Phosphatidylinositol 3-Kinase Activation Is Required for Stress Protocol-induced Modification of Hippocampal Synaptic Plasticity*§

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Stress dramatically affects the induction of hippocampal synaptic plasticity; however, the molecular details of how it does so remain unclear. Phosphatidylinositol 3-kinase (PI3K) signaling plays a crucial role in promoting neuronal survival and neuroplasticity, but its role, if any, in stress-induced alterations of long term potentiation (LTP) and long term depression (LTD) is unknown. We found here that inhibitors of PI3K signaling blocked the effects of acute restraint-tail shock stress protocol on LTP and LTD. Therefore, the purpose of the present study is to explore the signaling events involving PI3K in terms of its role in mediating stress protocol-induced alterations of LTP and LTD. We found that stress protocol-induced PI3K activation can be blocked by various inhibitors, including RU38486 for glucocorticoid receptors, LY294002 for PI3K, and dl-2-amino-5-phosphonopentanoic acid for N-methyl-D-aspartate receptors or brain-derived neurotrophic factor antisense oligonucleotides. Also, immunoblotting analyses revealed that stress protocol induced a profound and prolonged phosphorylation of numbers of PI3K downstream effectors, including 3-phosphoinositide-dependent protein kinase-1, protein kinase B, mammalian target of rapamycin (mTOR), p70 S6 kinase, and eIF4B in hippocampal CA1 homogenate, which was prevented by the PI3K inhibitor pretreatment. More importantly, we found that stress protocol significantly increased the protein expression of dendritic scaffolding protein PSD-95 (postsynaptic density-95), which is known to be increased the protein expression of dendritic scaffolding protein PSD-95 (postsynaptic density-95), which is known to be increased.

Stress has long been recognized to interfere with many aspects of brain functions through its activation of the hypothalamus-pituitary-adrenal axis. The hippocampal formation, an important structure involved in forming declarative, spatial, and contextual memory (1, 2), is a brain area particularly sensitive and vulnerable to stress and stress hormones (3). Stress dramatically affects hippocampal long term synaptic plasticity (3–7). Work in our laboratory and other laboratories has shown that a brief experience of acute restraint-tail shock stress protocol can impair high frequency stimulation (HFS)2-induced long term potentiation (LTP) (8–14), whereas low frequency stimulation (LFS)-induced long term depression (LTD) can be facilitated in the CA1 region of the hippocampus (11–14). However, the molecular mechanisms underlying the alterations of the inducibility of LTP and LTD by a stress protocol have only begun to be elucidated.

Phosphatidylinositol 3-kinases (PI3Ks) are a large family of intracellular signal transducers that catalyze the phosphorylation of the D-3 position of the inositol ring of phosphoinositides (15). The PI3K pathway is classically implicated in the regulation of cell growth, survival, proliferation, and movement (16–18). In the mammalian brain, in addition to its functions in neuronal survival and differentiation (19), a number of recent studies have implicated PI3K in synaptic plasticity and learning and memory. For example, it has been shown that activation of PI3K is required for the expression of LTP in the dentate gyrus (20) and CA1 region of the hippocampus (21, 22). Moreover, pharmacological inhibition of PI3K significantly impaired inhibitory avoidance (23), spatial learning (24–25), consolidation (26), retrieval, and extinction of fear-associated memory (27). PI3K may contribute to regulate LTP and memory formation by facilitating AMPA receptor insertion into the postsynaptic membrane (28), activating mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) (26, 29) and initiating de novo protein synthesis (30).

It has been proposed that stress may affect subsequent hippocampal synaptic plasticity by sharing some aspects of the same mechanisms underlying the support of LTP (4, 7, 10, 31, 32). Because PI3K is critical for the expression of LTP, we hypothesized that PI3K might also play a role in mediating the...
PI3K in Stress Protocol Effects

effects of stress on hippocampal synaptic plasticity. In this study, we show that PI3K signaling is activated in the hippocampal CA1 region following an acute restraint-tail shock stress protocol, and its activity is required for the effects of such a stress protocol on subsequent LTP and LTD induction.

EXPERIMENTAL PROCEDURES

Animals—Healthy adult male Sprague-Dawley rats weighing 250–300 g were used in these experiments. Animal care was consistent with the guidelines set by the Laboratory Animal Center of National Cheng Kung University. All experiments were approved by the National Cheng Kung University Institutional Animal Care and Use Committee governing the participating laboratories. Animals were housed in groups of four in a vivarium with a 12-h light-dark cycle (lights on at 7:00 a.m.), 50–60% humidity, and free access to food and water. Ambient temperature was controlled at 24 °C. All experiments were conducted during the light phase of the cycle.

Cannulation—Rats were cannulated as described previously (13, 14). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and 22-gauge cannulas were implanted bilaterally toward 1.0 mm above the stratum pyramidale of the dorsal CA1 region of the hippocampi (coordinates: anterior, ~4.0; lateral, ±4.0; ventral, 2.6), in accordance with the description by Paxinos and Watson (33). The cannulas were fixed to the skull with dental cement. The animals were allowed to recover from surgery for 7 days before the experiments started. To deliver the various drugs, we used a 26-gauge infusion cannula connected by a polyethylene tube to a 5-μl microsyringe. Bilateral injections were performed using an infusion pump (CAM/100; CAM Microdialysis, Solna, Sweden). A total volume of 0.5 μl was infused into each side over 1 min, and the infusion cannula was kept in place for an additional 2 min to minimize backflow of the injectant. Only slices from animals with accurate cannula placements, as verified microscopically by the cannula tips located within the hippocampal CA1 region, were taken for electrophysiological recordings or biochemical measurements. Although infusions spread with a radius of ~1.2–1.4 mm (as estimated by pilot experiments using a 0.5-μl solution of 4% methylene blue in saline), we made analyses from neurons within a radius 0.6–0.8 mm from the cannula tips to obtain better effects of infused drugs. We never observed any effects of cannulation itself or vehicle artificial CSF (aCSF; 10% Me₂SO) injection.

Stress Protocol—Animals were allowed to acclimate to the laboratory for 7 days before any experimental manipulation. Physical stress was evoked by 60 tail shocks (1 mA for 1 s, 30–90 s apart) while restrained in a Plexiglas tube, as described previously (11, 13). This restraint-tail shock stress protocol, adapted from the “learned helplessness” paradigm (i.e. animals are exposed to an unpredictable and uncontrollable aversive stimulus) (34), has been shown previously to reliably induce behavioral (freezing immobility, piloerection, urination, and defecation) and endocrine (elevated serum corticosterone levels) signs of stress in rats (11, 13). Control animals remained in their home cages. Control and stressed animals did not have available food and water during the experiments.

Plasma Corticosterone Assay—Blood samples were obtained from the tail before and after stress and immediately centrifuged at 1,000 × g, and plasma was separated and stored at −20 °C. Plasma corticosterone levels were determined by radioimmunoassay, as described previously (13).

Slice Preparation and Electrophysiology—Immediately after the stress protocol, animals were deeply anesthetized with halothane and decapitated with a guillotine, and hippocampal slices (400 μm thick) were prepared with a vibrating blade slicer (VT1000S; Leica Microsystems, Wetzlar, Germany). The slices were placed in a humidified interface-type holding chamber of aCSF oxygenated with 95% O₂, 5% CO₂ and kept at room temperature for at least 1 h before electrophysiological recording. For extracellular recordings, a single slice was transferred to a submerision-type recording chamber continually perfused with 30–32 °C oxygenated aCSF solution (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 11 mM glucose). Extracellular field potential recordings were carried out using an Axoclamp-2B amplifier (Axon Instruments, Union City, CA). The responses were low pass-filtered at 2 kHz, digitally sampled at 5–10 kHz, and analyzed using pCLAMP software (version 8.0; Axon Instruments). The evoked postsynaptic responses were induced in CA1 stratum radiatum by stimulation (0.02 ms duration) of Schaffer collateral/commissural afferents at 0.033 Hz with a bipolar stainless steel stimulating electrode. The stimulation strength was set to elicit a response having an amplitude that was 30–40% of the maximum spike-free response. Field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass pipette filled with 1 M NaCl (2–3 megaohms resistance), and the fEPSP slope was measured from ~20–70% of the rising phase using a least squares regression (13). The LTP was induced by HFS at the test pulse intensity, consisting of two 1-s trains of stimuli separated by an intertrain interval of 20 s at 100 Hz. LTD was induced using a standard protocol of 900 stimuli at 1 Hz (LFS). The stimulation intensity during LFS application was the same as the test pulse intensity.

Preparation of Synaptoneurosomes—Synaptoneurosome fractions were prepared as described previously (35, 36). Briefly, the microdissected CA1 regions were homogenized in ice-cold Ca²⁺-, Mg²⁺-free buffer (50 mM HEPES, 100 mM NaCl, and 3 mM KAc, pH 7.4) and were centrifuged at 2,000 × g for 1 min. Supernatants were passed through two 100-μm nylon mesh filters, followed by a 5-μm pore filter. The filtrate was then centrifuged at 1,000 × g for 10 min and then was gently resuspended with the same buffer at a protein concentration of 2 mg/ml.

Immunohistochemistry—Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneally) and transcardially perfused with physiological saline and subsequently with fresh 4% paraformaldehyde with 1.5% picric acid in 0.1 M phosphate buffer, pH 7.4 (4 °C). After the perfusion, brains were removed and postfixed in the same fixative for 4 h and then transferred into 30% sucrose in 0.1 M phosphate buffer overnight. Serial free floating sections (15-μm thickness) were obtained using a sliding microtome. For immunostaining, free floating sections were incubated for 30 min with 3% H₂O₂ in 0.1 M phosphate buffer to remove endogenous peroxidase activity,
washed in phosphate buffer three times, and then kept by blocking solution containing 5% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. The sections were incubated overnight at 4 °C with primary antibodies that recognize phosphorylated Akt at Ser473 (1:1000; Cell Signaling Technologies, Beverly, MA), MAP-2 (microtubule-associated protein-2; 1:100; Cell Signaling Technologies), or NeuN (neuron-specific nuclear protein; 1:100; Chemicon, Temecula, CA). After washing, secondary antibodies conjugated to the fluorescent markers Alexa-488 and Alexa-568 (1:400; Molecular Probes, Inc., Eugene, OR) were applied to sections for 2 h. Sections were then washed, mounted on slides, and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and images were taken using an upright fluorescence microscope (BX51; Olympus, Tokyo, Japan). Specific staining was abolished by omission of primary antibody.

**Western Blotting and Immunoprecipitation**—For each experimental group, homogenates from at least three slices were pooled. The microdissected subregions were lysed in ice-cold Tris-HCl buffer solution (TBS; pH 7.4) containing a mixture of protein phosphatase and proteinase inhibitors (50 mM Tris-HCl, 100 mM NaCl, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM microcystin-LR, 1 μM okadaic acid, 0.5% Triton X-100, 2 mM benzamidine, 60 μg/ml aprotinin, and 60 μg/ml leupeptin) to avoid dephosphorylation and degradation of proteins and ground with a pellet pestle (Kontes Glassware, Vineland, NJ). Samples were sonicated and spun down at 15,000 × g at 4 °C for 10 min. The supernatant was then assayed for total protein concentration using a Bio-Rad Bradford protein assay kit. Each sample was separated in 7.5% or 10% SDS-polyacrylamide gel. Following the transfer on nitrocellulose membranes, blots were blocked with Vectorshield mounting medium (Vector Laboratories, Burlingame, CA), and images were taken using an upright fluorescence microscope (BX51; Olympus, Tokyo, Japan). Specific staining was abolished by omission of primary antibody.

**Brain-derived Neurotrophic Factor (BDNF) Immunoassay**—The BDNF levels were measured with a conventional two-site enzyme-linked immunosorbent assay system according to the manufacturer's protocol (Promega, Charbonniere, France) (13). Briefly, each hippocampus was weighed and then homogenized in 20 volumes of ice-cold lysis TBS, and the homogenates were centrifuged for 20 min at 15,000 × g at 4 °C. Tissue samples were acidified for 15 min and neutralized, because this treatment has been reported to aid dissociation of bound trophic factors from their receptors (38). BDNF concentrations were calculated from regression analysis of human recombinant BDNF standard curve run in each assay and expressed as normalized per gram of tissue wet weight.

**Drugs**—The sequences of BDNF antisense and sense oligonucleotides were 5'-TCTTCCCTTCTTATGGT-3' and 5'-ACCATTAAAGGGGGAAGA-3', respectively, which correspond to all splice variants of BDNF (39). LY294002, LY305511, wortmannin, RU38486, RU28318, bi-2-amino-5-phosphonopentanoic acid (APV), and U0126 were purchased from Tocris Cookson (Bristol, UK); K252a, K252b, and U0124 were obtained from Calbiochem; rapamycin and ascomycin were obtained from Sigma.

**Drug Treatment**—Stock solutions of LY294002, LY305511, wortmannin, K252a, K252b, rapamycin, ascomycin, U0126, and U0124 were prepared by initially dissolving in Me2SO and then diluting in aCSF before infusion. Drug and vehicle solutions had a final concentration of 10% Me2SO. Drug doses of LY294002 (10 mM), LY305511 (10 mM), wortmannin (7.3 mM), K252a (0.5 μg/μl), K252b (0.5 μg/μl), rapamycin (1 ng/μl), ascomycin (1 ng/μl), U0126 (2 μg/μl), and U0124 (2 μg/μl) were selected on the basis of published studies (13, 27, 40, 41) and pilot experiments in our laboratory. APV was dissolved in ACSF to a concentration 25 mM, which has been shown to be effective to completely abolish the novelty-induced retrograde amnesia (42). The BDNF antisense and sense oligonucleotides were dissolved in ACSF and infused into the lateral ventricle at a dose of 50 pmol twice per day for 5 days (13). RU38486 was administered 30 min before stress by intraperitoneal injection with a dose of 40 mg/kg, which has been shown to be effective to completely abolish the glucocorticoid receptor-mediated functions (43). Control animals were injected with the vehicle propylene glycol for RU38486.

**Data Analysis**—Average data are presented as mean ± S.E. Student's t tests were used to calculate significance when two groups were compared. When more than two groups were compared, a one-way analysis of variance was used, and Bonferroni multiple comparisons were used to compare the signif-
**PI3K in Stress Protocol Effects**

Significance between groups. The number of animals used is indicated by *n*. Probability (p) values of less than 0.05 were considered statistically significant.

**RESULTS**

**PI3K Is Activated after the Restraint-Tail Shock Stress Protocol**—In response to stress protocol, significantly higher levels of circulating corticosterone concentrations (126.5 ± 7.1 ng/ml versus 9.2 ± 2.3 ng/ml in basal conditions, *n* = 10; *p* < 0.05) were observed, confirming that our experimental restraint-tail shock stress protocol activates hypothalamus-pituitary-adrenal responses as described previously (11, 13). To investigate whether such a stress protocol affects PI3K activity, we indirectly monitored the activation of PI3K by measuring the phosphorylation of its downstream target Akt on Ser473 using Western blot analysis (44). We found that phosphorylated Akt was readily detected in slices from control rats, and levels were greatly increased (~70%) in slices from stressed rats (Fig. 1A). Stress protocol showed no effect on total levels of Akt (supplemental Figs. S1A and S3A). Bilateral injection of two selective and structurally distinct PI3K inhibitors, LY294002 (10 nm, 0.5 μl/side; 30 min before stress) or wortmannin (7.3 μM, 0.5 μl/side), into the CA1 region of the hippocampus significantly reduced basal levels of phosphorylated Akt and completely prevented the increases in Akt phosphorylation induced by stress protocol (Fig. 1, A and B). Immunofluorescent staining was performed to extend these findings. Sections of hippocampus were immunostained for phosphorylated Akt to visualize activation of PI3K. As shown in Fig. 1C, stressed rats exhibited increased numbers of immunopositive cells and labeling intensity for phosphorylated Akt in the CA1 pyramidal layer compared with unstressed rats. In addition, stress protocol-induced phosphorylated Akt increases colocalized with the neuronal marker NeuN and the pyramidal cell makers MAP2. Together, these results indicate that PI3K in the hippocampal CA1 region is activated following the stress protocol application.

Previous findings from our laboratory demonstrated a parallel in time course of the increased ERK1/2 activation as well as the effects of stress protocol on LTP and LTD; additionally, a pharmacological blockade of the ERK1/2 signaling pathway completely prevented the stress protocol effects, suggesting a critical role of sustained ERK1/2 activation in mediating the effects of the stress protocol (7, 13). In addition, it has been shown that PI3K is essential for NMDA receptor-mediated ERK1/2 activation in cultured neurons (29, 45). Therefore, we examined the possible contribution of PI3K to stress protocol-induced increase of ERK1/2 activation. As expected, we found that the stress protocol induced a marked increase of ERK1/2 phosphorylation in the hippocampal CA1 region. This increase was severely impaired by prior intrahippocampal injection of LY294002 (Fig. 1A) or wortmannin (Fig. 1B), demonstrating that this event is mostly PI3K-dependent. There was no significant variation in total ERK1/2 in the hippocampal CA1 region of stressed rats when compared with unstressed rats (supplemental Fig. S1A).

A well established pathway for activation of ERK1/2 is via stimulation of Ras and formation of a Ras-Raf-1-MEK1/2 complex (46). Previous studies have shown that the cross-talk between the PI3K and ERK1/2 signaling pathways may occur at the level of Raf kinase (47, 48). We therefore tested whether PI3K inhibitors blocked the stress protocol-induced increases in the activation of ERK1/2 by suppressing Raf-1 and MEK1/2 activity. In agreement with our previous observations on the effects of the stress protocol (13), area CA1 excised from the hippocampal slices of stressed rats exhibited a significant increase in both Raf-1 and MEK1/2 phosphorylation in comparison with slices from unstressed control rats (supplemental Fig. S2, A and C). We found that bilateral injection of LY294002 or wortmannin into area CA1 of the hippocampus before stress
PI3K in Stress Protocol Effects

Glucocorticoid Receptors Mediate Stress Protocol-induced PI3K Activation—Having confirmed the role of PI3K in mediating the effects of stress protocol on hippocampal plasticity, we next investigated possible signaling events that underlie the stress protocol-induced PI3K activation. We have previously demonstrated that this acute restraint-tail shock stress protocol can modulate the subsequent hippocampal CA1 LTP and LTD through the corticosterone release to activate glucocorticoid receptors (13). To test the possible role of glucocorticoid receptor activation in mediating the stimulatory effect of stress protocol on PI3K activity, the specific glucocorticoid receptor antagonist RU38486 (40 mg/kg) was intraperitoneally injected 30 min before stress protocol application. As expected, RU38486 almost completely prevented the stress protocol effects, as indicated by normal LTP (48.6 ± 6.8%, n = 5) and LTD (5.7 ± 4.1%, n = 5) in slices from stressed rats (Fig. 3, A and B). In line with the electrophysiological observations, RU38486 also completely blocked the stress protocol-induced PI3K activation. We found no difference between vehicle-treated unstressed and RU38486-treated stressed rats in the levels of phosphorylated Akt at both Thr308 and Ser473 in the hippocampal CA1 region.

We next test whether PI3K activity is necessary for maintaining the effects of stress protocol on subsequent LTP and LTD induction in the hippocampal CA1 region. In accordance with previous results (11–13), the hippocampal slices from stressed rats exhibited impaired LTP (6.8 ± 5.7%, n = 6; p < 0.05) but facilitated LTD (33.5 ± 6.8% of baseline, n = 6; p < 0.05) compared with slices from unstressed control rats (LTP, 53.5 ± 8.2%, n = 6; LTD, 5.5 ± 5.7%, n = 6) (Fig. 2, A and B). Bilateral intrahippocampal injection of LY294002 almost completely prevented the blockade of LTP (48.7 ± 7.3%, n = 6) and the facilitation of LTD (6.3 ± 4.7%, n = 6) by stress protocol. Injection of LY294002 in unstressed rats had no effect on the induction of both LTP (51.9 ± 6.5%, n = 6) and LTD (4.4 ± 3.5%, n = 6). In contrast to LY294002, the inactive analog LY303511 (10 mM, 0.5 μM/side) did not significantly affect the effects of stress protocol (LTP, 11.3 ± 5.3%, n = 5; LTD, 32.8 ± 6.2%, n = 5). These results strongly support the view that the activation of PI3K signaling pathways is an obligatory component of biochemical bases that serves the effects of stress protocol on subsequent LTP and LTD induction.

Blockade of NMDA Receptors Prevents Stress Protocol-induced PI3K Activation—It has previously been shown that stress modifies hippocampal plasticity through the activation of NMDA receptors (11). It has been further evident that pharmacological blockade of NMDA receptors during stress prevents a stress-induced facilitation of the trace eye blink conditioning in rats (49). We therefore investigated whether the stress protocol exposure significantly suppressed the increases in Raf-1 and MEK1/2 phosphorylation by the stress protocol. The stress protocol showed no effect on total levels of Raf-1 or MEK1/2 (supplementary Fig. S2, B and D). These results indicate that activation of PI3K is required, at least in part, for the activation of Raf-1, MEK1/2, and ERK1/2 triggered by the stress protocol in the hippocampal CA1 region.

We next investigated whether the stress protocol modulates the PI3K activity through the release of corticosterone. We have previously shown that restraint-tail shock stress protocol modulates the subsequent hippocampal CA1 LTP and LTD through PI3K activation. We found no difference between vehicle-treated unstressed and RU38486-treated stressed rats in the levels of phosphorylated Akt at both Thr308 and Ser473 in the hippocampal area CA1 (Fig. 3C). In contrast, prior administration of specific mineralocorticoid receptor antagonist RU28318 (10 mg/kg) did not affect the effects of stress protocol on LTP (9.7 ± 4.6%, n = 5) and LTD (26.3 ± 6.1%; n = 5) (Fig. 3, A and B). Likewise, the ability of stress protocol to increase the Akt phosphorylation on either the Thr308 or Ser473 site was not significantly affected by RU28318 pretreatment (Fig. 3C). Injection of either RU28318 or RU38486 in unstressed rats had no effects on the induction of LTP or LTD or the levels of Akt phosphorylation. In addition, neither RU28318 nor RU38486 treatment showed an effect on total levels of Akt (supplemental Fig. S1B). These results support the hypothesis that stress protocol modulates the PI3K activity through the release of corticosterone to activate glucocorticoid receptors.

FIGURE 2. Activation of PI3K mediates the effects of stress protocol on LTP and LTD. A, stressed rat slices displayed a deficit in HFS-induced LTP, whereas HFS induced a robust LTP in slices from stressed rats administered with LY294002 (LY2). Administration of the inactive analog LY303511 (LY3) did not significantly affect the effect of stress protocol. The bar graphs show a comparison of the magnitude of LTP 50 min after HFS. B, LFS induced a reliable LTD in slices from stressed rats but not in slices from stressed rats administered with LY294002. The bar graphs show a comparison of the magnitude of LTD 50 min after LFS (1 Hz for 15 min). The number of experiments per group is indicated by n. *, significant difference compared with the control-vehicle slices (p < 0.05).
col-induced PI3K activation also depends on the activation of NMDA receptors by using bilateral injection of a competitive NMDA receptor antagonist, APV, into the CA1 region of the hippocampus. The advantages of using APV as a NMDA receptor antagonist include the reversibility of its effect and its ability to selectively inhibit NMDA receptors. Confirming and extending previous findings (11), when administered 30 min before the start of the stress protocol, APV (25 mM, 0.5 μl/ side) almost completely prevented the effects of stress protocol on LTP (58.7 ± 6.9%, n = 5) and LTD (6.2 ± 3.5%, n = 5) (Fig. 4, A and B). A similar blockade of the enhancing effect of stress protocol on PI3K activation was also observed in APV-treated stressed rats (Fig. 4C). When APV was applied to a group of unstressed rats, it showed no effects on the induction of LTP or LTD or the levels of Akt phosphorylation. APV treatment showed no effect on total levels of Akt (supplemental Fig. S1C). These results suggest that stress protocol activates PI3K through a NMDA receptor-dependent mechanism.

In subsequent experiments, we investigated the downstream signaling components activated by NMDA receptors to increase PI3K activity. Because BDNF was shown to play an important role as a mediator of stress response in the hippocampus (50) and the activation of PI3K signaling is one of key signaling responsible for binding of BDNF to its receptor TrkB (tyrosine kinase B) (51, 52), the rapid increase in BDNF expression during stress protocol exposure could result in the activation of PI3K signaling pathways by NMDA receptors. To evaluate this possibility, we examined the activation of PI3K in slices from rats that received intracerebroventricular injection of BDNF antisense, sense, or scrambled oligonucleotide (50 pmol twice per day, bilateral injection for 5 consecutive days). As shown in Fig. 5A, treatment with BDNF antisense oligonucleotide completely prevented PI3K activation by the stress protocol. In contrast, BDNF sense or scrambled oligonucleotide treatment did not show any significant influence on the effects of the stress protocol on PI3K activity. Furthermore, BDNF antisense, sense, or scrambled oligonucleotide treatment showed no effects on total levels of Akt (supplemental Fig. S1D). To confirm the effect of intracerebroventricular injection of BDNF antisense oligonucleotide on BDNF synthesis, the BDNF protein levels of stressed rats were also determined. Fig. 5B shows that the BDNF protein levels in the hippocampi of antisense oligonucleotide-treated rats were significantly lower than those in vehicle, sense, or scrambled oligonucleotide-treated rats. Remarkably, microinjection of APV into the bilateral hippocampus area CA1 before stress protocol application also completely prevented the stress protocol-induced increase in BDNF synthesis, further supporting these effects were specifically attributable to the activation of NMDA receptors.

Stress Protocol Stimulates PSD-95 Protein Translation through the PI3K-Akt-mTOR Signaling Pathway—How does activation of PI3K lead to the effects of stress protocol on hippocampal synaptic plasticity? Protein synthesis is required for effects of stress protocol on LTP and LTD (53). In addition, it
has been shown that BDNF can increase protein synthesis by activating initiation and elongation steps in mRNA translation through a TrkB-PI3K-mTOR-dependent mechanism (54). We therefore hypothesized that a PI3K–regulated translational activation is involved in mediating the effects of the stress protocol. To further ascertain that the stress protocol can rapidly activate the PI3K-Akt-mTOR signaling pathway, we conducted a series of experiments to measure the PDK1, Akt, and mTOR activities in hippocampal slices from stressed rats. For PDK1, we used an antibody specific for phosphorylated Ser241, which is on the activation loop of PDK1 and is essential for kinase activity (55). For mTOR, we used an antibody specific for phosphorylated Ser2448, which has been shown to be important in the control of translation and to promote ribosome binding to mRNA (59). eIF4B is an RNA-binding protein that has the ability to stimulate translation and may mediate some of the effects of the S6K on translation (60). In hippocampal slices from stressed rats administered NMDA receptor antagonist APV, the bar graphs show a comparison of the magnitude of LTP 50 min after HFS. B, LFS failed to induce LTD in slices from stressed rats administered APV. The bar graphs show the comparison of the magnitude of LTD 50 min after LFS. C and D, representative immunoblot and corresponding densitometric analysis showing that APV administration specifically prevented the stress protocol-induced increase in Akt phosphorylation at Ser473. The number of experiments per group is indicated by n. *, significant difference compared with the control-vehicle slices (p < 0.05).

PI3K in Stress Protocol Effects

stress protocol and 0.5 h later (Fig. 6B). Furthermore, we found a significant increase of mTOR phosphorylation at 0–12 h after the stress protocol (Fig. 6C). The stress protocol showed no effect on total levels of Akt, PDK1, or mTOR (supplemental Fig. S3, A–C).

Because mTOR activation may regulate translation by direct or indirect phosphorylation of ribosomal protein S6K (57), we used an antibody that recognizes S6K dually phosphorylated on Thr229 and Thr389 sites after stress protocol. Phosphorylation of S6K on Thr229 and Thr389 is crucial for kinase activities (58). As shown in Fig. 6B, the levels of phosphorylation of S6K were strongly elevated within 0–24 h after the stress protocol. The total S6K levels did not exhibit any change after stress protocol (supplemental Fig. S3D).

eIF4B is an RNA-binding protein that has the ability to stimulate translation and to promote ribosome binding to mRNA (59). eIF4B is a key downstream target of S6K (57) and may mediate some of the effects of the S6K on translation (60). In addition, eIF4B hyperphosphorylation on Ser422 stimulated by serum or mitogen correlates well with increased translation rates (61). We then asked whether stress protocol-induced S6K activation was accompanied by enhanced eIF4B phosphorylation on Ser422. Consistent with the time course of S6K activation after stress protocol, we found a strong increase of eIF4B phosphorylation within 0–24 h after the stress protocol (Fig. 6E). Total levels of eIF4B were unaffected after stress protocol, supporting a specific effect on the activation of eIF4B, supporting a specific effect on the activation of eIF4B (supplemental Fig. S3E). These data strongly suggest that stress protocol can trigger a translation initiation in the hippocampal CA1 region through the activation of PI3K-Akt-mTOR signaling pathways.

To further explore a role of mTOR–coupled mechanism mediating stress effects, we performed parallel electrophysiological studies to examine the inducibility of LTP and LTD in slices from rats treated with the mTOR inhibitor, rapamycin (1 ng/μl; bilateral intrahippocampal injection 30 min before stress). These effects of the stress protocol were inhibited by the prior administration of rapamycin. Reliable LTP was elicited (56.3 ± 7.6%, n = 5), and LTD was no longer induced (5.3 ± 3.6%, n = 6) in slices from stressed rats (Fig. 7, A and B). Injection of rapamycin in unstressed rats had no effect on the induction of both LTP (53.2 ± 6.7%, n = 5) and LTD (5.1 ± 3.2%, n = 5).
PI3K in Stress Protocol Effects

**FIGURE 5. BDNF signaling pathway mediates the effects of stress protocol.** A, representative immunoblot and corresponding densitometric analysis showing that administration of BDNF antisense oligonucleotide specifically prevented the stress protocol-induced increased Akt phosphorylation at Ser473. B, administration of BDNF antisense oligonucleotide or APV specifically suppressed the stress protocol-induced increase in BDNF protein levels in the whole rat hippocampi. The number of experiments per group is indicated by n. *, significant difference compared with the control-vehicle slices (p < 0.05).

However, the inactive analogue ascomycin (1 ng/μl) (62) infused bilaterally into the CA1 before stress did not produce changes in the effects of stress protocol on the induction of both LTP (10.5 ± 6.3%, n = 5) and LTD (32.2 ± 5.8%, n = 4).

In subsequent experiments, we investigated which plasticity-related protein synthesis is possibly involved in mediating the effects of the stress protocol on hippocampal synaptic plasticity. PSD-95 (postsynaptic density-95) is suggested as a scaffolding protein containing multiple PSD-95/Discs large/Zona occluens-1 domains to anchor and associate glutamate receptors with other functional proteins in the PSD (63). The activation of PI3K-Akt-mTOR signaling pathway by estrogen or insulin has been shown to rapidly stimulate dendritic PSD-95 synthesis in NG108-15 neuroblastoma cells (64) and the hippocampal CA1 region (36). Furthermore, like stress protocol does, it has been reported recently that overexpression of PSD-95 impairs LTP but enhances LTD induction in the hippocampal slice cultures (65). We therefore examined whether stress protocol may stimulate new PSD-95 protein synthesis. Consistent with this notion, we found a profound increase in the levels of PSD-95 protein in hippocampal CA1 homogenates within 0–24 h after stress protocol (Fig. 6F). The highest level was found at 0.5 h after stress. Given that PSD-95 is more centrally located in the postsynaptic density compartment, it was important to establish that the stress protocol also increases the expression of PSD-95 in the postsynaptic component. In order to test this, we generated synaptoneurosome, a biological preparation that enriched for postsynaptic fractions (66) and contained untranslated PSD-95 mRNA (36). Consistent with results obtained with total homogenates, an increase in the PSD-95 protein levels was observed within 0–24 h after stress protocol in our hippocampal CA1 synaptoneurosome preparations (supplemental Fig. S4A). Noticeably, the stress protocol-induced up-regulation of PSD-95 protein in total homogenates was higher than in the synaptoneurosomes. In addition, in the synaptoneurosomes, this effect of the stress protocol was specifically inhibited by APV, LY294002, K252a, and rapamycin but not by inactive analogs, LY303511, K252b, and ascomycin pretreatment (supplemental Fig. 4, B–D). However, infusion of the permeable MEK1/2 inhibitor U0126 (2 μg/μl, 0.5 μl/side) bilaterally into the hippocampal area CA1 region 30 min before the stress protocol application did not significantly affect the up-regulation of PSD-95 protein expression by stress. These findings strongly suggest that PI3K-Akt-mTOR signaling pathway is a major pathway that couples TrkB receptors to enhance PSD-95 translation during stress protocol exposure.

Finally, we investigated the plausible mechanisms by which stress protocol-induced up-regulation of PSD-95 protein modulates the induction of hippocampal synaptic plasticity. PSD-95 is known to inhibit NMDA receptor function through suppression of the tyrosine kinase activity of Src by interacting directly with the Src SH2 domain (67). Because the NR2B subunit of the NMDA receptors is the major tyrosine-phosphorylated protein in the brain (68) and the level of Src-mediated tyrosine phosphorylation of the NR2B subunit has been found to increase in LTD in CA1 (69) and dentate gyrus (70) of the hippocampus, it is therefore possible that the changes in the level of NR2B tyrosine phosphorylation by a stress protocol may contribute to its effects on LTP and LTD. To test this prediction, we therefore assessed the effect of a stress protocol on tyrosine phosphorylation of the NR2B subunit in the CA1 region by immunoprecipitation with NR2B-specific antibody followed by immunoblotting with antiphosphotyrosine antibody. We found that the basal tyrosine phosphorylation level of the NR2B subunit was consistently reduced in slices from stressed rats compared with slices from unstressed rats (Fig. 8A). There is a clear inverse
correlation between the levels of PSD-95 and NR2B tyrosine phosphorylation. In accordance with previous findings (71), the tyrosine phosphorylation level of the NR2B subunit was elevated after LTP induction. The amount of LTP-induced increase of tyrosine phosphorylation of NR2B was found to be significantly lower in slices from stressed rats than in slices from unstressed rats (Fig. 6B). Furthermore, prior administration of LY294002 prevented the stress-induced up-regulation of PSD-95 and was effective to reverse the inhibitory effect of stress on tyrosine phosphorylation of NR2B. Total protein levels of NR2B were unaffected after stress protocol with or without LY294002.

**DISCUSSION**

In this study, we have identified a critical role of PI3K in the stress protocol-induced alterations in the inducibility of LTP and LTD in the CA1 region of the hippocampus. We have found that an acute restraint-tail shock stress protocol increases the circulating corticosterone levels, leading to the activation of glucocorticoid receptors, which in turn facilitates the activation of NMDA receptors of hippocampal CA1 neurons. This then results in provoking the synthesis and release of BDNF acting on the TrkB receptors, which leads to activation of the PI3K-Akt-mTOR signaling pathway. mTOR, through its downstream translation regulatory molecules, p70 S6K and eIF4B, activates translational machinery and stimulates the synthesis of dendritic scaffolding protein PSD-95 (Fig. 9). Furthermore, we have shown that stress protocol-induced up-regulation of PSD-95 inhibits the enhanced tyrosine phosphorylation of the NR2B subunit of NMDA receptors in LTP.

PI3K is an unusual lipid signaling kinase that has been implicated in a wide range of biological functions most commonly associated with cell growth and survival (16–18). Beyond its role in neuronal growth and survival (19), the PI3K also directly regulates the neuritic outgrowth (71) and synaptic plasticity (20–22). Using the specific inhibitors of PI3K, recent studies have shown that activation of PI3K is necessary for the induction (72) or, in contrast, the expression (21, 22) of LTP in hippocampal area CA1. In addition, PI3K has also been shown to be implicated in the induction of a metabotropic glutamate receptor-dependent form of LTD (73). In the present study, the fact that the parallel time course of the increased PI3K activation and the effects of the stress protocol on LTP and LTD and pharmacological blockade of PI3K completely prevented the stress effects strongly suggests...
PI3K in Stress Protocol Effects

To explore the functional consequences of activation of PI3K and to identify intracellular signaling events implicated in mediating the effects of the stress protocol on synaptic plasticity, we investigated the regulation of different upstream activators and downstream effectors of the PI3K signaling pathway. Consistent with a role of glucocorticoid receptor in mediating the effects of stress (11, 13, 74, 75), the glucocorticoid receptor antagonist RU38486, when administered before stress protocol application, completely prevented the effects of the stress protocol on both PI3K activation and hippocampal synaptic plasticity. Moreover, we have demonstrated that the competitive NMDA receptor antagonist APV, when administered before stress, completely prevented the stress protocol-induced PI3K activation. Thus, the activation of NMDA receptors can initiate the molecular process that contributes to activate PI3K signaling. This is in accordance with an early report (11) indicating that pharmacological blockade of NMDA receptors during stress prevents the effects of stress protocol on subsequent LTP and LTD induction. Moreover, previous studies reported that NMDA receptor stimulation can activate PI3K-Akt signaling pathways in cultured cortical (76) and striatal (29) neurons. How glucocorticoid receptor enhances the activation of NMDA receptors remains an important question which needs to be addressed. In cultured rat hippocampal neurons, previous work has shown that corticosterone can induce a rapid and nongenomic prolongation of NMDA receptor-mediated Ca\(^{2+}\) elevation via putative membrane surface glucocorticoid receptors (77).

How might NMDA receptor activation lead to the sustained PI3K activation? The NMDA receptor could regulate PI3K through a direct activation process or indirectly through stimulating the release of an endogenous mediator. A number of studies have pointed toward an important role for BDNF in a variety of NMDA receptor-mediated biological activities. For example, NMDA has been shown to promote BDNF expression in cerebellar granule cells, and the neuroprotective activity of NMDA in cerebellar granule cells has been associated with increased BDNF release and TrkB receptor activation (78–80). Our results also suggest this possibility, since stress protocol application induced a significant increase in BDNF protein expression in the whole rat hippocampus in a NMDA receptor-dependent manner, and BDNF antisense oligonucleotide intracerebroventricular injection completely prevented this increase and the effects of stress protocol on PI3K activation. Moreover, we also found that the stress protocol-induced PI3K activation was suppressed by the TrkB receptor inhibitor K252a.

that the PI3K pathway is an important cascade implicated in mediating the blockade of LTP and the facilitation of LTD induced by stress protocol.

![Image 1](https://example.com/image1.png)

**FIGURE 7.** The protein kinase mTOR signaling mediates the effects of the stress protocol on LTP and LTD. A, HFS induced robust LTP in slices from stressed rats administered the mTOR inhibitor rapamycin but not ascomycin. The bar graphs show a comparison of the magnitude of LTP 50 min after HFS. B, LFS failed to induce LTD in slices from stressed rats administered rapamycin. The bar graphs show a comparison of the magnitude of LTD 50 min after LFS.

![Image 2](https://example.com/image2.png)

**FIGURE 8.** The stress protocol suppresses the increase in tyrosine phosphorylation of NR2B subunits after LTP. A, representative immunoblot and corresponding densitometric analysis showing the basal levels of tyrosine phosphorylation of NR2B subunits in slices from control and stressed rats. The NR2B subunit was immunoprecipitated with anti-NR2B antibody, and phosphorylation of NR2B was detected with anti-phosphotyrosine antibody. Stress decreased the phosphorylation state of NR2B subunits. B, representative immunoblot and corresponding densitometric analysis showing stress protocol application inhibited the increased tyrosine phosphorylation of NR2B subunits 20 min after LTP induction. Administration of LY294002 blocked the effects of the stress protocol on LTP-induced increase in tyrosine phosphorylation of NR2B subunits. The number of experiments per group is indicated by n. *, significant difference in comparison with slices from unstressed control rats (p < 0.05).
PI3K can stimulate multiple signaling pathways via PDK1 and Akt in both neuronal and nonneuronal cells (17, 81). One major downstream target of this signaling pathway is protein kinase mTOR, which can stimulate translational initiation and elongation. Using a specific mTOR inhibitor, rapamycin, we confirm a significant role of mTOR-coupled signaling in mediating the stress protocol effects. Furthermore, our results also reveal that stress protocol increases the phosphorylation of S6K and eIF4B, which can result in an enhanced translational rate of 5’-oligopyrimidine tract-containing mRNAs that encode numerous components of the translational machinery (57, 60). Our results suggest that a stress protocol can promote translation of specific target proteins in the hippocampal CA1 region through the activation of PI3K-Akt-mTOR-S6K-eIF4B signaling pathway. This is in line with the previous finding of a role of new protein synthesis in mediating the effects of stress on hippocampal synaptic plasticity (53). In search of likely protein synthesis for the alterations of LTP and LTD by a stress protocol, our data suggest that one such candidate is dendritic spine scaffolding protein PSD-95. The increase was observed immediately after the stress protocol, in agreement with previous evidence showing that mRNA encoding PSD-95 is present at the synapses and can be rapidly stimulated following the activation of the PI3K-Akt-mTOR signaling pathway (36, 64). It is noteworthy that a significantly higher stress protocol-induced PSD-95 expression was found in the hippocampal CA1 homogenates than in the synaptoneurosomes within 0.5–24 h after stress (Fig. 6F and supplemental Fig. S4A). A possible explanation for this difference is that stress protocol-induced rapid PSD-95 protein synthesis in the synaptoneurosomes is primarily dependent on the existing population of PSD-95 mRNA already transcribed, whereas some stress protocol-induced new PSD-95 mRNA transcription in the neuronal cell body also participates in subsequent new protein synthesis observed in the homogenates. In support of this assertion, we have found that stress protocol-induced PSD-95 expression in the homogenates was significantly inhibited by the administration of trancitional inhibitor actinomycin-D. However, we cannot rule out the increases in PSD-95 trafficking to the synapses involved in this difference. Indeed, it has been shown that both synaptic NMDA receptor stimulation and BDNF applied to cultured cortical neurons can increase the trafficking of PSD-95 to dendrites and synapses through the activation of PI3K-Akt signaling pathways (82).

The precise mechanism by which PSD-95 interacts with synaptic plasticity remained to be elucidated. We found that stress protocol-induced up-regulation of PSD-95 is accompanied by a reduction in the amount of basal and LTP-induced increase in tyrosine phosphorylation of NR2B subunits of NMDA receptors. Furthermore, pharmacological blockade of stress protocol-induced up-regulation of PSD-95 also effectively reversed the effects of the stress protocol on NR2B subunit tyrosine phosphorylation and the induction of long term synaptic plasticity. Given that tyrosine phosphorylation of NR2B subunits is essential for the induction and maintenance of hippocampal...
CA1 LTP (69, 70), it seems possible that the increased PSD-95 induced by the stress protocol may negatively regulate NMDA receptor activity and thereby synaptic plasticity, but additional studies will be necessary to examine this issue. Related to this idea, recent work demonstrates that PSD-95 may suppress, via its binding to the SH2 domain of Src, Src catalytic activity with the NMDA receptor complex and suppresses the induction of LTP (67).

We have recently shown an obligatory role of the Ras-ERK1/2 signaling pathway in the stress protocol-induced alterations in the inducibility of LTP and LTD in the CA1 region of the hippocampus (13, 83). In the present study, we have extended these findings by showing that PI3K-Akt signaling pathways can cross-talk with Ras-ERK1/2 signaling pathways in the hippocampal CA1 neurons to mediate the stress protocol effects. Our results indicate that stress protocol-induced activation of Raf-1 in the hippocampal CA1 neurons appears to be partially dependent on PI3K activation, since inhibition of PI3K prevented activation of Raf-1 and its downstream MEK1/2-ERK1/2. In fact, there is some evidence indicating that PI3K can, through its protein kinase activity, regulate Raf kinase activity (47, 48). Whether or not PI3K may lie upstream or downstream of Ras in our observed stress protocol-induced ERK1/2 activation is currently unknown.

In conclusion, this study has underscored the importance of activation of PI3K signaling pathways not only directly mediating synaptic plasticity but also serving to determine the polarity of subsequent synaptic plasticity via a metaplastic function. Our results also support the hypothesis that stress may affect subsequent hippocampal plasticity by sharing the same molecular machinery required to support LTP. These findings provide new insights into the molecular mechanisms underlying stress-related memory disorders, which, in turn, might provide a new avenue for development of more selective medication that targets these pathways and prevents their malfunctioning.

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