Corticosterone, the major stress hormone, plays an important role in regulating neuronal functions of the limbic system, though the cellular targets and molecular mechanisms of corticosteroid signaling are largely unknown. Here we show that a short treatment of corticosterone significantly increases AMPAR-mediated synaptic transmission and AMPAR membrane trafficking in pyramidal neurons of prefrontal cortex (PFC), a key region involved in cognition and emotion. This enhancing effect of corticosterone is through a mechanism dependent on Rab4, the small GTPase controlling receptor recycling between early endosome and plasma membrane. Guanosine nucleotide dissociation inhibitor (GDI), which regulates the cycle of Rab proteins between membrane and cytosol, forms an increased complex with Rab4 after corticosterone treatment. Corticosterone also triggers an increased GDI phosphorylation at Ser-213 by the serum- and glucocorticoid-inducible kinase (SGK). Moreover, AMPAR synaptic currents and surface expression and their regulation by corticosterone are altered by mutating Ser-213 on GDI. These results suggest that corticosterone, via SGK phosphorylation of GDI at Ser-213, increases the formation of GDI:Rab4 complex, facilitating the functional cycle of Rab4 and Rab4-mediated recycling of AMPARs to the synaptic membrane. It provides a potential mechanism underlying the role of corticosteroid stress hormone in up-regulating excitatory synaptic efficacy in cortical neurons.

**Keywords:** corticosterone, SGK, GDI, Rab4, phosphorylation, AMPA receptor, mEPSC, prefrontal cortex

Adrenal corticosterone, the major stress hormone, has a potent impact on the function of several key limbic regions including hippocampus, prefrontal cortex (PFC) and amygdala (1, 2, 3). Emerging evidence suggests that corticosterone influences the neurophysiology of limbic cells by modifying synaptic transmission and ion channels (4). For example, it has been found that corticosterone enhances AMPAR-mediated miniature EPSC (mEPSC) amplitude and surface expression of GluR1 and GluR2 subunits in hippocampal neurons (5, 6); increases the surface mobility and synaptic content of AMPAR GluR2 subunits (7); and enhances L-type calcium currents in CA1 pyramidal neurons (8). The molecular mechanisms underlying these regulatory effects of corticosteroid signaling remain to be identified.

Corticosterone operates through mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), both of which belong to the family of nuclear receptors which bind to response elements in the DNA, thus modifying the activity of responsive genes (9, 10). One of the immediate early genes transcriptionally stimulated by corticosteroid stress hormones is the Serum- and Glucocorticoid-Inducible Kinase (SGK) (11), a family of serine/threonine kinase consisting of three isomers. Studies in *Xenopus oocytes* have found that SGK can activate certain ion channels and transporters by increasing protein abundance in the plasma membrane (12). However, the cellular targets and functional significance of SGKs in central nervous system are far from being understood.

Recently we have found that in vivo acute stressor induces a prolonged potentiation of AMPAR-mediated synaptic currents in PFC pyramidal neurons (13), which was mimicked by in...
vitro short-term corticosterone treatment. The GR-triggered increase in AMPAR membrane trafficking seems to be responsible for the acute stress-induced plasticity in PFC (13). To determine how corticosterone/SGK regulates AMPARs, we focused on the Rab family small GTPases, which is a key coordinator of intracellular transport steps in exocytic and endocytic pathways (14).

In addition to cycling between inactive (GDP-bound) and active (GTP-bound) states, many Rab proteins also cycle between a membrane-bound and a cytosolic state (15), which is dependent on the GDP dissociation inhibitor (GDI, 16). GDI has the capacity to extract the inactive GDP-bound Rab from membranes and functions as a cytosolic chaperone of Rab (17). The formation of GDI-Rab complex can be stimulated by phosphorylation of GDI (18, 19), leading to accelerated exocytosis or endocytosis. In this study, we examined whether corticosterone could increase the Rab-mediated AMPAR membrane traffic via SGK-induced phosphorylation of GDI.

MATERIALS AND METHODS

DNA Constructs. Rat GDI-1 and Rab4 open reading frame (ORF) were cloned from rat brain cDNA by PCR, and HA or Flag tag was added to the N-terminal of GDI in frame. For expression in HEK 293 cell or cultured neurons, GDI and Rab4 were subcloned to pcDNA3.1 vector (Invitrogen) from T/A vector. Generation of GDI mutants (S45A, S121A, S213A, S213D) and Rab4 mutants (S27N, Q72L) was carried out with the Quick-Change Site-Directed Mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing.

Primary Neuronal Culture. Rat PFC cultures were prepared as previously described (20, 21). In brief, frontal cortex was dissected from E18 rat embryos, and cells were dissociated using trypsin and trituration through a Pasteur pipette. Neurons were plated on coverslips coated with poly-L-lysine in Dulbecco's modified Eagle's medium with 10% fetal calf serum at a density of 1×10^5 cells/cm². When neurons attached to the coverslip within 24 hr, the medium was changed to Neurobasal medium with B27 supplement (Invitrogen). Cytosine arabinoside (ARAC, 5 μM) was added at DIV3 to stop glial proliferation. Culture neurons (DIV 18-25) were transfected with various plasmids using Lipofectamine 2000 (Invitrogen).

Synaptic Current Recording in Neuronal Cultures. Cultured PFC neurons (DIV20-27) were used for recording AMPAR-mediated miniature excitatory postsynaptic currents (mEPSC) as previously described (21). The external solution contained (mM): 127 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 12 glucose, 10 HEPES, 0.001 TTX, pH 7.3-7.4, 300-305 mOsm. The NMDA receptor antagonist D-aminophosphonovalerate (APV, 20 μM) and GABA_A receptor antagonist bicuculline (10 μM) were added to the external solution. The internal solution consisted of (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 10 HEPES, 1 MgCl₂, 5 EGTA, 2.2 QX-314, 12 phosphocreatine, 5 MgATP, 0.5 Na₂GTP, 0.1 leupeptin, pH 7.2-7.3, 265-270 mOsm. The membrane potential was held at -70 mV during recording. The Mini Analysis program (Synapsoft) was used to analyze the frequency, amplitude, and decay time (the amount of time decaying from 90% to 10% of the peak) of spontaneous synaptic events over a 30 sec period.

Immunostaining in Neuronal Cultures. Surface AMPA receptors were measured as previously described (21, 22). In brief, 2-3 days after transfection, PFC cultures were fixed in 4% paraformaldehyde (20 min, room temperature), but not permeabilized. Following the incubation with 3% bovine serum albumin (BSA, 1 hr) to block nonspecific staining, neurons were incubated with anti-NT-GluR1 (1:500, Upstate) overnight at 4°C. After three washes, neurons were incubated with Alex594 (red)-conjugated secondary antibody (Molecular Probe) for 2 hr at room temperature. After washing in PBS three times, the coverslips were mounted on slides with VECTASHIELD mounting media. Fluorescent images were captured with a 100X objective and a cooled CCD camera mounted on a Nikon microscope using identical parameters and
quantified with the Image J software. To define dendritic clusters, a single threshold was chosen manually, so that clusters corresponded to puncta of 2-3 fold greater intensity than the diffuse fluorescence on the dendritic shaft. On each coverslip, the cluster density, size, and fluorescence intensity of 4-6 GFP-positive neurons (2-3 dendritic segments of ~50 μm per neuron) were compared. Quantitative analyses were conducted blindly.

Co-immunoprecipitation. HEK293 cells transfected with various Flag-tagged GDI constructs were collected and homogenized in cold NP-40 lysis buffer (0.5% NP-40, 10% glycerol, 50 mM Tris pH 7.6, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM EDTA, and 0.1 mM Na3VO4, 10 nM Calyculin, 1 mM PMSF, 10 μg/ml leupeptin, and protease inhibitor tablet). Lysates were ultracentrifuged (100,000 x g) at 4°C for 60 min. Supernatant fractions were incubated with anti-Flag (1:1000, Sigma) for 4 hr at 4°C, followed by incubation with 50 μl of protein A/G plus agarose (Santa Cruz) for 2 hr at 4°C. Immunoprecipitates were washed three times with lysis buffer, then boiled in 2x SDS loading buffer for 5 min, and separated on 12% SDS-polyacrylamide gels. Western blotting experiments were performed with anti-Rab4 (1:1000, Santa Cruz).

Expression and purification of GST fusion proteins. Wild-type GDI and its mutants S45A, S121A, S213A were subcloned to pGEX-4T1 vector from T/A vector, and transformed to BL21 E. Coli bacterial (Stratagene). Expression of GST fusion proteins was induced by treatment with 1 mM IPTG for 3.5 h at 25°C. Cells were collected and lysed in BugBuster protein extraction reagent (Novagen). The resulting lysate was centrifugated, and GST fusion proteins were purified with GSTrapTM HP columns (GE).

Phosphorylation analysis. HEK293 cells were grown in 6 cm dishes in 10% FBS DMEM medium. When cells were 90% confluent, the medium were changed to 0.5% FBS DMEM to limit serum-induced up-regulation of SGK. For in vivo phosphorylation analysis, the following approach was used. HA-tagged wild-type GDI or its mutants were transfected to HEK293 cells using the calcium phosphate method. One day after transfection, HEK293 cells were treated without or with 100 nM corticosterone for 30 min. Cell were then washed 3 times with phosphate-free DMEM medium (Invitrogen). After washing, phosphate-free DMEM medium containing 0.5% FBS, 0.2 mCi/ml of 32P orthophosphate (PerkinElmer), 100 nM calyculin, and 1 μM okadaic acid were added to cell dishes and incubated at 37 °C for 1.5 hr. After incubation, the medium were aspirated, and cells were washed twice with ice-cold phosphate-free DMEM medium. Then cells were lysed on ice with 0.5% NP-40 lysis buffer. Cell lysates were centrifuged at 16,000×g at 4 °C for 20 min, supernatants were immunoprecipitated with anti-HA (1:1000, Roche). After washing, SDS-PAGE was carried in 7.5% gel, and transferred membrane was subjected to autoradiography for visualizing radiolabeled proteins.

For in vitro phosphorylation analysis, the following approach was used. HEK293 cells were transfected with or without SGK1 siRNA. One day after transfection, cells were treated without or with 100 nM corticosterone for 30 min, washed and maintained in 0.5% FBS DMEM for 1.5 hr. Then cells were lysed in the CytoBuster Protein Extraction Reagent (Novagen) containing protease inhibitors. Cell lysates were centrifuged at 16,000×g at 4 °C for 20 min. The supernatants (40 μl, ~50 μg total protein) were incubated with 1 μg purified GST fusion protein of wild-type GDI or its mutants for 30 min at 30 °C in the reaction buffer (30 mM HEPES pH 7.5, 10 mM MgCl2, 30 μM ATP, 1 μCi γ-32P ATP, 100 nM calyculin, 1 μM okadaic acid). SDS-PAGE was carried out, and phosphorylated GDI was visualized with autoradiography.

RESULTS
Corticosterone treatment increases synaptic AMPAR activity via Rab4-mediated membrane trafficking of AMPARs.
First, we examined whether corticosterone influences AMPAR-mediated synaptic currents in
cultured PFC pyramidal neurons. Cells were exposed to a short treatment of corticosterone (100 nM, 30 min), and recorded at 1.5-4 hrs post-treatment. Miniature EPSC (mEPSC), which represents the postsynaptic response to release of individual vesicles of glutamate, was measured. As shown in Fig. 1A, corticosterone caused a significant enhancement of mEPSC amplitude, as indicated by a rightward shift in the distribution (control: 26.1 ± 1.0 pA, n = 21; CORT: 32.8 ± 0.83 pA, n = 23, p < 0.01, ANOVA, Fig. 1E). The frequency of mEPSC was not changed by corticosterone treatment (Fig. 1F). Corticosterone did not significantly change the mEPSC decay kinetics (control: 3.47 ± 0.18 ms, n = 18; CORT: 3.63 ± 0.22 ms, n = 17, p > 0.05, ANOVA).

The corticosterone-induced increase in glutamatergic transmission could be due to increased excytosis/recycling of AMPARs. Thus, we investigated the potential involvement of Rab4, a member of the Rab family controlling early sorting and recycling of proteins to the cell surface from early endosomes (14, 23, 24). Two Rab4 mutants, dominant negative Rab4 (DN-Rab4, Rab4-S27N) or constitutively activate Rab4 (CA-Rab4, Rab4-Q72L) (25) were transfected to PFC cultures. As shown in Fig. 1B, compared to control neurons, transfecting DN-Rab4 caused a significant decrease of mEPSC amplitudes, whereas CA-Rab4 led to a significant enhancement of mEPSC amplitudes. The mEPSC frequency was not changed by DN-Rab4 or CA-Rab4 (Fig. 1F). Moreover, corticosterone failed to increase mEPSC amplitude in neurons transfected with DN-Rab4 (Fig. 1C, DN-Rab4: 20.6 ± 0.95 pA, n = 13; DN-Rab4+CORT: 21.3 ± 0.75 pA, n = 15, Fig. 1E) or CA-Rab4 (Fig. 1D, CA-Rab4: 32.0 ± 0.9 pA, n = 17, CA-Rab4+CORT: 33.1 ± 0.97 pA, n = 15, Fig. 1E). Taken together, these data suggest that Rab4 is involved in AMPAR recycling, and corticosterone increases synaptic AMPAR currents via a Rab4-dependent mechanism.

Corticosterone treatment increases GDI:Rab4 complex.

Next, we sought to determine the mechanism underlying corticosterone enhancement of Rab4-mediated AMPAR synaptic delivery. It is known that GDI retrieves GDP-bound Rab proteins from membranes to cytosol, thus plays a key role in the recycling of Rab proteins (26, 27). Previous studies have found that GDI can be phosphorylated in cytosol, and Rab proteins predominantly interact with phosphorylated GDI (28). Thus, we tested whether corticosterone induces GDI phosphorylation, leading to increased formation of the GDI:Rab4 complex.
GDI contains 26 Ser residues, and 3 residues have been predicted to face the outer surface of the molecule based on its 3-D structure (19, 29), Ser-45, Ser-121 and Ser-213. Thus, we transfected HEK293 cells with Flag-tagged wild-type GDI or non-phosphorylatable GDI mutants, S45A, S121A, and S213A. After transfection, cells were treated with corticosterone (100 nM) for 30 min. After washing for 1.5 hr, cell lysates were subjected to co-immunoprecipitation assay to detect the GDI:Rab4 complex. As shown in Fig. 3A and 3B, corticosterone treatment significantly increased the amount of Rab4 that binds to WT-GDI, S45A-GDI, or S121A-GDI, but not S213A-GDI. The level of total Rab4 was not altered. These data indicate that corticosterone increases the formation of GDI:Rab4 complex, which requires an intact S213 phosphorylation site.

**Corticosterone treatment induces GDI phosphorylation at Ser-213 through SGK.**

The corticosteroid stress hormone exerts its function by activating MRs and GRs, both of which are nuclear transcription factors that induce or repress the expression of target genes (9). Serum- and glucocorticoid-inducible kinase (SGK) is one of the downstream targets of corticosteroid signaling (11). SGK phosphorylates serine and threonine residues in the motif R-X-R-X-X-(S/T), but the -3 site arginine is not quite critical for SGK (30). To examine whether GDI is a potential substrate of SGK, we analyzed the flank sequences of S45, S121, and S213 on GDI. We found that only the flank sequence R-I-K-L-Y-S of S213 matched the substrate motif of SGK (Fig. 4A).

Next, we performed an in vivo phosphorylation assay to detect the potential GDI phosphorylation by corticosterone and the putative phosphorylation site on GDI. HA-tagged wild-type GDI and three mutants of GDI, S45A, S121A, and S213A, were transfected to HEK293 cells. After transfection, cells were treated with corticosterone (100 nM) for 30 min. Following washing off corticosterone, cells were incubated with $32^P$ orthophosphate-containing medium for 1.5 hr. As shown in Fig. 4B and 4C, $32^P$-labeled GDI was increased remarkably by corticosterone treatment in cells transfected with WT-GDI, S45A-GDI, and S121A-GDI, but not in cells transfected with S213A-GDI. These data suggest that corticosterone induces GDI phosphorylation, and Ser-213 is likely to be the phosphorylation site.

We further examined whether SGK is involved in the corticosterone-induced GDI phosphorylation. There are three SGK isforms, SGK1, SGK2 and SGK3, and they share the same substrate motif (12, 31). The mRNAs of SGK1 and SGK3 are widely and highly expressed in all tissues, whereas SGK2 is expressed at a lower level in the brain. Furthermore, SGK1 mRNA is strongly enhanced by stimulation of glucocorticoids (31). To detect the role of SGK1 in GDI phosphorylation, we transfected SGK1 siRNA in HEK293 cells to knockdown its expression. After transfection and corticosterone treatment (30 min), an in vitro phosphorylation assay was performed by adding cell lysates to tubes containing $\gamma^{32}P$ ATP and one of the purified recombinant GST fusion proteins, GST-WT-GDI, GST-S45A-GDI, GST-S121A-GDI or GST-S213A-GDI. As shown in Fig. 4D, the lysates from corticosterone-treated cells induced the phosphorylation of GST-WT-GDI, GST-S45A-GDI, and GST-S121A-GDI, but not GST-S213A-GDI, and this effect was blocked in cells transfected with SGK1 siRNA. These data suggest that corticosterone induces GDI phosphorylation via SGK1.

**GDI phosphorylation at Ser-213 is required for corticosterone regulation of AMPAR synaptic activity and surface expression.**

Given that corticosterone increases GDI phosphorylation and the formation of GDI:Rab4 complex, we further investigated whether GDI phosphorylation could influence synaptic AMPAR activity and its regulation by corticosterone. Since Ser-213 on GDI is likely to be the SGK phosphorylation site, two constructs with mutated Ser-213 were generated. Ser-213 was mutated to Alanine to represent the non-phosphorylatable form (S213A), and Ser-213 was mutated to Aspartic acid to represent the phosphomimetic form (S213D). As shown in Fig. 5A, compared to neurons transfected
with GFP alone, transfecting S213AGDI caused a significant decrease of mEPSC amplitude, whereas transfecting S213DGDI led to a significant enhancement of mEPSC amplitude (Fig. 5D), suggesting that GDI phosphorylation at Ser-213 facilitates synaptic AMPAR activity. Furthermore, the enhancing effect of corticosterone on mEPSC amplitude was blocked by the non-phosphorylatable S213AGDI (Fig. 5B, S213AGDI: 21.3 ± 0.84 pA, n = 17; S213AGDI+CORT: 21.8 ± 0.79, n = 15, p > 0.01, ANOVA, Fig. 5D), and occluded by the phosphomematic S213DGDI (Fig. 5C, S213DGDI: 31.7 ± 1.2 pA, n = 15; S213DGDI+CORT: 32.5 ± 1.5, n = 18, p > 0.01, ANOVA, Fig. 5D). The mEPSC frequency was not altered by these GDI mutants (Fig. 5E). These data suggest that corticosterone increases synaptic AMPAR currents by inducing GDI phosphorylation at Ser-213.

To confirm the role of GDI phosphorylation in corticosterone-induced AMPAR membrane delivery, we performed immunocytochemical experiments to measure AMPAR surface expression in neurons transfected with non-phosphorylatable or phosphomematic GDI mutants. As shown in Fig. 6A, in GFP-transfected neurons, corticosterone treatment (100 nM, 30 min) significantly increased surface GluR1 cluster density (number of clusters/50µm dendrite) (control: 17.5 ± 2.2, n = 18; CORT: 33.6 ± 3.1, n = 17, p < 0.01, ANOVA, Fig. 6D). Transfecting S213AGDI caused a significant decrease of surface GluR1 cluster density and blocked the enhancing effect of corticosterone (Fig. 6B, S213AGDI: 10.3 ± 1.8, n = 15; S213AGDI+CORT: 11.0 ± 1.9, n=17, p > 0.01, ANOVA, Fig. 6D). On the other hand, transfecting S213DGDI significantly increased surface GluR1 cluster density and occluded the enhancing effect of corticosterone (Fig. 6C, S213DGDI: 31.0 ± 3.5, n = 15; S213DGDI+CORT: 32.7 ± 3.1, n=18, p > 0.01, ANOVA, Fig. 6D). These data suggest that GDI phosphorylation at Ser-213 facilitates the membrane trafficking of AMPARs, and corticosterone increases AMPAR surface expression via a mechanism dependent on GDI phosphorylation.

**DISCUSSION**

After stress, the level of stress hormones such as corticosterone is markedly increased. Corticosterone exerts a time- and region-specific action on cellular physiology of limbic neurons (4). In this study, we have investigated the potential molecular mechanisms underlying corticosterone regulation of AMPARs in PFC neurons. The combined electrophysiological, biochemical and immunocytochemical evidence suggest that corticosterone facilitates excitatory synaptic transmission by increasing the Rab4-mediated recycling of AMPARs to synaptic membrane via SGK1-induced phosphorylation of GDI at Ser-213.

It is well recognized that the trafficking of AMPARs plays a key role in controlling excitatory synaptic efficacy (32, 33). The internalization, recycling or spine delivery of AMPA receptors are under the control of Rab family of small GTPases, a key regulator for all stages of membrane traffic (34). For example, Rab5, which controls the transport from plasma membrane to early endosomes (35), is involved in clathrin-dependent AMPAR internalization (36, 37). Rab11, which mediates recycling from recycling endosomes to plasma membrane (38), controls the supply of AMPARs for Long-Term Potentiation (LTP) induced by electrical stimulation (39). Rab8, which is associated with TGN (Trans-Gorgi Network) membranes, plays a role in AMPAR transport to the spine surface (40). Rab4, which controls a rapid direct recycling route from early endosomes to cell surface (24, 41), is critical for maintaining spine size (42). By using dominant-negative and constitutively-active Rab4, we have demonstrated that Rab4 is not only involved in the membrane trafficking of GluR1, but also mediates the corticosterone-induced potentiation of glutamatergic transmission. Corticosterone, via activated Rab4, increases AMPAR recycling by causing its redistribution from early endosomes to the plasma membrane.

A key question is how corticosteroid signaling leads to the activation of Rab4. One possibility is through GDI, an important class of regulatory protein involved in the functional cycle and recycling of Rab GTPases (14). GDI is abundantly expressed in the brain, and the two isoforms, GDI-1
and GDI-2, share 86% identical amino acid sequence and exhibit no major functional differences (43). Biochemical assays show that GDI is able to solubilize the membrane-bound forms of Rab4 and Rab5 in a GDP/GTP-dependent manner (44). Interestingly, the phosphorylation of GDI controls its interaction with Rab proteins (28). It has been found that tyrosine phosphorylation of GDI increases the Rab4 soluable form and the GDI:Rab4 complex (18). Acute insulin treatment of cultured adipocytes, presumably by activating the downstream protein kinase B (PKB), increases cytosolic levels of Rab4 due to the formation of GDI:Rab4 complex (45). Moreover, p38 MAPK activates GDI and stimulates the formation of GDI:Rab5 complex by phosphorylating GDI on Ser-121, therefore facilitating the delivery of Rab5 from endosomes to the plasma membrane and accelerating endocytosis (19). Our biochemical data suggest that corticosterone activates Rab4 by stimulating the formation of GDI:Rab4 complex via SGK1 phosphorylation of GDI.

SGKs phosphorylate serine and threonine residues lying in the motif R-X-R-X-X-(S/T) (46). Because SGK has an absolute requirement for the presence of an arginine residue five residues N-terminal (n-5) to the site of phosphorylation (30, 31), and Ser-213 has an arginine residue at the n-5 position, Ser-213 is the most likely phosphorylation site by SGK. Our mutation experiments confirm that corticosterone/SGK-induced phosphorylation of GDI is at Ser-213.

To further demonstrate that the GDI phosphorylation at Ser-213 underlies corticosterone-induced synaptic plasticity, we have directly tested the impact of non-phosphorylatable or phosphomematic GDI mutants on the AMPAR and its regulation by corticosterone. The non-phosphorylatable S213A GDI reduces AMPAR trafficking/function and blocks the enhancing effect of corticosterone, while phosphomematic S213D GDI increases AMPAR trafficking/function and occludes the enhancing effect of corticosterone. It suggests that corticosteroid signaling facilitates excitatory synaptic transmission by increasing GDI phosphorylation at Ser-213.

In summary, we have revealed a potential mechanism for corticosterone regulation of AMPARs. This stress hormone-induced changes in glutametric transmission could alter cognitive functions subserved by PFC (13). Understanding molecular and cellular mechanisms underlying the actions of corticosterone will provide valuable targets for designing novel therapies that modify the neuronal stress response (47).
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

FIG. 1. Rab4 is involved in corticosterone regulation of synaptic AMPAR currents. A, Cumulative plots of the distribution of mEPSC amplitudes in untreated (control) or corticosterone (100 nM, 30 min)-treated cortical neurons transfected with GFP. B, Cumulative plots of the distribution of mEPSC amplitudes in cultured neurons transfected with GFP alone, or GFP plus dominant-negative Rab4 (DN-Rab4), or GFP plus constitutively-active Rab4 (CA-Rab4). C, D, Cumulative plots of the distribution of mEPSC amplitudes in untreated (control) or corticosterone-treated neurons transfected with DN-Rab4 (C) or CA-Rab4 (D). Inset (A, C, D), representative mEPSC traces. Scale bars, 30 pA, 1 s. E, F, Bar graphs (mean ± S.E.) showing the mEPSC amplitude (E) and frequency (F) in control vs. corticosterone-treated neurons transfected with different constructs. *, p < 0.01, ANOVA.

FIG. 2. Rab4 is involved in corticosterone regulation of AMPAR surface expression. A-C, Immunocytochemical images of surface GluR1 staining in untreated (control) or corticosterone (100 nM, 30 min)-treated cortical neurons cultures (DIV20) transfected with GFP alone (A), or GFP plus DN-Rab4 (B), or GFP plus CA-Rab4 (C). Enlarged versions of the boxed regions of dendrites are shown beneath each of the images. D-F, Cumulative data (mean ± S.E.) showing the clusters density (D), clusters size (E), fluorescence intensity (F) of surface GluR1 in control vs. corticosterone-treated neurons transfected with different constructs. *, p < 0.01, ANOVA.

FIG. 3. Corticosterone treatment increases the formation of GDI:Rab4 complex, which is blocked by mutating Ser-213. A, Co-immuno precipitation blots showing the level of Rab4 that binds to GDI in HEK293 cells transfected with Flag-tagged wild-type GDI or its three mutants, S45A, S121A, S213A. After transfection, cells were treated without or with corticosterone (100 nM) for 30 min. A control for the amount of Flag-GDI effectively immunoprecipitated is also shown. HC, heavy chain. B, Quantification showing the normalized level of GDI-bound Rab4 in control vs. corticosterone-treated HEK293 cells transfected with different GDI constructs. p < 0.01, ANOVA.

FIG. 4. Corticosterone treatment increases GDI phosphorylation by SGK1, which is blocked by mutating Ser-213. A, Diagram of rat GDI-1 protein showing the three serine sites that face the outer surface, and the substrate motif of SGK1. Only Ser-213 matches the motif. B, Representative autoradiography of GDI in vivo phosphorylation assay in HEK293 cells transfected with HA-tagged wild-type GDI or its three mutants. After corticosterone treatment (100 nM, 30 min), 32P orthophosphate was incorporated into the cells. Cell lysates were immunoprecipitated with the HA antibody, and 32P-labeled proteins were subjected to SDS-PAGE and visualized with autoradiography (upper panel). A loading control was shown in the HA blots (lower panel). HC, heavy chain. C, Quantification showing the normalized radioactive intensity of 32P-labeled GDI in control vs. corticosterone-treated HEK293 cells transfected with different GDI constructs. *, p < 0.01, ANOVA. D, Representative autoradiography of GDI in vitro phosphorylation assay in HEK293 transfected without or with SGK1 siRNA. After corticosterone treatment (100 nM, 30 min), cell lysates were added to reaction tubes including 1 µCi γ-32P ATP and 1 µg purified GST fusion protein of wilde-type GDI or its three mutants. Phosphorylated GDI proteins were subjected to SDS-PAGE and visualized with autoradiography.
FIG. 5. Phosphorylation of GDI at Ser-213 is required for corticosterone regulation of synaptic AMPAR currents. A, Cumulative plots of the distribution of mEPSC amplitudes in cultured cortical neurons transfected with GFP alone, or GFP plus S213A GDI (non-phosphorylatable mutant), or GFP plus S213D GDI (phosphomimetic mutant). B, C, Cumulative plots of the distribution of mEPSC amplitudes in untreated (control) or corticosterone (100 nM, 30 min)-treated neurons transfected with S213A GDI (B) or S213D GDI (C). Inset: Representative mEPSC traces. Scale bars: 30 pA, 1 s. D, E, Bar graphs (mean ± S.E.) showing the mEPSC amplitude (D) and frequency (E) in control vs. corticosterone-treated neurons transfected with different constructs. *, p < 0.01, ANOVA.

FIG. 6. Phosphorylation of GDI at Ser-213 is required for corticosterone regulation of AMPAR surface expression. A, B, C, Immunocytochemical images of surface GluR1 staining in control or corticosterone (100 nM, 30 min)-treated neurons transfected with GFP alone (A), or GFP plus S213A GDI (B), or GFP plus S213D GDI (C). D, Cumulative data (mean ± S.E.) showing the surface GluR1 cluster density in control vs. corticosterone-treated neurons transfected with different constructs. *, p < 0.01, ANOVA.
Fig. 1

A. GFP transfected

B. Controls and CORT

C. DN-Rab4 transfected

D. CA-Rab4 transfected

E. mEPSC Amp (pA)

F. mEPSC Freq (HZ)
Fig. 2

A. GFP control CORT

B. DN-Rab4 control CORT

C. CA-Rab4 control CORT

D. Control CORT

E. Control CORT

F. Control CORT

Surface GluR1 cluster size (µm²)
Fig. 3

A

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IP: Flag
WB: Rab4

WB: Rab4

WB: Flag

IP: Flag
WB: Flag

Flag-GDI, ~57kD
IgG HC

B

![Graph showing normalized GDI-binding Rab4](image)

Control
CORT

WT-GDI
S45A-GDI
S121A-GDI
S213A-GDI

* Indicates significant difference.
Fig. 4

A

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Substrate motifs of SGK1: R-X-R-X-X-(S/T)

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32P HA-GDI

IP: HA

WB: HA

HA-GDI, ~57kD

IgG HC

C

D

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<th>GST-S45AGD</th>
<th>GST-S121AGD</th>
<th>GST-S213AGD</th>
</tr>
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<td>-</td>
<td>+</td>
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</tbody>
</table>

32P GST-GDI

 normalized 32P HA-GDI
Fig. 5

A

B

C

D

E

mEPSC Amp (pA)

mEPSC Freq (Hz)

GFP

S213A-GDI

S213D-GDI

controls

CORT

controls

CORT

controls

CORT

- +

- +

- +

- +

- +

- +

- +

- +

- +

- +

- +

- +

- +
Fig. 6

A  GFP-transfected
   control  CORT

B  \(S^{213A}\)GDI-transfected
   control  CORT

C  \(S^{213D}\)GDI-transfected
   control  CORT

D  

\(\#\) surface GluR1 clusters/50 µm dendrite

GFP  S213A  S213D

control  CORT  CORT

* * *
The stress hormone corticosterone increases synaptic AMPA receptors via SGK regulation of the GDI:Rab4 complex
Wenhua Liu, Eunice Y. Yuen and Zhen Yan

J. Biol. Chem. published online January 5, 2010

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