1.2 mM NaH_2PO_4 , and 11 mM glucose, pH 7.3–7.4). Appropriate stock solutions of drugs were made and diluted with aCSF just before application. Drugs, including wortmannin, LY294002, and rapamycin, were dissolved in dimethyl sulfoxide (DMSO) stock solution and stored at -20°C until the day of the experiment. The concentration of DMSO in the aCSF was 0.05%.

Electrophysiological recordings

A total of 14 adult rats were anesthetized with urethane (1.25 g/kg, *i.p.*) and mounted on a stereotaxic frame (SN-2; Narishige, Japan). Small holes were drilled in the skull to allow insertion of electrodes in the brain. The stereotaxic coordinates (bregma and lambda in the same horizontal plane) were derived from Paxinos and Watson (1986). A concentric bipolar stainless-steel stimulating electrode with a tip separation of 0.5 mm, and a monopolar stainless-steel recording electrode, 0.1 mm in diameter, was placed into the coordinates (2.7–3.2 mm anterior to bregma; 0.7–1.0 mm lateral to sagittal line; 3.0–3.7 mm ventral to the cortical surface) in the contralateral hemispheres, respectively (Fig. 8A). Electrode positions were optimized to record maximal field excitatory postsynaptic potentials (fEPSPs). After placement of the electrodes, rats were left for a minimum period of 30 min for stability of baseline recordings. Evoked field responses were amplified and monitored with an oscilloscope (Nihon Kohden, VC-10), and sampled by a personal computer. Two animals with unstable baseline responses were excluded from further experimentation. Stimulation of the prefrontal cortex in the contralateral hemisphere *in vivo* evokes a characteristic fEPSP, which was a triphasic waveform, consisting of an initial positive deflection followed by a trough followed by a second positive deflection (Gemmell and O'Mara 2000; Roder et al. 2003). The slope and the amplitude of the evoked fEPSPs were measured. The fEPSPs slope was calculated as the rate of amplitude change for the initial fEPSPs positive deflection to the negative peak, and the fEPSPs amplitude was estimated by calculating the voltage difference between the most negative and the most positive peak of the fEPSPs.

To examine basal synaptic transmission, an input–output

Figure 8. Coronal sections showing (A) a representative placement of a stimulating electrode (thick vertical line in one hemisphere) and of several recording sites (black dots in the contralateral hemisphere) in the mPFC in the electrophysiological recordings; (B) the location of the injection needles (black dots) in the mPFC in the neurobehavioral testing. All electrodes and injection needles were located in the prelimbic cortex. (Acg) Anterior cingulate cortex, (PL) prelimbic cortex, (IL) infralimbic cortex. Drawings based on Paxinos and Watson (1986).

function for each animal was determined by delivering an ascending series of eight stimulus intensities (50–750 μA), ranging from subthreshold for elicitation of a field response to those eliciting maximal responses. Six evoked field potentials were averaged at each stimulus's intensity. For analysis of input–output functions, response was normalized to the percentage of maximal fEPSPs slope or amplitude. After collection of the input–output function was completed, paired-pulse facilitation (PPF) of the field response was measured at 10 interpulse intervals (20–700 msec), with the stimulus intensity that produced a 50%–60% maximal response. Six stimulus pairs delivered at 10-sec intervals were averaged at each interpulse interval. PPF was expressed as the ratio of the mean slope or amplitude of the second response to the first one (pulse 2/pulse 1 $\times$ 100). For induction of long-term potentiation (LTP), a probe stimulus was chosen, with intensity sufficient to produce a field response ~50%–60% of maximal response. The baseline responses to the probe stimulus (two per min) were recorded for 15 min. Thereafter, high-frequency stimulations (HFS), consisting of 20 trains of 150 pulses at 250 Hz taken 10 sec apart, were delivered with stimulus intensity the same as the baseline probe pulse. After high-frequency stimula-

tion was delivered, single-pulse recording resumed for 180 min. LTP was expressed as the percent change from the mean of 10 pre-tetanus baseline recordings taken just before the high-frequency stimulation was delivered.

For time course experiments of testing PI3K/Akt signaling molecules during LTP induction, a different set of rats was used. Rats were killed by decapitation at different indicated times, the brains were removed, the mPFC dissected on ice. mPFC tissues of both hemispheres were pooled together, immediately frozen by liquid nitrogen, and stored at -80°C until protein extracts were prepared. Six rats were used at each time point in the test stimulation group and in the HFS-inducing groups, respectively. Rats in the test stimulation group were continuously exposed to the test stimulation, without receiving HFS, for an equivalent amount of time as that in the HFS-inducing group.

Intra-mPFC infusion for electrophysiological recordings in vivo

Rats were also anesthetized with urethane and placed in a stereotaxic frame (SN-2). Two small holes which were bilaterally located at 2.7–3.2 mm anterior to bregma; 0.7–1.0 mm lateral to sagittal line; and 3.0–3.7 mm ventral to the cortical surface were drilled on the skull for insertion of a stimulating electrode and an infusion-recording combined electrode, respectively (Fig. 8A). The infusion-recording combined electrode was manually constructed by gluing a Teflon-coated monopolar stainless-steel electrode (for field-potential recording) to a guide cannula (400 μm in inner diameter and 650 μm in outer diameter, for infusion of drug solution). The injection needle was inserted into the guide cannula and was extended 0.5 mm beyond the tip of the guide cannula. The distance between the tip of the injection needle and that of the recording electrode was 0.3–0.5 mm.

For intra-mPFC drug infusion electrophysiological recording studies, 44 rats, excluding three with unstable baseline responses, were examined. Optimal fEPSPs were first established and input-output function and PPF were monitored. Then, inhibitors, including 4 ng wortmannin, 1.5 μg LY294002, 7 ng rapamycin, or vehicle (0.05% DMSO in the aCSF, as the control group) of 1 μL were infused into the mPFC at a rate of 0.25 $\mu\text{L}/\text{min}$ by a motorized microinfusion pump (ALZA Pharmaceuticals), and the injection needle was left in place throughout subsequent recordings. At the same time, fEPSPs were evoked and monitored continuously for 30 min. After that, a second input-output function and PPF were examined, and LTP induction experiments were carried out. After the whole experiment, the mPFC tissues were dissected and frozen for protein extracts.

Western blotting

mPFC tissues were homogenized with ice-cold lysis buffer containing 1% Triton X-100, 5 mM EDTA, 10 mM Tris, 1% SDS, 1% NP-40 (nonidet P-40), 10 mM deoxycholate, 60 mM CHAPS, 1 mM NaF, 1 mM Na_3VO_4 , 1 mM PMSF, and complete protease inhibitor cocktail for 30 min. Samples were sonicated and spun down at 15,000g for 10 min at 4°C . Protein concentrations were determined by a BCA protein assay. The supernatant protein samples were boiled for 3 min at 100°C in loading buffer containing 10% SDS, 1 M Tris-HCl (pH 6.8), 20% glycerol, 0.2 M dithiothreitol (DTT), and 0.02% bromophenol blue. Equal protein extracts were then separated by denaturing SDS-polyacrylamide gels on a 10% resolving gel with a 4% stacking gel and transferred onto a PVDF membrane. The PVDF membranes were blocked in 5% nonfat dry milk for 2 h at room temperature in Tris-buffered saline containing Tween 20 (TBST, 0.1% Tween 20, 50 mM Tris-HCl pH 7.5, 150 mM NaCl). The membranes were then hybridized overnight with primary antibodies in primary buffer (5% bovine serum albumin in TBST). This was followed by three washes in TBST and incubation for 2 h with a secondary horseradish peroxidase-conjugated goat antibody diluted 1:2000 in primary buffer. The membranes were washed in TBST and blots were developed using ECL and exposed to X-ray film. Multiple exposures of each membrane were taken to ensure the linearity of the immunoreactive bands. For reuse of membranes that had

been Western blotted, the blots then were incubated in Restore Western blot stripping buffer for 1 h followed by incubation in TBST for 30 min. The stripped blots were incubated with an antibody directed against total levels of the respective protein. For the detection of phospho- and total levels of the proteins, the dilution of all the antibodies was 1:1000. Densitometric analysis of phospho- and total immunoreactivity for each protein was conducted using Quantity One software (Bio-Rad). Phosphorylated immunoreactivity was normalized to total immunoreactivity for each kinase.

Surgery and intra-mPFC infusion for neurobehavioral training and testing

The surgical procedures were performed under aseptic conditions under anesthesia (sodium pentobarbital, 40 mg/kg, i.p.). Stainless steel guide cannulae (22-gauge) were implanted bilaterally into the mPFC (2.7–3.2 mm anterior to bregma; 0.7–1.0 mm lateral to sagittal line; 1.5–2.2 mm ventral to the cortical surface) with a stereotaxic device (SN-2). A dummy cannula was placed in the guide cannula during non-use. Animals were given a 7- to 10-d rest period after surgery before behavioral experiments. Stylettes that extended 1.5 mm past the end of the cannula were used for drug infusions (Fig. 8B). Rats that displayed signs of distress such as weight loss or apparent behavioral abnormalities were excluded from the experiments.

For the post-training intra-mPFC infusion experiment, the animals first were trained, and then 1 μL of 4 ng of wortmannin, 1.5 μg of LY294002, 7 ng of rapamycin, or vehicle (0.05% DMSO in the aCSF, as the control group) prepared as mentioned above was infused bilaterally into the mPFC immediately after training. All infusions were performed in freely moving animals at a rate of 0.25 $\mu\text{L}/\text{min}$ by a motorized microinfusion pump (ALZA Pharmaceuticals). After all infusions, the needles were left in place for 2 min to allow for diffusion of the drug, and the sterilized dummy cannula was replaced. Animals were allowed to recover from the infusion in their home cages.

Object recognition

Apparatus and behavioral measures

The object recognition tasks were performed according to the protocol reported (Hannesson et al. 2004; Barker et al. 2007). A square open-field box (50 $\times$ 90 $\times$ 100 cm) was constructed from plastic and was surrounded with a black cloth to a height of 1.5 m so that no external stimuli could be seen during the experiment (the black cloth was removed for the object-in-place and object location tasks). An overhead camera and a video recorder were used to monitor and record the animal's behavior for subsequent analysis. The stimuli presented were copies of objects that varied in shape, color, and size (9 $\times$ 8 $\times$ 7 cm to 25 $\times$ 15 $\times$ 10 cm) and were too heavy for the animal to remove. All objects were balanced in terms of physical complexity and were emotionally neutral. Before training, the rats were handled for 1 wk, and then were habituated to the open-field box without stimuli for 30 min daily for 2 d. Moreover, the open-field box and objects were thoroughly cleaned by 70% alcohol after each session to avoid possible instinctive odorant cues. For object recognition tasks training and testing, a total of 135 rats received surgeries, and each animal received only one type of training and testing, and only one type of drug infusion.

Exploring the object was considered positive when the head of the animal was facing within 2 cm of the object or any part of the body except the tail was touching the object. The time spent exploring each object was recorded.

Novel object preference task

The procedure comprised an acquisition or sample phase, followed by a preference test after a delay of 2 h and 24 h (Fig. 4A). In the sample phase, duplicate copies of an object were placed near the two corners of the open-field box (15 cm from the wall). The rat was then placed into the box facing the wall and allowed a total exploration of 5 min in the arena. For the test (3 min

duration), the animal was replaced into the box and presented with two objects in the same positions: One was the same object used in the sample phase, and the other was a novel object. The position of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals.

Object location task

In this task, the rat's ability to recognize that an object it had experienced before had changed location was assessed. In the sample phase, the rat was exposed to objects that were placed near the two corners of the open-field box (Fig. 4B) and was allowed a total exploration of 5 min. After a delay of 2 h and 24 h, the test phase (3 min duration) began. Two objects in the test phase were equally familiar, but one remained in the same position as the previous one in the sample phase, and the other was moved to a new position. The position of the moved object was counterbalanced between the rats.

Object in place task

This task also comprised a sample phase and a test phase separated by delays of 2 h and 24 h (Fig. 4C). In the sample phase, the rats were presented with four different objects, which were placed in the corners of the open-field box 15 cm from the walls. Each rat was placed in the center of the arena and allowed to explore the objects for 5 min. In the test phase, two of the objects, which were both on the left or right of the open-field box exchanged positions, and the animals were allowed to explore the objects for 3 min. The objects moved, and the position of the objects in the sample phase was counterbalanced between the rats. If object-in-place memory is intact, the subject will spend more time exploring the two objects that are in different locations compared with the two objects that are in the same locations.

Temporal order task

This task comprised two sample phases and one test phase (Fig. 4D). In each sample phase, the rats were allowed to explore two copies of an identical object for a total of 5 min. Different objects were used for two sample phases, with a delay between the sample phases of 2 h. The test phase (3 min duration) was given 2 h and 24 h after the second sample phase. In the test phase, one object from the first sample phase and the other object from the second sample phase were used. The position of the objects in the test phase and the objects used in two sample phases were counterbalanced between the animals. If temporal order memory is intact, the subjects will spend more time exploring the object from the first sample phase compared with the object from the second sample phase.

Trace fear conditioning

Apparatus

The trace fear conditioning experiments were performed in two fear conditioning chambers (chambers A and B) connected to a computerized freezing monitor system (San Diego Instruments). The fear conditioning context chamber (chamber A) was a transparent Plexiglas cage (36 × 23 × 18 cm) and a floor lined with 14 stainless steel rods (0.5 cm in diameter, 1.2 cm spacing) that can be electrified by a shock generator (ShockStim, San Diego Instruments). Chamber A was placed within a constantly illuminated dark gray environment. The shock steel rods and the pan underneath the rod floor were cleaned with 70% alcohol before each individual rat was placed into the chamber. The fear conditioning cue chamber (chamber B) was an identically sized cage with a Plexiglas panel that was opaque on four sides and a plain floor (no shock rods) in a white surrounding with the lights in the experimental room always on. The floor and the pan underneath the floor of chamber B were cleaned with 1% acetic acid to ensure that chamber B was different from chamber A. The infrared photo beams along the sides of the chambers, which were linked to a computer for assessment of the freezing response,

automatically monitored the behavior of the rats. Freezing response was recorded during conditioning training and testing. Freezing behavior was measured at 2-sec intervals. Freezing behavior is defined as the absence of all movements, excluding movement caused by respiration and heartbeat.

Trace fear conditioning training

The experimental protocol was adopted from Runyan's studies with a slight modification (Runyan and Dash 2004; Runyan et al. 2004). Briefly, on the conditioning day, rats were brought into a holding room and allowed to sit undisturbed in their home cages for 30 min. For the trace fear conditioning training, rats were placed in the context chamber (chamber A) and given a 120-sec habituation period. Conditioning trials began with a 10-sec tone (CS, 96 dB, 2200 Hz, 5 msec rise/fall time), followed by a 20-sec trace period before the animal received a 0.5-sec (1-mA) foot shock (US). Then, a pseudorandom intertrial interval (ITI) that varied between 1 and 4 min was followed. Conditioning trials were repeated 10 times to strengthen the CS–trace–US association. After the final trial, the rats were returned to their home cages. Delay fear conditioning training, pseudorandom (unpaired), US-alone, and CS-alone fear conditioning were used for experimental control groups. For delay fear conditioning training, another set of rats received the delay conditioning, which consisted of four 10-sec tone and 0.5-sec (1-mA) foot shock pairings in which the tone and the foot shock co-terminated, separated by a pseudorandom ITI that varied between 1 and 4 min. For pseudorandom conditioning, the chamber, the CS, the US, and the trace period were the same as those used in trace fear conditioning. Pseudorandom fear conditioning trials consisted of the same number of tone and foot shock exposures as the trace fear conditioning group within the same time frame. However, the tone and the foot shock were presented in a pseudorandom manner so that the offset of the tone could not be used as a predictor of the foot shock onset. This was achieved by varying the order as well as the time interval separating the two stimuli. After the final trial, the rats were returned to their home cages. The chambers were also cleaned by 70% alcohol, which is the same as that used in the trace fear conditioning between the conditioning of each rat. The US-alone group was exposed to the same protocol as the trace fear conditioning group minus the tone (CS). The CS-alone group was exposed to the same protocol as the trace fear conditioning group minus the foot shock (US). A different set of 157 rats was under the surgical procedure and received drug infusion for trace, delay, pseudorandom (unpaired), US-alone, and CS-alone fear conditioning training and testing. Each animal received only one kind of fear memory training, and only one drug infusion.

Contextual fear memory testing

Contextual fear memory was measured 2 h, 24 h, 3 d, and 6 d after training by scoring the animals' freezing behavior to contextual cues only. The trained rats were placed back into the original training context (chamber A) for a 60-sec period without receiving the CS or the US. Furthermore, the context was identical to the training conditions.

Retention fear memory testing

Two hours after the contextual fear memory testing, the trained animals were tested for a freezing response to the tone CS only. For the retention fear memory testing, the animal was placed in a novel context (chamber B) and given a 120-sec habituation period. In the absence of foot shock, a 10-sec tone was given, followed by a varied ITI between 1 and 4 min, which was repeated a total of four times.

Histology

After the electrophysiological recording and all behavioral testing, animals were overdosed with sodium pentobarbital (100 mg/kg) and perfused transcardially with saline (0.9% NaCl) followed by a 4% paraformaldehyde solution. Brains were removed and

postfixed in 4% paraformaldehyde for a minimum of 2 h before being transferred to 30% sucrose in 0.2 M phosphate buffer and left for 48 h. Coronal brain sections were used to verify the precise target sites (Fig. 1). Every tenth coronal section (50- μ m thick) through the mPFC was mounted on gelatin-coated slides and stained with neural red. Rats that had received proper insertion of electrodes and proper bilateral infusions into the mPFC were accepted for data analysis.

Statistical analysis

All data were expressed as mean $\pm$ standard error of mean (S.E.M.). Input-output functions, PPF data, and LTP data were subjected to repeated measures analysis of variance (ANOVA). Where significant interactions were found, step-down ANOVAs and mean contrast tests using Tukey's *t*-test were performed. For Western blotting experiments, the "*n*" used for statistical analysis was the number of animals. Because the *F* test demonstrated a significant nonhomogeneous variance between groups, a Kruskal-Wallis one-way nonparametric ANOVA test was selected for multiple statistical comparisons. The Mann-Whitney *U*-test was used to determine significant differences between two independent groups.

For the object recognition task, any subjects that failed to complete a minimum of 15 sec of exploration in the sample phase or 10 sec of exploration in the test phase (a total of four) were excluded from the analysis. A preference index was used to measure recognition memory. In the novel object preference task, the object location task, and the temporal order task, preference index was calculated as the ratio of the amount of time spent exploring any one of the two items (sample period) or the novel object (test period) over the total time spent exploring both objects. In the object in place task, preference index was calculated as the ratio of the time spent exploring the two objects that had changed position or the time spent exploring the two objects that had remained in the same position over the total time spent exploring the four objects. Previous systematic studies of discrimination performance in a range of object recognition tests have demonstrated that the data obtained from the first 1 min were the most sensitive measure of recognition memory (Dix and Aggleton 1999; Bussey et al. 2000; Barker et al. 2007). Therefore, in the present study, we compared the preference index of the drug or vehicle-infusion groups over the first minute only, and group comparisons were performed using one-way ANOVA, followed by post hoc Newman-Keuls tests. For trace fear conditioning training and testing data, freezing responses throughout the training session were analyzed with repeated measures ANOVA. Percent freezing in the testing was analyzed by one-way ANOVA. For each significant *F*-ratio, post hoc analyses were performed with Fisher's protected least significant difference test. In this study, a confidence level of *P* < 0.05 was adopted for statistical significance.

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Role of the phosphoinositide 3-kinase-Akt-mammalian target of the rapamycin signaling pathway in long-term potentiation and trace fear conditioning memory in rat medial prefrontal cortex

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