Myosin IIb-dependent Regulation of Actin Dynamics Is Required for N-Methyl-d-aspartate Receptor Trafficking during Synaptic Plasticity*

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Background: The motor protein myosin IIb has been detected in the N-methyl-d-aspartate receptor (NMDAR)-associated protein complex.

Results: Myosin IIb is essential for NMDAR synaptic incorporation during synaptic plasticity.

Conclusion: The myosin light chain kinase (MLCK)- and myosin IIb-dependent regulation of actin dynamics is required for NMDAR trafficking during synaptic plasticity.

Significance: This study provides new insight into how the actin cytoskeleton underpins NMDAR plasticity.

N-Methyl-d-aspartate receptor (NMDAR) synaptic incorporation changes the number of NMDARs at synapses and is thus critical to various NMDAR-dependent brain functions. To date, the molecules involved in NMDAR trafficking and the underlying mechanisms are poorly understood. Here, we report that myosin IIb is an essential molecule in NMDAR synaptic incorporation during PKC- or β burst stimulation-induced synaptic plasticity. Moreover, we demonstrate that myosin light chain kinase (MLCK)-dependent actin reorganization contributes to NMDAR trafficking. The findings from additional mutual occlusion experiments demonstrate that PKC and MLCK share a common signaling pathway in NMDAR-mediated synaptic regulation. Because myosin IIb is the primary substrate of MLCK and can regulate actin dynamics during synaptic plasticity, we propose that the MLCK- and myosin IIb-dependent regulation of actin dynamics is required for NMDAR trafficking during synaptic plasticity. This study provides important insights into a mechanical framework for understanding NMDAR trafficking associated with synaptic plasticity.

The N-methyl-D-aspartate (NMDA)-type glutamate receptor plays a central role in various physiological processes in the central nervous system, including neural development, learning and memory, sensory perception, and synaptic plasticity (1, 2). Accordingly, a change in NMDAR-mediated neurotransmission may exert an important influence on these brain functions. An alteration in the number of NMDARs at the postsynaptic membrane, which may be achieved via the regulation of NMDAR trafficking at postsynaptic sites, is an important molecular mechanism that underlies changes in synaptic efficacy in NMDAR-mediated neurotransmission. Notably, the insertion or internalization of NMDARs at the postsynaptic membrane is tightly regulated, both during development and in response to synaptic activity and sensory experience (3). PKC activation promotes new NMDARs to the surface of hippocampal neurons (4) and has been used as an important model to investigate NMDAR trafficking (5–7). Rapid channel insertion occurs through SNARE-dependent exocytosis and does not require the direct phosphorylation of NMDAR subunits; instead, it requires a receptor anchoring and/or trafficking protein (8), which suggests that PKC indirectly exerts its effect through interactions with NMDAR-associated signaling and/or protein trafficking (3).

The motor protein myosin IIb has been detected in the NMDAR-associated protein complex (NRC) (9, 10), which indicates its potential role in the trafficking of NMDAR or NRC between the postsynaptic membrane and intracellular com-

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3 The abbreviations used are: NMDAR, NMDA receptor; aMLCK, constitutively active form of MLCK; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-f[1]quinoxaline-2,3-dione; NRC, NMDAR-associated protein complex; LTP, long term potentiation; CaMKII, calcium/calcmodulin-dependent protein kinase II; TBS, β burst stimulation; AMPAR, AMPA receptor; PMA, phorbol 12-myristate 13-acetate; TIF, Triton X-100-insoluble fraction; EGFP, enhanced green fluorescence protein; ROI, region of interest; ANOVA, analysis of variance; TeTx, tetanus toxin; rAAV, recombinant adenovirus-associated virus; EPSC, excitatory postsynaptic current; Blebb, blebbistatin; p-cofilin, phosphorylated cofilin; LatA, latrunculin A.

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dements (11). Class II myosin includes motors that constitute the thick filaments of smooth, skeletal, and cardiac muscles. Members of this class were initially referred to as “conventional myosin” largely because myosin II isoforms are present in these tissues and were thus the first contractile proteins investigated (12, 13). However, a more recent study demonstrated that myosin IIb, a cytoplasmic myosin II that is particularly enriched in the central nervous system, shares a number of features with “unconventional” myosin isoforms, which suggests that myosin IIb exhibits differential functions compared with its conventional myosin II counterparts. This suggestion received substantial supporting evidence from subsequent studies (12, 14).

Myosin IIb was subsequently demonstrated to be involved in growth cone motility (15) and the regulation of actin dynamics during synaptic plasticity and memory formation (16, 17). Because dendritic spine morphology typically involves the dynamic modulation of actin polymerization (18–20), this finding can be explained, at least in part, by the critical role of myosin IIb in dendritic spine morphology and synaptic function (21). As a result of its short coil-coil domain and its lack of a global tail domain, however, it is unlikely that myosin IIb is directly employed in the loading and transport of NMDARs. Therefore, the potential role of myosin IIb in NMDAR trafficking remains unclear.

Cofilin is an actin-binding protein that can regulate actin cytoskeleton dynamics and, in turn, intracellular receptor trafficking during synaptic plasticity (12, 14, 22, 23). Actin reorganization underlies the bidirectional plasticity in various models, including long term potentiation (LTP) (24, 25), a cellular model for learning and memory (26). It has been proposed that F-actin polymerization is not only required for the stabilization of early LTP (16) but also supports the eventual capture of plasticity-related proteins involved in the stabilization of late phase LTP (27). NMDAR combined with autophosphorylated CaMKII translocates to the postsynaptic membrane following exposure to LTP-inducing stimuli, including PKC activation and θ burst stimulation (TBS) (7). Importantly, this NMDAR-CaMKII complex is proposed to serve as a “seed” for anchoring other plasticity-related proteins, including AMPA receptors (AMPARs) (28, 29). We thus hypothesize that the myosin IIb-dependent regulation of actin dynamics is crucial for NMDAR trafficking during synaptic plasticity.

In the present study, we aimed to identify the potential role of myosin IIb in NMDAR trafficking during synaptic plasticity. We demonstrated that the knockdown of myosin IIb or the pharmacological blockade of myosin IIb activity suppresses the potentiation in postsynaptic NMDAR expression and function induced by PKC activation or TBS. We further demonstrated that myosin light chain kinase (MLCK)- and myosin IIb-dependent actin reorganization is required for NMDAR trafficking. These findings facilitate the elucidation of a mechanical framework for understanding NMDAR trafficking associated with synaptic plasticity.

Materials and Methods

Hippocampal Slices—The protocols for animal care and use were approved by the Experimental Animal Ethics Committee at Nanjing Medical University. Sprague-Dawley rats, aged 13–18 days, were anesthetized with ethyl ether and decapitated. Their entire brains were then quickly removed. Three hundred fifty-μm-thick brain slices were cut using a vibratome (VT1000S, Leica, Germany) in ice-cold artificial cerebrospinal fluid that contained 126 mmol/liter NaCl, 2.5 mmol/liter KCl, 1 mmol/liter MgCl2, 1 mmol/liter CaCl2, 1.25 mmol/liter KH2PO4, 26 mmol/liter NaHCO3, and 20 mmol/liter glucose at pH 7.4, which was gassed with 95% O2 and 5% CO2. The fresh slices were incubated in a chamber with oxygenated artificial cerebrospinal fluid, followed by recovery at 34°C for 1 h.

Electrophysiological Recordings in Acute Slices—The recovered hippocampal slices were used for electrophysiological recordings. Conventional whole-cell recordings were obtained with patch pipettes that contained 132.5 mmol/liter cesium gluconate, 17.5 mmol/liter CsCl, 2 mmol/liter MgCl2, 0.5 mmol/liter EGTA, 10 mmol/liter HEPES, 4 mmol/liter ATP, and 5 mmol/liter QX-314 with the pH adjusted to 7.2 with CsOH. LTP was induced with phosphol 12-myristate 13-acetate (PMA; 0.5 μM) or TBS (10 bursts of 4,100-Hz single pulses with a 200-ms interburst interval) at Schaffer collateral CA1 synapses. The data were collected with pClamp version 9.2 software and analyzed using Clampfit version 9.2 software (Molecular Devices, Palo Alto, CA).

Western Blotting—The homogenized slices were stored in cold 0.32 M sucrose that contained 1 mmol/liter HEPES, 1 mmol/liter MgCl2, 1 mmol/liter NaHCO3, 20 mmol/liter sodium pyrophosphate, 20 mmol/liter β-phosphoglycerol, 0.2 mmol/liter dithiothreitol, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 50 mmol/liter NaF, 1 mmol/liter Na3VO4, and 1 mmol/liter p-nitrophenil phosphate at pH 7.4 in the presence of the following protease and phosphatase inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 g/ml aproatin, 5 g/ml leupeptin, 5 g/ml pepstatin A, and 16 g/ml benzamidine. The homogenate was centrifuged at 1,000 × g for 10 min, and 200–400 μl of the resulting supernatant was collected as the total protein fraction. The remainder was centrifuged at 3,000 × g for 15 min, and its supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in 8 ml of hypotonic buffer (in the presence of protease inhibitors) and centrifuged at 100,000 × g for 1 h. The resultant pellet was resuspended in 8 ml of buffer that contained 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000 × g for 1 h. The final pellet was resuspended in 20 μl HEPES and homogenized three times (10 s × 3, intertrial interval of 10 s). This fraction was referred to as the Triton X-100-insoluble fraction (TIF). The TIF was used instead of the classical postsynaptic density preparation because of the limited amount of hippocampal slices (7). The protein content was measured using a BCA protein assay (Pierce).

Twenty μg of protein were boiled and separated with 7.5% SDS-PAGE and transferred onto a PVDF membrane. The membranes were blocked in 3% (w/v) BSA (fraction V) with TBST (0.1% Tween 20) for 1 h at room temperature and incubated with primary antibodies at room temperature for 2 h. After five washes with TBST for 30 min, the membranes were incubated for 1 h at room temperature with secondary antibodies and washed again using the same protocol. The ECL Western blotting detection reagents (GE Healthcare) were added, and the intensity of the bands was quantified using ImageJ software.
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(National Institutes of Health). Tubulin was employed as the internal reference to normalize the volume of the samples. The mean intensity of each individual band was calculated after background value subtraction, followed by normalization to the mean intensity of the loading tubulin band for each sample in the same lane. Thus, tubulin intensity was normalized as 1, and the relative values of other samples were determined. Subsequently, these normalized values were normalized again to the mean intensity of the loading control band for each sample. The control of each experiment was thus normalized as 1, and the relative values of other groups under various treatments were determined.

**shRNA Design, Packaging, and Stereotactic Virus Injection**—The shRNA sequence that targeted the rat isoform of MyoH10 and the sequence for the MyoH10-specific shRNA was gataacagttggccgagat. This sequence was subsequently cloned into plasmids for packaging into recombinant adeno-associated virus (rAAV1) particles constructed with rAAV2 coat proteins (rAAV2/1). The virus titer was 1–1.5 × 10^12 genome copies/ml, and the EGFP rAAV2/1 virus was a generous gift from Biomiao Biological Technology Co. (Beijing, China). Rats at 4–5 days after birth were anesthetized with 1% trichloroacetaldehyde hydrate and fixed in a stereotaxic frame that contained a needle against GluN1 under membrane-permeable conditions. To further examine these findings following PKC activation, we performed an immunofluorescence assay with hippocampal cultured cells to determine the influence of PMA (0.5 μM) on the postsynaptic localization, expression, and function of NMDARs. Using PSD-95 as a postsynaptic marker, we first performed an immunofluorescence assay with hippocampal cultured cells to determine the influence of PMA (0.5 μM) on the postsynaptic localization of NMDARs. The postsynaptic localization of GluN1 was determined by quantifying the degree of colocalization of GluN1 and PSD-95 using a C-terminal antibody against GluN1 under membrane-permeable conditions. We identified an increased GluN1 density at synapses following 15 min of PMA (0.5 μM) treatment (1.34 ± 0.01; n = 19, from 19 ROIs imaged in 19 neurons from eight independent cultures; p < 0.01, one-way ANOVA; Fig. 1, A1 and A2). To further examine these findings following PKC activation, we performed a Western blot assay of the TIF of hippocampal sliced

**Results**

**PKC Activation Triggers NMDAR Insertion at Postsynaptic Sites**—Before we investigate the potential role of myosin IIb in NMDAR trafficking, we must establish a reliable model of NMDAR trafficking at postsynaptic sites. It has been reported that PKC activation promotes NMDAR trafficking to the cell surface (3, 4). However, it remains unclear whether PKC activation triggers NMDAR insertion and subsequent enhancement of NMDAR function at postsynaptic sites. Thus, we examined the effect of the PKC agonist PMA (0.5 μM) on the postsynaptic localization, expression, and function of NMDARs. Using PSD-95 as a postsynaptic marker, we first performed an immunofluorescence assay with hippocampal cultured cells to determine the influence of PMA (0.5 μM) on the postsynaptic localization of NMDARs. The postsynaptic localization of GluN1 was determined by quantifying the degree of colocalization of GluN1 and PSD-95 using a C-terminal antibody against GluN1 under membrane-permeable conditions. We identified an increased GluN1 density at synapses following 15 min of PMA (0.5 μM) treatment (1.34 ± 0.01; n = 19, from 19 ROIs imaged in 19 neurons from eight independent cultures; p < 0.01, one-way ANOVA; Fig. 1, A1 and A2). To further examine these findings following PKC activation, we performed a Western blot assay of the TIF of hippocampal sliced

**Immunofluorescence**—On days 17 and 18, the neurons were treated with drugs (rAAV2/1 virus was added 1 week earlier) and fixed in 4% paraformaldehyde for 20 min at −20 °C. The cells were washed five times with PBS in a shaker and blocked with 10% fetal calf serum (Invitrogen) without Triton X-100 at room temperature for 2 h. The detection and secondary antibodies were diluted in 10% fetal calf serum with 0.5% Triton X-100. The cells were incubated with primary antibodies at 4 °C overnight, washed as described previously, and incubated with secondary antibodies at room temperature for 2 h. After the cells were washed, glass coverslips were fixed with gold antifade reagent (Invitrogen). Nail polish was subsequently added around the glass coverslips. The experiment was conducted in the dark when the secondary antibodies were used.

**Image Acquisition and Quantification**—All immunofluorescence images were analyzed using Fiji software (National Institutes of Health). The statistical analyses were performed on a number of regions of interest (ROIs) that contained numerous spines from various cultures. The colocalization was calculated using the Coloc 2 plugin in Fiji following the instructions provided on the Coloc 2 Web site. The Pearson r values obtained from the plugin were automatically selected as the “colocalization values”. Furthermore, the n values indicate n different images, and in every image, one ROI was selected. The ROIs comprised random areas of a 50-μm dendritic shaft located ∼30 μm from the cell body.

**MLCK-knock-out Mice**—The MLCK floxed mice were generated as briefly described elsewhere (32). Neural specific MLCK-knock-out mice were generated by crossing MLCK floxed mice with Nestin-Cre transgenic mice (33) in the laboratory of Prof. Minsheng Zhu at Nanjing University. The animals were maintained in specific pathogen-free standard animal rooms at the National Resource Center for Mutant Mice of China.

**Antibodies and Drugs**—Anti-tubulin (ComWin Biotech), anti-GluN1 (Santa Cruz Biotechnology, Inc.), anti-myosin II (Covance), anti-phosphorylated coflin (p-coflin) (Abcam), anti-coflin (Abcam), anti-phosphorylated MLCK (CST), anti-MLC (CST), anti-mouse IgG-HRP (GE Healthcare), rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP (SunShine Biotechnology), poly-d-lysine (Sigma), B27 (Life Technologies, Inc.), glutamaxTM (Life Technologies), β-mercaptoethanol (Amresco), PMA (Sigma), BMI (TOCRIS), NBQX (Sigma), tetanus toxin (TeTx) (Sigma), blebbistatin (TOCRIS), Iatrunculin A (Invitrogen), and ML-7 (Biomol) were used.

**Data Analysis**—All population data are expressed as the means ± S.E. Paired sample t tests were used to assess statistical significance, and independent sample t tests and analysis of variance (ANOVA) were performed for comparisons between multiple groups. p values of <0.05 were considered statistically significant.
tissue (34), which roughly represents the subcellular fraction at postsynaptic sites (34, 35). We identified a significant increase in the postsynaptic GluN1 expression (1.30 ± 0.07; n = 6, from six samples of 36 independent animals; p < 0.01, one-way ANOVA; Fig. 1, B1 and B2). These findings support the notion that PKC activation promotes NMDAR trafficking to postsynaptic sites.

To further determine whether this enhancement in the level of postsynaptic NMDARs leads to a corresponding enhancement in NMDAR function, we examined the effects of PKC.
activation on evoked NMDAR-mediated excitatory postsynaptic currents (NMDA EPSCs) in CA1 hippocampal neurons from acute brain slices. CA1 pyramidal cells were held at +40 mV, which allows relief of the Mg\(^{2+}\) blockage of NMDARs; NMDA EPSCs were subsequently recorded under a whole-cell patch clamp configuration. We identified an increase in NMDA EPSCs after 20 min of PMA treatment (PMA, 1.91 ± 0.33; n = 6, from six whole-cell recordings of five independent animals; p < 0.01, paired sample t test; Fig. 1, C1 and C2). This enhancement of NMDA EPSCs was largely attenuated via the application of the SNARE-dependent exocytosis blocker TeTx (composed of a heavy chain (100 kDa) and a light chain (57 kDa) connected through an interchain disulfide bond, 0.1 μM) in the recording pipette solution (TeTx + PMA, 1.13 ± 0.11; n = 6, from six whole-cell recordings of five independent animals; p > 0.05, paired sample t test). These findings suggest that the insertion of new NMDARs into the postsynaptic membrane accounts for this enhancement in NMDAR function.

Myosin IIb Is Required for NMDAR Trafficking—Myosin IIb has been identified in the NMDA NRC (9), which suggests that this actin-dependent motor may be involved in NRC trafficking between the postsynaptic membrane and intracellular compartments or the facilitation of NRC movements within the postsynaptic membrane (11). To investigate the potential role of myosin IIb in NMDAR trafficking, we designed and packaged rAAV particles that express shRNA in vitro or in vivo and selectively targeted the myosin IIb heavy chain MyH10 (16). A recombinant adeno-associated virus (rAAV2) particle constructed with rAAV1 coat proteins (rAAV2/1) is a particularly effective tool for selectively transducing hippocampal neurons (36). These virus particles were in the same virus package (rAAV2/1) but contained two expression cassettes. The resulting EGFP expression facilitates the location of the transfected neurons or regions of CA1 to be studied. For the in vivo experiments, we injected the MyH10 shRNA virus into the dorsal hippocampus (Fig. 2C) and used the uninjectected side as an internal control (Naive) for hairpin expression. The effect of myosin IIb knockdown by the virus was evaluated via Western blot assay in both transfected cultured cells (Fig. 2A) and hippocampal tissues (Fig. 2C). Myosin IIb shRNA driven by these particles caused an ~36% reduction in myosin IIb expression in the cultured cells and a 46% reduction in the homogenate collected from the dorsal hippocampus closest to the injection point compared with the naive hippocampal samples.

We subsequently employed immunofluorescence assays to determine the influence of myosin IIb knockdown on the PMA-induced enhancement in the postsynaptic localization of NMDARs. PMA (0.5 μM) application elicited an enhancement in the postsynaptic localization of NMDARs (PMA, 1.25 ± 0.01; n = 16, from 16 ROIs imaged in 16 neurons from seven independent cultures; p < 0.01, one-way ANOVA; Fig. 2B), as indicated via the calculation of GluN1 colocalization with the postsynaptic marker PSD-95 (37, 38). This enhancement in the postsynaptic localization of NMDARs, however, was not identified in the cells transfected with rAAV2/1 that targeted the myosin IIb heavy chain MyH10 (MyH10 + PMA, 1.08 ± 0.07; n = 19, from 19 ROIs imaged in 19 neurons from seven independent cultures; p > 0.05, one-way ANOVA; Fig. 2, B1 and B2). The KO of myosin IIb did not affect the amplitude of the evoked NMDA EPSCs (normalized amplitude, control 1.00 ± 0.07, myosin IIb KO 1.01 ± 0.05; n = 5, from five whole-cell recordings of four independent animals; p > 0.05; Fig. 2, D1 and D2). Moreover, the hippocampal neurons in slices obtained from the myosin IIb-knockdown tissues failed to exhibit an enhancement in NMDA EPSCs following PMA application (PMA, 1.71 ± 0.19; n = 4, from four whole-cell recordings of four independent animals; p < 0.05, paired sample t test; MyH10 + PMA, 1.00 ± 0.10; n = 9, from nine whole-cell recordings of seven independent animals; p > 0.05, paired sample t test; Fig. 2, D3 and D4). Combined with previous findings that indicate the contribution of NMDAR trafficking to PKC-induced potentiation in surface NMDAR expression and NMDAR-mediated currents (4, 6), these results suggest that myosin IIb plays an essential role in PMA-induced NMDAR trafficking.

To further investigate the involvement of myosin IIb in NMDAR trafficking, we applied blebbistatin (Blebb), a specific inhibitor of myosin II ATPase activity (39), to cultured cells to determine how the inhibition of myosin IIb activity would affect PMA-induced NMDAR trafficking. Blebb has been reported to selectively inhibit the myosin II ATPase motor without affecting the function of other classes of myosin (e.g. myosin V and myosin VI) (40). Moreover, it does not alter the basic properties of synaptic transmission or spine morphology (16). We demonstrated that cultured CA1 hippocampal neurons pretreated with Blebb (10 μM) for 30 min failed to exhibit PMA-induced enhancement in the postsynaptic localization of NMDARs (PMA, 1.26 ± 0.01; n = 17, from 17 ROIs imaged in 17 neurons from seven independent cultures; p < 0.01, one-way ANOVA; Blebb + PMA, 0.96 ± 0.03; n = 16, from 16 ROIs imaged in 16 neurons from seven independent cultures; p > 0.05, one-way ANOVA; Blebb, 1.08 ± 0.04; n = 7, from 7 ROIs imaged in 7 neurons from three independent cultures; p > 0.05, one-way ANOVA; Fig. 3A). Consistently, the PMA-induced enhancements in postsynaptic NMDAR and myosin IIb expression were also not observed, as indicated by a Western blot assay of the TIF preparation obtained from hippocampal slices, which roughly represents the postsynaptic fraction (GluN1: PMA, 1.22 ± 0.05; n = 6; p < 0.01, one-way ANOVA; Blebb + PMA, 1.09 ± 0.04; n = 6; p > 0.05, one-way ANOVA; Blebb, 1.08 ± 0.06; n = 6; p > 0.05, one-way ANOVA; myosin IIb: PMA, 1.65 ± 0.19; n = 6; p < 0.01, one-way ANOVA; Blebb + PMA, 1.14 ± 0.12; n = 6; p > 0.05, one-way ANOVA; Blebb, 1.07 ± 0.05; n = 6; p > 0.05, one-way ANOVA; n values were obtained from six samples of 36 independent animals; Fig. 3B). Accordingly, the PMA-induced enhancement in postsynaptic NMDAR function, which was denoted by NMDA EPSCs in this study, was also suppressed in hippocampal slices pretreated with Blebb (PMA, 1.96 ± 0.19; n = 4, from four whole-cell recordings of four independent animals; p < 0.01, paired sample t test; PMA + Blebb, 1.10 ± 0.21; n = 6, from six whole-cell recordings of seven independent animals; p > 0.05, paired sample t test; Fig. 3C). Taken together, these findings provide solid support for the hypothesis that myosin IIb is required for NMDAR trafficking induced by PKC activation.
PMA-induced Actin Reorganization Contributes to NMDAR Trafficking—How does myosin IIb undertake its role in NMDAR trafficking following PKC activation? PKC activation with PMA can elicit a chemical form of LTP (41), which represents a cellular model that is widely considered the substrate of synaptic plasticity and learning and memory (25, 26). This form of synaptic plasticity is typically accompanied by changes in dendritic spine morphology (18, 42, 43). Accumulating evidence suggests that these morphological changes involve the dynamic reorganization of the actin cytoskeleton (44, 45). Cofilin is an actin-binding protein that can regulate actin cytoskeleton dynamics through its filament-severing and monomer-binding activities (12, 14). Importantly, cofilin-mediated actin dynamics regulate intracellular receptor trafficking during synaptic plasticity (22, 23). Moreover, myosin IIb regulates actin dynamics in response to LTP-inducing stimuli (16). We hypothesize that NMDAR trafficking following PKC activation involves both the myosin IIb-dependent and cofilin-mediated
regulation of actin dynamics. To examine this hypothesis, we first determined how PKC activation affects the levels of p-cofilin (the inhibited form of cofilin) and total cofilin. Using an antibody specific to p-cofilin, we demonstrated that the amount of p-cofilin in the cytoplasmic fraction of CA1 neurons in hippocampal slices immediately decreased (~3 min) after the onset of PMA treatment but recovered and even exceeded the baseline level at 40 min after PMA washout (Fig. 4, A1 and A2). In contrast, the total cofilin detected with a pan-cofilin antibody did not significantly change following PMA treatment (PMA 3 min, 0.94 ± 0.05; n = 4, from four samples of 16 independent animals; p > 0.05, one-way ANOVA; PMA 40 min, 1.00 ± 0.01; n = 5, from five samples of 20 independent animals; p > 0.05, one-way ANOVA). The change in the p-cofilin levels at 3 and 40 min after PMA washout was not identified in the CA1 neurons in hippocampal slices that were pretreated with Blebb, an inhibitor of myosin IIb ATPase activity (10 μM; Fig. 4, A3 and A4). To better describe the changes in cofilin phosphorylation, we quantified the Western blot data and normalized the p-cofilin signal to the total cofilin level. We determined that PKC activation by PMA caused a short term decrease in the p-cofilin/total cofilin ratio, which indicates transient cofilin dephosphorylation and activation (PMA 3 min, 0.79 ± 0.05; n = 6, from six samples of 24 independent animals; p < 0.01, one-way ANOVA; PMA 40 min, 1.03 ± 0.09; n = 6, from six samples of 24 independent animals; p < 0.01; Fig. 4A5). When these neurons were allowed to recover for 40 min after 15 min of PMA treatment, the p-cofilin/total cofilin exceeded the control level (PMA 40 min, 1.14 ± 0.02; n = 5, from five samples of 20 independent animals; p < 0.01, one-way ANOVA). This temporal sequence of cofilin dephosphorylation and phosphorylation following PKC activation was not identified following blockade of myosin IIb (PMA + Blebb 3 min, 1.00 ± 0.13; n = 6, from six samples of 24 independent animals; p > 0.05, one-way ANOVA; PMA + Blebb 40 min, 0.91 ± 0.09; n = 9, from nine samples of 36 independent animals; p > 0.05, one-way ANOVA; Fig. 4A5). Notably, similar biphasic changes were identified in the p-cofilin level during chemical LTP, which was induced by briefly exposing cells to the potassium channel blocker tetraethylammonium (22, 46).

The temporal sequence of cofilin dephosphorylation and phosphorylation suggests that actin undergoes depolymerization and polymerization consecutively following chemical LTP. To determine whether actin polymerization is required for NMDAR trafficking following PKC activation, we used latrunculin A (LatA), an agent known to suppress actin polymerization via actin monomer sequestration, to investigate whether the inhibition of actin polymerization (without disrupting the existing actin network) could affect the surface addition of NMDARs during PMA-induced LTP. We pretreated 16–18-day in vitro hippocampal cultured neurons with LatA (10 μM) for 10 min, followed by PMA for 15 min. Immunofluorescence assays were performed before and after PMA or PMA plus LatA treatment. We demonstrated that the co-application of PMA (0.5 μM) and LatA failed to exhibit an addition of NMDARs at postsynaptic sites (PMA, 1.29 ± 0.03; n = 20, from 20 ROIs imaged in 20 neurons from nine independent cultures; p < 0.01, one-way ANOVA; LatA + PMA, 1.00 ± 0.03; n = 16, from 16 ROIs imaged in 16 neurons from seven independent cultures; p > 0.05, one-way ANOVA; Fig. 4B). This observation further supports the findings obtained from the examination of NMDAR function. In contrast to the facilitation of NMDA EPSCs following PMA treatment, we did not identify significant changes in the NMDA responses in acute hippocampal cells pretreated with LatA (PMA, 2.03 ± 0.50; n = 4, from four whole-cell recordings of four independent animals; p > 0.05, paired sample t test; LatA + PMA, 1.06 ± 0.10; n = 7, from seven whole-cell recordings of five independent animals; p > 0.05, paired sample t test; Fig. 4C). These results are consistent with a previous finding that indicated that depolymerization of the cytoskeleton modulates NMDAR activity (47) and suggest that PMA-induced actin reorganization is required for NMDAR trafficking at postsynaptic sites.

**Actin-dependent Regulation of NMDAR Trafficking by MLCK—** Because myosin IIb regulates actin dynamics and its activity is required for NMDAR trafficking following PKC activation, it is tempting to further examine whether the reorganization of actin required for NMDAR trafficking is achieved via the PMA-induced regulation of myosin IIb activity. MLCK is a kinase activated by Ca²⁺-calmodulin and will, in turn, phos-

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**FIGURE 2. Knockdown of myosin IIb inhibits PKC-induced NMDAR surface incorporation at postsynaptic sites.** A, myosin IIb knockdown in cultured hippocampal neurons. A1, examination of the effect of myosin IIb knockdown in vitro. Cultured cells transfected with MyH10 shRNA, which selectively targets the myosin IIb heavy chain MyH10, exhibit a decreased myosin IIb level. A2, plot summarizing the data from the experiments shown in A1. B, PKC-induced potentiation in postsynaptic NMDAR localization was absent in myosin IIb knockdown cultured cells. B1, representative images of cell surface NMDARs (GluN1; red) at postsynaptic sites (PSD-95; blue) in naïve cultures and cultures transfected with MyH10 shRNA (green with EGFP). B2, plot summarizing the data (mean ± S.E.) from the experiments shown in B1. MyH10, 1.25 ± 0.01; n = 16, from 16 ROIs imaged in 16 neurons from seven independent cultures; p < 0.01, one-way ANOVA; MyH10 + PKC, 1.08 ± 0.07; n = 19, from 19 ROIs imaged in 19 neurons from seven independent cultures; p > 0.05, one-way ANOVA; bar, 10 μm. Higher magnification images of dendritic branches are shown in the bottom panels. Bar, 2 μm. The boxes in the Merge column indicate the corresponding magnified regions. C, in vivo myosin IIb knockdown in the dorsal hippocampus. C1, diagram showing the injection of MyH10 shRNA-EGFP-containing rAAV2/1 virus into one side of the rat dorsal hippocampus at 5–6 days after birth. The side without virus injection is used as the naïve control. Images showing the EGFP expression in the dorsal hippocampal slices prepared from the contralateral ( naïve) and injected (bottom right) hemispheres following unilateral injections (4 μl) of a rAAV2/1 that coexpressed MyH10 shRNA and EGFP are displayed. Bar, 10 μm. C2, examination of the effect of myosin IIb knockdown in vivo. Two weeks after injection, local virus-injected hippocampal regions that contained approximately one-third of the whole hippocampus were collected. Hippocampal slices transfected with MyH10 shRNA exhibited a decreased myosin IIb level. C3, plot summarizing the data from the experiments shown in C2. The PKC-induced increase in NMDA EPSCs was not observed in the myosin IIb knockdown slices. D1, sample traces of averaged NMDA EPSCs in naïve slices and slices transfected with MyH10 shRNA. The myosin IIb KO, did not affect the amplitude of the evoked NMDA EPSCs. D2, plot summarizing the data from the experiments shown in D1; normalized amplitude, control 1.00 ± 0.07, myosin IIb KO 1.01 ± 0.05; n = 5, from five whole-cell recordings of four independent animals; p > 0.05. D3, sample traces indicate the averaged NMDA EPSCs before and after PMA treatment in naïve slices and slices transfected with MyH10 shRNA. Cells recorded in the myosin IIb knockdown slices failed to exhibit PMA-induced potentiation of NMDA EPSCs. D4, plot summarizing the data from the experiments shown in D3. PMA, 1.71 ± 0.19; n = 4, from four whole-cell recordings of four independent animals; p < 0.05, paired sample t test; MyH10 + PMA, 1.00 ± 0.10; n = 9, from nine whole-cell recordings of seven independent animals; p > 0.05, paired sample t test. **, p < 0.05; ***, p < 0.01 compared with the control. Error bars, S.E.
phorylate myosin IIb to increase myosin-ATPase activity. As a result, it drives the cycling of myosin across bridges along actin filaments and causes the contraction of smooth muscle (48). Interestingly, MLCK is not restricted to smooth muscle. It can also regulate various functions related to motility in non-muscle cells, including neurons. For example, MLCK is expressed in hippocampal neurons (49) and increases synaptic vesicle mobilization (50). More importantly, MLCK is a highly substrate-specific kinase; myosin II, particularly the regulatory light chain, is the only known physiological substrate of MLCK (47, 51). As a result, we investigated the potential role of MLCK in the regulation of postsynaptic expression and function. Through immunofluorescence assays in cultured hippocampal cells, we determined that pretreatment with a MLCK antagonist, ML-7 (10 μM), completely abolished the PMA-induced enhancement in NMDAR localization at postsynaptic sites.
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(PMA, 1.34 ± 0.03; n = 23, from 23 ROIs imaged in 23 neurons from 11 independent cultures; p < 0.01, one-way ANOVA; ML-7 + PMA, 1.06 ± 0.03; n = 20, from 20 ROIs imaged in 20 neurons from 10 independent cultures; p > 0.05, one-way ANOVA; Fig. 5A). Consistently, the PMA-induced increase in actin polymerization in the cytoplasmic fraction of hippocampal cells in slices, as indicated by an increase in the cofilin phosphorylation level, was also suppressed by ML-7 application (PMA, 1.17 ± 0.03; n = 4, from four samples of 16 independent animals; p < 0.01, one-way ANOVA; ML-7 + PMA, 1.00 ± 0.04; n = 4, from four samples of 16 independent animals; p > 0.05, one-way ANOVA; Fig. 5B). Additional supporting evidence was obtained from the observation in acute hippocampal slices that the PMA-induced increase in NMDAR function (NMDA EPSCs) was abolished by ML-7 application (PMA, 1.82 ± 0.28; n = 4, from four whole-cell recordings of four independent animals; p < 0.05, paired sample t test; ML-7 + PMA, 1.07 ± 0.17; n = 4, from four whole-cell recordings of four independent animals; p > 0.05 compared with the control, paired sample t test; Fig. 5C). Combined with previous findings that indicated the contribution of NMDAR trafficking to PKC-induced potentiation in surface NMDAR expression and

![Graphs and images](https://example.com/graphs/1.png)
NMDAR-mediated currents (4, 6), these results suggest that MLCK is essential in the regulation of NMDAR trafficking following PKC activation.

To further confirm the role of MLCK in NMDAR trafficking, we created MLCK-knock-out (MLCK-KO) mice and determined how the absence of MLCK function affected postsynaptic NMDAR expression and function. MLCK-KO mice were created by crossing Mylk floxed mice (52) with Nestin-Cre transgenic mice (49) to specifically delete MLCK in the nervous system. The efficiency of the MLCK knockout was verified via a Western blot assay (Fig. 6A1). We identified a 75% reduction in the MLCK levels in the dorsal hippocampus of the MLCK KO (Mylk<sup>lox/lox</sup>; Nestin-Cre) mice compared with their littermate controls (Mylk<sup>lox/lox</sup>; Nestin-Cre). Consistent with the previously described findings obtained using the pharmacological agent ML-7, we demonstrated that the enhancements in postsynaptic NMDAR expression and NMDAR function induced by PKC activation were not observed in the hippocampal sliced tissues obtained from the MLCK-KO mice (NMDAR expression: PMA, 1.22 ± 0.07; n = 6, from six samples of 36 independent animals; p < 0.01, one-way ANOVA; KO + PMA, 0.93 ± 0.06; n = 6, from six samples of 36 independent animals; p > 0.05, one-way ANOVA; Fig. 6A2). NMDAR function: PMA, 1.74 ± 0.41; n = 5, from five whole-cell recordings of four independent animals; p < 0.05, paired sample t test; KO + PMA, 0.94 ± 0.17; n = 5, from five whole-cell recordings of four independent animals; p > 0.05, paired sample t test; Fig. 6, B1 and B2). Taken together, our findings provide substantial evidence that supports the notion that MLCK activity is essential for the myosin IIb-mediated and actin-dependent regulation of NMDAR synaptic incorporation during PMA- or TBS-induced synaptic plasticity.

PKC and MLCK Share a Common Pathway to Regulate NMDA EPSCs—Although it has been reported that PKC activation can indirectly trigger downstream MLCK activation (33) and that the activation of MLCK can, in turn, activate its only substrate, myosin IIb (47, 51), it remains unknown whether these signaling molecules act in series following the regulation of NMDAR function in the present study. To test this possibility, we performed whole-cell patch clamp recordings of NMDA currents to determine how NMDAR function is affected by various inhibitors of these molecules. Consistent with previous studies (5, 7), PKC activation with PMA (0.5 μM) induced a chemical form of LTP in the evoked NMDA EPSCs in acute hippocampal slices (PMA, 1.90 ± 0.02; n = 7, from seven whole-cell recordings of five independent animals; p < 0.01, paired sample t test; Fig. 7A). Similarly, the application of a constitutively active form of MLCK (aMLCK) in the recording pipette solution also induced LTP<sup>NMDA</sup> with a potentiation magnitude comparable with PMA (aMLCK, 1.64 ± 0.03; n = 7, from seven whole-cell recordings of five independent animals; p < 0.01, paired sample t test). This potentiation in NMDA EPSCs was abolished by the pretreatment of brain slices with the broad-spectrum PKC inhibitor chelerythrine chloride (10 μM, chelerythrine chloride + aMLCK, 0.94 ± 0.02; n = 5, from five whole-cell recordings of five independent animals; p > 0.05, paired sample t test). Combined with the previously described findings that the inhibition of myosin IIb (Fig. 4C) or MLCK (Fig. 6C) activity suppresses the PKC-induced enhancement in NMDA EPSCs, these results strongly suggest that MLCK and myosin IIb act downstream of PKC.

To further examine our previously discussed hypothesis, we performed mutual occluding experiments to investigate whether LTP<sup>NMDA</sup> induced by PKC and that induced by MLCK share a common pathway. We pretreated slices with PMA (0.5 μM) for 20 min, which produces LTP<sup>NMDA</sup> with a saturated potentiation level (7). When the potentiation level reached a stable plateau after 20 min, the loading of recorded neurons with the active form of MLCK through the recording pipette further increase MLCK activation failed to produce additional potentiation (1.00 ± 0.02; n = 7, from seven whole-cell recordings of six independent animals; p > 0.05, paired sample t test; Fig. 7B). Similarly, after LTP<sup>NMDA</sup> was induced via MLCK activation (2.11 ± 0.02; n = 8, from eight whole-cell recordings of six independent animals; p > 0.05, paired sample t test; Fig. 7C), additional PKC activation via PMA treatment failed to exhibit additional potentiation (p > 0.05, paired sample t test). These mutual occlusion experiments provide evidence that supports the hypothesis that the potentiation of NMDAR function induced by PKC and MLCK activation uses a common cellular mechanism. Because PKC-induced NMDAR trafficking is the main cause of potentiation in NMDA EPSCs (Fig. 1C), we conclude that PKC, MLCK, and myosin IIb share a common path-

**FIGURE 4.** Actin reorganization is required for PKC-induced NMDAR surface incorporation. A, temporal sequence of cofilin dephosphorylation and phosphorylation following PKC activation. The amount of p-cofilin in the cytoplasmic fraction of CA1 neurons in hippocampal slices immediately decreased (~3 min) after the onset of PMA treatment (A1); however, it recovered and even exceeded the baseline level at 40 min after PMA washout (A2). The total cofilin detected with a pan-cofilin antibody did not significantly change following PMA treatment. The changes in the p-cofilin levels at 3 min (A3) and 40 min (A4) after PMA washout were not identified in the CA1 neurons in hippocampal slices pretreated with the myosin IIb ATPase activity inhibitor Blebb (10 μM), A5. PKC activation by PMA caused a short term decrease in the p-cofilin/total cofilin ratio, which indicates transient cofilin dephosphorylation and activation. PMA 3 min, 0.94 ± 0.05; n = 4, from four samples of 16 independent animals; p = 0.05, one-way ANOVA; PMA 40 min, 1.00 ± 0.01; n = 5, from five samples of 20 independent animals; p > 0.05, one-way ANOVA; PMA 3 min, 0.79 ± 0.05; n = 6, from six samples of 24 independent animals; p < 0.01, one-way ANOVA; PMA 40 min, 1.03 ± 0.09; n = 6, from six samples of 24 independent animals; p < 0.01; PMA 40 min, 1.14 ± 0.02; n = 5, from five samples of 20 independent animals; p < 0.01, one-way ANOVA; PMA + Blebb 3 min, 1.00 ± 0.13; n = 6, from six samples of 24 independent animals; p > 0.05, one-way ANOVA; PMA + Blebb 40 min, 0.91 ± 0.02; n = 9, from nine samples of 36 independent animals; p > 0.05, one-way ANOVA. 8, actin polymerization is required for the PMA-induced increase in postsynaptic localization of NMDARs. B1, example images of cell surface NMDARs (GluN1-green) at postsynaptic sites (PSD-95-red) under various conditions. The inhibition of actin polymerization with LatA (10 μM) suppresses the PMA-induced increase in the postsynaptic localization of NMDARs in cultured hippocampal cells. B2, plot summarizing the data from the experiments shown in B1. PMA, 1.29 ± 0.03; n = 20, from 20 ROIs imaged in 20 neurons from nine independent cultures; p < 0.01, one-way ANOVA; LatA + PMA, 1.00 ± 0.03; n = 16, from 16 ROIs imaged in 16 neurons from seven independent cultures; p > 0.05, one-way ANOVA. C, LatA suppresses the PMA-induced increase in NMDA EPSCs. C1, sample traces showing averaged NMDA EPSCs in LatA-untreated (untreated) and LatA-treated (LatA) slices. Hippocampal slices in which actin polymerization was inhibited failed to exhibit PMA-induced potentiation in NMDA EPSCs. C2, plot summarizing the data from the experiments shown in C1. PMA, 2.03 ± 0.50; n = 4, from four whole-cell recordings of four independent animals; p < 0.05; LatA + PMA, 1.06 ± 0.10; n = 7, from seven whole-cell recordings of five independent animals; p < 0.05. *, p < 0.05,**, p < 0.01 compared with the control. Error bars, S.E.
way in the regulation of NMDA trafficking induced by PKC activation.

Regulation of TBS-induced NMDAR Synaptic Incorporation—Although NMDAR trafficking induced by PKC activation has previously been used as a model to investigate NMDAR trafficking (6, 7), PKC activation may also exert broad and non-specific effects on downstream effectors unrelated to NMDAR trafficking (53). Therefore, we examined whether the previously described observations obtained following PKC activation could be reproduced under more physiological conditions. In a recent study, we confirmed that TBS, which is used to induce a classical form of LTP of AMPAR-mediated synaptic responses, also induces potentiation in the postsynaptic expression of NMDARs. Accordingly, TBS induced a similar increase in NMDA EPSCs that could be reserved by the SNARE-dependent exocytosis blocker TeTx (0.1 μM), which suggests that the

FIGURE 5. MLCK-dependent actin reorganization contributes to PKC-induced NMDAR trafficking. A, MLCK activity is required for the PMA-induced increase in the postsynaptic localization of NMDAR. A1, example images of cell surface NMDARs (GluN1; green) at postsynaptic sites (PSD-95; red) under various conditions. The inhibition of MLCK activity with ML-7 (10 μM) in cultured hippocampal cells suppresses the PMA-induced increase in the postsynaptic localization of NMDAR. A2, plot summarizing the data from the experiments shown in A1. PMA, 1.34 ± 0.03; n = 23, from 23 ROIs imaged in 23 neurons from 11 independent cultures; p < 0.01, one-way ANOVA; ML-7 + PMA, 1.06 ± 0.03; n = 20, from 20 ROIs imaged in 20 neurons from 10 independent cultures; p > 0.05, one-way ANOVA. B, ML-7 (10 μM) abolishes the PKC-induced enhancement in postsynaptic NMDAR expression. B1, a Western blot assay of the cytoplasmic fraction of hippocampal cells in the slices indicates that the PKC-induced enhancement in postsynaptic NMDAR expression is inhibited by slice pretreatment with ML-7 (10 μM). B2, plot summarizing the data from the experiments shown in B1. PMA, 1.17 ± 0.03; n = 4, from four samples of 16 independent animals; p < 0.01, one-way ANOVA; ML-7 + PMA, 1.00 ± 0.04; n = 4, from four samples of 16 independent animals; p > 0.05, one-way ANOVA; C, ML-7 reserves the PMA-induced increase in NMDA EPSCs. C1, sample traces showing the averaged NMDA EPSCs in ML-7-untreated (untreated) and ML-7-treated (ML-7) slices. Hippocampal slices in which MLCK activity was inhibited failed to exhibit PMA-induced potentiation in NMDA EPSCs. C2, plot summarizing the data from the experiments shown in C1. PMA, 1.82 ± 0.28; n = 4, from four whole-cell recordings of four independent animals; p < 0.05, paired sample t test; ML-7 + PMA, 1.07 ± 0.17; n = 4, from four whole-cell recordings of four independent animals; p > 0.05 compared with the control, paired sample t test; *, p < 0.05; **, p < 0.01 compared with the control. Error bars, S.E.
insertion of additional NMDARs is responsible for increased NMDAR function (7). Moreover, TBS induced a physiological form of LTP_{NMDA} in hippocampal slices, which was abolished by pretreatment of the slices for 10 min with the myosin IIb inhibitor Blebb (10μM), the actin polymerization inhibitor LatA (10μM), or the MLCK inhibitor ML-7 (10μM) (TBS, 1.74 ± 0.41; n = 5, from five whole-cell recordings of four independent animals; p < 0.05, paired sample t test; KO + PMA, 0.94 ± 0.17; n = 5, from five whole-cell recordings of four independent animals; p > 0.05, paired sample t test; * p < 0.05; ** p < 0.01 compared with the control. Error bars, S.E.)

These findings indicate that similar to the previously discussed findings obtained in the PKC-induced NMDAR trafficking model, myosin IIb activity, actin polymerization, and MLCK activity are all required for TBS-induced NMDAR synaptic incorporation. Moreover, LTP in evoked NMDA EPSCs (LTP_{NMDA}) induced by TBS was also not observed in the MLCK-knock-out mice (0.93 ± 0.02; n = 6, from six whole-cell recordings of five independent animals; p < 0.01, one-way ANOVA; KO + PMA, 0.93 ± 0.06; n = 6, from six samples of 36 independent animals; p > 0.05, one-way ANOVA. The mice in the control and MLCK groups are wild-type mice. PKC and MLCK share a common pathway in LTP_{NMDA} induction. When the potentiation level reached a stable plateau after pretreatment for 10 min with the broad-spectrum PKC inhibitor chelerythrine chloride (10μM), PKC activation by PMA treatment failed to exhibit additional potentiation above basal level. When the potentiation was prevented by pretreatment for 10 min with the broad-spectrum PKC inhibitor chelerythrine chloride (10 μM), PMA-induced LTP of NMDA-mediated EPSCs is occluded by aMLCK preloading in recorded cells. Following LTP_{NMDA} induced by aMLCK activation, further PKC activation by PMA treatment failed to exhibit additional potentiation of NMDA EPSCs: 2.11 ± 0.02; n = 8, from eight whole-cell recordings of six independent animals; p > 0.05, paired sample t test. Error bars, S.E.

**FIGURE 6.** PKC-induced potentiations in postsynaptic NMDAR expression and NMDAR function are not observed in MLCK-knock-out mice. A1, PKC-induced potentiations in postsynaptic NMDAR expression are not observed in MLCK-knock-out mice. A1, the efficiency of the knock-out was verified via Western blot assay. A2, MLCK-KO mice fail to exhibit an enhancement in postsynaptic NMDAR expression in hippocampal sliced tissues. NMDAR expression: PMA, 1.22 ± 0.07; n = 6, from six samples of 36 independent animals; p < 0.01, one-way ANOVA; KO + PMA, 0.93 ± 0.06; n = 6, from six samples of 36 independent animals; p > 0.05, one-way ANOVA. The mice in the control and PKC groups are wild-type mice. B1, sample traces showing the averaged NMDA EPSCs in control (CON) and PMA-treated (PMA) hippocampal slices from wild-type (WT) and MLCK-KO (KO) mice. B2, plot summarizing the data from the experiments shown in B1. PMA, 1.74 ± 0.41; n = 5, from five whole-cell recording of four independent animals; p < 0.05, paired sample t test; KO + PMA, 0.94 ± 0.17; n = 5, from five whole-cell recordings of four independent animals; p > 0.05, paired sample t test; * p < 0.05; ** p < 0.01 compared with the control. Error bars, S.E.

**FIGURE 7.** PKC and MLCK share a common pathway in the regulation of NMDA EPSCs. A, the application of aMLCK in the recording pipette solution induces LTP_{NMDA} with a potentiation magnitude comparable with that of PMA. This potentiation in NMDA EPSCs was abolished by the pretreatment of slices with the broad-spectrum PKC inhibitor chelerythrine chloride (10μM). PMA, 1.90 ± 0.02; n = 7, from seven whole-cell recordings of five independent animals; p < 0.01, paired sample t test; aMLCK, 1.64 ± 0.03; n = 7, from seven whole-cell recordings of five independent animals; p < 0.01, paired sample t test; chelerythrine chloride + aMLCK, 0.94 ± 0.02; n = 5, from five whole-cell recordings of five independent animals; p > 0.05, paired sample t test. B and C, mutual occlusion experiments demonstrated that PKC and MLCK share a common pathway in LTP_{NMDA} induction. B, aMLCK-induced LTP of NMDA-mediated EPSCs is occluded by the pretreatment of slices with PKC inhibitor chelerythrine chloride (10 μM) for 15 min prior to LTP_{NMDA} induction. C, when the potentiation level reached a stable plateau after pretreatment for 20 min, further MLCK activation via the loading of recorded neurons with aMLCK through the recording pipette failed to produce additional potentiation in NMDA EPSCs. The time line of the mutual occlusion experiment is shown in the diagram above the plot. 1.00 ± 0.02; n = 7, from seven whole-cell recordings of six independent animals; p > 0.05, paired sample t test. C, PMA-induced LTP of NMDA-mediated EPSCs is occluded by aMLCK preloading in recorded cells. Following LTP_{NMDA} induced by aMLCK activation, further PKC activation by PMA treatment failed to exhibit additional potentiation of NMDA EPSCs: 2.11 ± 0.02; n = 8, from eight whole-cell recordings of six independent animals; p > 0.05, paired sample t test. Error bars, S.E.
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FIGURE 8. Regulation of TBS-induced potentiation in NMDA EPSCs. A, myosin IIb, MLCK activity and actin polymerization are required for the TBS-induced increase in NMDAR function. A1, sample traces showing the averaged NMDA EPSCs in hippocampal slices under various conditions. Before TBS was delivered, the slices were pretreated with inhibitors of myosin IIb, MLCK activity, or actin polymerization. The NMDA EPSC traces prior to manipulation (untreated) and after manipulation (treated) during baseline recording before TBS are displayed. Overlay traces are shown in the right panel. These NMDA EPSCs were recorded by holding cells at −65 mV and perfusing slices with low magnesium (0.25 mM) artificial cerebrospinal fluid that contained the AMPAR blocker NBQX (10 μM) and the GABA<sub>A</sub> receptor blocker BMI (10 μM) to isolate NMDAR EPSCs. A2, the TBS-induced physiological form of LTP<sub>NMDA</sub> was abolished by the pretreatment of hippocampal slices with various inhibitors. A3, plot summarizing the data from the experiments shown in A2. TBS, 1.74 ± 0.02; n = 4, from four whole-cell recordings of four independent animals; p < 0.01, paired sample t test; ML-7 0.03; n = 4, from four whole-cell recordings of four independent animals; p > 0.05, paired sample t test; LatA + TBS, 1.01 ± 0.01; n = 5, from five whole-cell recordings of five independent animals; p > 0.05, paired sample t test; ML-7 + TBS, 1.01 ± 0.03; n = 4, from four whole-cell recordings of four independent animals; p > 0.05, paired sample t test; LatA + TBS, 1.01 ± 0.01; n = 5, from five whole-cell recordings of five independent animals; p > 0.05, paired sample t test; ML-7 + TBS, 1.01 ± 0.03; n = 4, from four whole-cell recordings of four independent animals; p > 0.05, paired sample t test. B, LTP<sub>NMDA</sub> induced by TBS is not observed in MLCK-KO mice. B1, TBS induces LTP<sub>NMDA</sub> in WT but not MLCK-KO mice. The overlaid traces above the graph indicate the changes in the averaged NMDA EPSCs at the times indicated. B2, plot summarizing the data from the experiments shown in B1. MLCK-KO, 0.93 ± 0.02; n = 6, from six whole-cell recordings of five independent animals; p > 0.05, paired sample t test; **, p < 0.01 compared with the baseline. Error bars, S.E.

Because the entire LTP recording procedure typically takes 1 h and cells that are voltage-clamped for such a long period of time tend to be unhealthy, we held the cells at −65 mV and perfused the slices with low magnesium (0.25 mM) artificial cerebrospinal fluid that contained the AMPAR blocker NBQX (10 μM) and the GABA<sub>A</sub> receptor blocker bicuculline methiodide (10 μM) to isolate NMDAR EPSCs (Fig. 8A1). Combined with previous findings that demonstrated the contribution of NMDAR trafficking to the PKC-induced potentiation in surface NMDAR expression and NMDAR-mediated currents, these results support an essential role of myosin IIb in PMA-induced NMDAR trafficking. Because both PKC activation and TBS are conventional protocols used to induce chemical or physiological LTP, these findings suggest that MLCK- and myosin IIb-dependent actin reorganization represents a general mechanism that underlies NMDAR trafficking during synaptic plasticity.

Discussion

Because the NMDAR plays a central role in various brain functions, the regulation of NMDAR activity is essential to NMDAR-dependent neural processes, including synaptic plasticity. A previous study reported MLCK-dependent regulation of NMDAR-mediated synaptic responses in cell-attached patches (47). The active form of MLCK enhances the NMDAR-mediated whole-cell and synaptic currents in acutely isolated CA1 pyramidal and cultured hippocampal neurons, whereas MLCK inhibitors depress these currents. Intriguingly, this regulation of NMDA responses is lost after excision to inside-out
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patches, which suggests that unknown intracellular components or certain processes involved in intracellular molecules may contribute to this regulation. Our present study further demonstrates that MLCK can implement this regulation via its action on intracellular NMDAR trafficking, which can, in turn, alter the number of NMDARs at postsynaptic sites. Moreover, we provide new evidence that supports the hypothesis that this MLCK-dependent regulation is achieved by the action of MLCK on its only known physiological substrate, myosin Iib, and subsequent actin reorganization. Thus, our findings provide an important molecular mechanism through which MLCK is involved in the regulation of NMDAR activity.

The presence of myosin Iib in NRC suggests its potential role in the trafficking of NMDAR or NRC between the postsynaptic membrane and intracellular compartments (11). However, myosin Iib is unlikely to be the motor protein directly responsible for NMDAR transportation for at least two reasons: the presence of a short coiled-coil domain and the lack of a global tail domain. The short coiled-coil domain limits the steps of motor movement, whereas the global tail of a motor protein is the domain essential for cargo-upon-cargo transportation. Therefore, myosin Iib may be involved in other processes or steps that are critical for NMDAR trafficking. One potential scenario is that myosin Iib-dependent actin reorganization during synaptic plasticity may serve to pave the “track” for the transportation of receptor-containing vesicles undertaken by a certain motor protein. Additional studies are needed to identify the motor protein responsible for NMDAR transportation.

NMDARs are transported selectively to dendrites by the dendrite-specific motor protein KIF17 (14, 54). KIF17 transports GluN2B-carrying vesicles via association with a scaffolding complex that comprises LIN10 (Mint1), LIN2, and LIN7. When NMDARs are transported to the spine base where synapses are located, they are released by KIF17 (55) and associate with an actin-based motor protein, which is responsible for its further transportation to the postsynaptic membrane. To date, the specific motor protein that implements this task of NMDAR transportation to spines remains unclear. Nevertheless, the rule of subunit-specific NMDAR trafficking to synapses has been elucidated to some extent. The GluN2 subunit directs the delivery of receptors to synapses with different rules controlling GluN2A and GluN2B. The synaptic incorporation of GluN2A—but not GluN2B-containing NMDARs requires synaptic activity (56). Moreover, the binding affinity of GluN2 with CaMKII determines the magnitude of LTP (57). The composition of GluN2 controls synaptic plasticity via the regulation of binding to CaMKII.

Synaptic plasticity, such as LTP, is typically accompanied by actin reorganization (25, 31, 58). The actin dynamics may involve a temporal sequence of cofilin dephosphorylation and phosphorylation that underlies receptor trafficking and spine enlargement (23). Similar observations were observed during exposure to LTP-inducing stimuli by PKC activation in the present study. However, unlike the complete recovery of cofilin phosphorylation following brief tetraethylammonium treatment and washout, an increased level of cofilin phosphorylation was still detected 40 min after PMA washout. This inconsistency may be ascribed to the different features of actin dynamics caused by different LTP-inducing stimuli. A stable but increased level of actin polymerization may be essential for the maintenance of LTP induced by PKC activation (28, 59). At least two distinct roles of F-actin polymerization have been proposed for LTP-inducing synaptic stimulation (18). One role is to stabilize early LTP (31), whereas the other role is to capture plasticity-related proteins that stabilize the late phase of LTP. The NMDAR may represent a plasticity-related protein during exposure to LTP-inducing stimuli, and the NMDAR-CaMKII complex has been proposed to serve as a “seed” for anchoring other plasticity-related proteins, including AMPARs (29, 34). Moreover, because the trafficking of both AMPARs and NMDARs during synaptic plasticity requires actin dynamic regulation (23), the disruptions in myosin Iib may exert a more widespread effect on synaptic function in addition to the NMDAR-mediated synaptic function. It is likely that the MLCK- and myosin Iib-dependent actin reorganization represents a general mechanism that underlies receptor forward trafficking.

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