

RESEARCH ARTICLE

Microtubule-associated protein 2 mediates induction of long-term potentiation in hippocampal neurons

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Abstract

Microtubule-associated protein (MAP) 2 has been perceived as a static cytoskeletal protein enriched in neuronal dendritic shafts. Emerging evidence indicates dynamic functions for various MAPs in activity-dependent synaptic plasticity. However, it is unclear how MAP2 is associated with synaptic plasticity mechanisms. Here, we demonstrate that specific silencing of high-molecular-weight MAP2 in vivo abolished induction of long-term potentiation (LTP) in the Schaffer collateral pathway of CA1 pyramidal neurons and in vitro blocked LTP-induced surface delivery of AMPA receptors and spine enlargement. In mature hippocampal neurons, we observed rapid translocation of a subpopulation of MAP2, present in dendritic shafts, to spines following LTP stimulation. Time-lapse confocal imaging showed that spine translocation of MAP2 was coupled with LTP-induced spine enlargement. Consistently, immunogold electron microscopy revealed that LTP stimulation of the Schaffer collateral pathway promoted MAP2 labeling in spine heads of CA1 neurons. This translocation depended on NMDA receptor activation and Ras-MAPK signaling. Furthermore, LTP stimulation led to an increase in surface-expressed AMPA receptors specifically in

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APV, (2R)-amino-5-phosphonovaleric acid; BIS, bisindolylmaleimide; CA1, Cornu Amonis 1; CA3, Cornu Amonis 3; CaMKII, calcium/calmodulin-dependent protein kinase II; cLTP, chemical long-term potentiation; DIV, days in vitro; ERK, extracellular signal-regulated kinase; fEPSP, field excitatory postsynaptic potential; FTS, farnesyl thiosalicylic acid; GABA, gamma-aminobutyric acid; GFP, green fluorescent protein; GluA1, glutamate ionotropic receptor, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type subunit 1; GTP, guanosine triphosphate; HFS, high-frequency stimulation; HMW, high-molecular weight; JNK, c-Jun N-terminal kinase; LMW, low-molecular-weight; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MTs, microtubules; NMDA, N-methyl D-aspartic acid; PKA, protein kinase A; PKC, protein kinase C; PSD, postsynaptic density; RFP, red fluorescent protein; SPAR, spine-associated Rap GTPase-activating protein; STS, staurosporine.

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the neurons with MAP2 spine translocation. Altogether, this study indicates a novel role for MAP2 in LTP mechanisms and suggests that MAP2 participates in activity-dependent synaptic plasticity in mature hippocampal networks.

KEYWORDS

dendritic spine, electron microscopy, MAP2, synaptic plasticity

1 | INTRODUCTION

In the brain, information is stored by experience-dependent modifications of synaptic weights.^{1,2} This activity-dependent refinement of neuronal circuits is accomplished by structural and functional synaptic plasticity. Long-term potentiation (LTP) at the hippocampal area CA1, the best-studied cellular mechanism of synaptic plasticity,³ requires activation of NMDA receptors (NMDARs) and involves synaptic insertion of AMPA-type glutamate receptors (AMPA receptors),^{4,5} enlargement of existing dendritic spines (the postsynaptic sites at which most excitatory synapses are formed), and new spine formation.⁶⁻¹⁰ However, many of the molecules that mediate synaptic remodeling and trafficking in dendritic spines during LTP remain to be discovered. Since synaptic functions are known to be impaired in various neurological and neurodegenerative disorders,¹¹⁻¹³ identifying the molecular mechanisms involved in synaptic remodeling could advance our understanding of cognitive function in physiological and pathological conditions.

Microtubule-associated protein (MAP) 2 is a neuron-specific cytoskeletal protein that stabilizes microtubules (MTs) and is implicated in establishing dendritic arborization during development.¹⁴ Long considered as a mono-functional protein that regulates microtubule properties, MAP2 has been extensively used as a reliable dendritic marker, based on its tight association with MTs and static immunoreactivity that is restricted to the dendritic shaft.¹⁵⁻¹⁷ This conceptual barrier has largely excluded MAP2 from the mainstream of synaptic plasticity mechanisms.¹⁸ In the rodent brain, at least three isoforms of MAP2 are produced by alternative splicing of a single gene: the high-molecular-weight (HMW, ~280 kDa) MAP2A and 2B, and the low-molecular-weight (LMW, ~70 kDa) MAP2C.^{19,20} MAP2C is predominantly expressed during early dendritic development and is gradually replaced by high-molecular-weight MAP2.²¹ However, a potential role for HMW MAP2 in mature hippocampal networks has not been determined. Accumulating evidence indicates that neuronal MAPs play a role in synaptic plasticity mechanisms.²²⁻²⁴ Furthermore, some ultrastructural studies have reported that MAP2 is observed in dendritic spines and interacts with actin filaments.²⁵⁻²⁸ These studies suggest that MAP2

may act as a dynamic element in response to extracellular stimulation. However, the possible role of MAP2 in activity-dependent synaptic plasticity has remained unaddressed.

Here, we addressed this issue to determine whether MAP2 participates in LTP mechanisms in mature hippocampal networks. Specific silencing of HMW MAP2 abolished LTP induction in the Schaffer collateral-to-CA1 synapses and prevented the surface insertion of AMPARs. Surprisingly, we observed LTP-induced translocation of a subpopulation of MAP2 to dendritic spines in mature hippocampal neurons, which is correlated with LTP-induced spine enlargement as well as surface delivery of AMPARs. In addition, LTP elicited by high-frequency stimulation (HFS) at the Schaffer collateral pathway increased the immunogold labeling of MAP2 in spine heads of CA1 neurons. This spine translocation of MAP2 requires activation of NMDARs and the Ras-MAPK signaling pathway. Taken together, our findings suggest a novel dynamic role for MAP2 in LTP-dependent synaptic plasticity.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of Korea Brain Research Institute (IACUC-2018-0016).

2.2 | Neuron culture and transfection

Primary rat hippocampal cultures were prepared from E18-19 Sprague-Dawley fetal rats as previously described.²⁹ Briefly, dissociated hippocampal neurons were plated onto glass coverslips coated with 100 µg/mL of poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) and 2 µg/mL of laminin (Roche, Indianapolis, IN, USA). Hippocampal cultures (~200 cells mm⁻²) were maintained in Neurobasal medium (Invitrogen, San Diego, CA, USA) supplemented with 2% of B27 (Invitrogen) or 2% of SM1 (Stem Cell Technologies, Vancouver, Canada), 0.5 mM of glutamine, and 12.5-25 µM of glutamate. Neurons were analyzed at 10-14 days in

vitro (DIV) for young stages and DIV 20–24 for mature stages. DNA constructs were transfected into neurons using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or calcium phosphate³⁰ 2–3 days prior to treatment with pharmacological reagents or vehicle control.

The following plasmids with fluorescent protein expression were used in the study: pLV-mCherry (#54764) and pLV-hSyn-RFP (#22909), which were obtained from Addgene (Cambridge, MA, USA). MAP2-GFP constructs were kindly provided by Dr Stefanie Kaech (Oregon Health & Science University, Portland, Oregon). DNA corresponding to rat MAP2B was subcloned into pPAGFP-N1 plasmid (a kind gift from Dr Sunghoe Chang, Seoul National University, South Korea). The fidelity of all the constructs was verified by sequencing and protein expression was also confirmed by western blot analysis and immunostaining.

2.3 | Chemical LTP induction and pharmacological reagents

Under baseline conditions, neurons were incubated in their original growth medium. To induce chemical LTP (cLTP) in the primary hippocampal neuron culture, we used three well-established cLTP protocols: glycine-induced LTP,⁴ treatment with forskolin/rolipram,³¹ and APV withdrawal.³² Briefly, coverslips were gently transferred to conditioned medium containing 200 μ M glycine and 1 μ M of strychnine for 3 minutes and immediately returned to the original growth medium and incubated for 5 minutes. For cyclic adenosine monophosphate (cAMP)-mediated LTP induction, neurons at DIV 21 were incubated with 10 μ M of forskolin and 100 nM of rolipram for 15 minutes, returned to the original growth medium, and then, incubated for 5 minutes. For APV withdrawal, neurons were initially maintained in the presence of 200 μ M of APV, added to the media 5 days after plating. We transferred coverslips to conditioned medium without APV for 40 minutes, and then, placed them back into original medium for 5 minutes.

Neurons were treated with the following pharmacological reagents: farnesyl thiosalicylic acid (Ras inhibitor, 10 μ M); PD98059 (MEK inhibitor, 10 μ M); FR180204 (ERK inhibitor, 0.5 μ M); KN62 (CaMKII inhibitors, 2 μ M); H89 and KT5720 (PKA inhibitors, 1 μ M each); staurosporine and bisindolylmaleimide I (PKC inhibitors, 1 μ M each); JNK inhibitor II (5 μ M); cycloheximide (protein synthesis inhibitor, 10 μ g/mL); APV (competitive NMDAR antagonist, 100–200 μ M); TTX (sodium channel blocker, 1–2 μ M); PTX (GABA_A receptor antagonist, 100 μ M); KCl (20 mM); EGTA (1 mM); NMDA (50 μ M); forskolin (10 μ M); rolipram (100 nM); Taxol (10 nM). All reagents were purchased from Sigma (St. Louis, MO, USA), Tocris Bioscience (Bristol, UK), Cayman Chemical

(Ann Arbor, MI, USA), and Calbiochem (San Diego, CA, USA), respectively.

2.4 | Antibodies

The following antibodies were used in the study: mouse MAP2 HM-2 (Sigma-Aldrich, St. Louis, MO, USA) and MAP2 AP20 (Millipore, Burlington, MA, USA), chicken MAP2 (PhosphoSolutions, Aurora, CO, USA), rabbit GluA1 N-terminal (Calbiochem, San Diego, CA, USA), rat tyrosinated tubulin (Millipore, Darmstadt, Germany), mouse TUJ1 (Covance, Princeton, NJ, USA), mouse PSD95 K28/43 (NeuroMab, Davis, CA, USA), rabbit PSD95 D27E11 (Cell Signaling Technology, Boston, MA, USA), rabbit SPAR,³³ mouse GFP monoclonal 3E6 (Quantum Biotechnologies, St. Cloud, MN, USA), rabbit polyclonal GFP and Alexa 488, 594, or 647 conjugated goat/donkey anti-chicken/mouse/rat/rabbit secondary antibodies (Invitrogen, Carlsbad, CA, USA).

2.5 | RNA interference

The small hairpin RNAs (shRNAs) for RNA interference against HMW (shMAP2^{HMW}) or all isoforms (shMAP2^{pan}) of MAP2 mRNA were designed on the basis of MAP2 cDNA sequence (Gene bank accession number NM_013066.1, NM_001310634.1, respectively), targeting the region of nucleotides 5'-GACAAAGTCCGTAGAGATT-3' (shMAP2^{HMW}, nucleotides 2701–2719 for rat and 2710–2728 for mouse) and 5'-GGAGAAGGCCCAAGCTAAA-3' (shMAP2^{pan}, nucleotides 5026–5044, all isoforms of rat MAP2). Complementary oligonucleotides were synthesized separately with the addition of an HpaI site at the 5' end and an XhoI site at the 3' end. The annealed cDNA fragments were cloned into the HpaI-XhoI sites of pLL3.7 vector (#11795, Addgene, Cambridge, MA, USA). The fidelity of all the constructs was verified by sequencing. For validation of isoform-specific knock-down (KD) of MAP2, shRNAs were tested on protein levels of GFP-tagged MAP2 isoforms transfected into Lenti-X 293T cells and of endogenous MAP2 in cultured hippocampal neurons using the lentiviral vector-mediated shRNA expression. Neurons were transduced at DIV 13 and harvested at DIV 22 for KD of HMW MAP2. For production of MAP2 KD lentiviruses, pLL3.7 plasmid containing shRNAs against MAP2 was cotransfected with ViraPower Packaging Mix (Invitrogen) into Lenti-X 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The viral supernatant was collected 48 hours after transfection, concentrated by ultra-centrifugation using Beckman ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA), and aliquoted until use.

2.6 | Immunocytochemistry and confocal microscopy

Hippocampal neurons were fixed either in 4% of paraformaldehyde/4% of sucrose/PBS (pH 7.4) for 10 minutes at RT or in methanol at -20°C for 10 minutes. Neurons were permeabilized with 0.01% of Triton X-100 in PBS for 15 minutes, and then, processed for standard immunocytochemistry. Antibodies for immunostaining were diluted in GDB buffer (0.1% of gelatin, 0.3% of Triton X-100, 16 mM of sodium phosphate pH 7.4, 450 mM of NaCl). For confocal microscopy, we used a Nikon A1R confocal microscope (Nikon Instruments, Melville, NY, USA) mounted onto a Nikon Eclipse Ti-E body.

For immunostaining of cell-surface GluA1 receptors, live neurons were incubated with N-terminal-specific anti-GluA1 antibody under nonpermeabilizing conditions for 10 minutes, then fixed, washed, and treated with saturating concentrations of secondary antibody. Cells were permeabilized, and then, immunostained for endogenous MAP2. For the intensity measurements, images were acquired using an Axiovert 200M (Carl Zeiss, Oberkochen, Germany), and then, analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) or Image J software (NIH, Bethesda, MD, USA). The numbers and integrated intensity of punctate signals were measured from at least three dendritic segments for each neuron. The mean values for each neuron were then averaged to obtain a group mean.

For dendritic spine analysis, z-stacks confocal images were visualized as maximum intensity projections. One to four dendritic segments of hippocampal pyramidal neurons were randomly chosen. Morphological parameters including spine density and size were measured using Imaris v9.0.2 (Bitplane, Zurich, Switzerland).

2.7 | Live-cell imaging and image analysis

For time-lapse live imaging, hippocampal neurons transfected with relevant DNA constructs for 3 days were imaged using a Nikon A1R confocal microscope equipped with digital-zooming Nikon imaging software (NIS-element AR 64-bit version 4.40.00; Laboratory Imaging, Praha, Czech Republic) or a delta 512 $\times$ 512 EMCCD camera (Bruker, Billerica, MA, USA). A Chamlide TC system was used to maintain conditions of 37°C and 5% of CO_2 (Live Cell Instruments, Seoul, Korea). To visualize MAP2 translocation dynamics to spines, we performed cLTP induction with glycine in cultured rat hippocampal neurons. Before cLTP induction, neurons were imaged three times every 30 seconds. Following bath application of glycine for 5 minutes, 59 images were taken every 30 seconds.

Individual time-lapse images (512 $\times$ 512 pixel) were obtained by acquiring three z-stacks at 1 μm intervals for GFP and for RFP or mCherry. For image analysis, the fluorescence intensities of three z-stacks were processed by maximum intensity projection to obtain a single collapsed z-stack image. Spine volumes and MAP2 protein levels were assumed to be proportional to the integrated intensities of the RFP and GFP signals, respectively. To quantify the changes in synaptic fluorescence during cLTP, spine intensity measurements were normalized to a dendrite shaft intensity factor, which consisted of the average mean pixel value from regions of interest ($\sim 0.3 \mu\text{m}^2$) on the associated dendrite shaft in the red (RFP) and green (MAP2B-GFP) channels. The integrated fluorescence intensity was calculated using MATLAB (MathWorks, Natick, MA, USA) and fitted to a Gaussian curve using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

For photoactivation experiments, hippocampal neurons were cotransfected with MAP2B-PAGFP and mCherry constructs at DIV 17. After 3–4 days of transfection, we performed time-lapse live imaging to visualize photoactivation and spine translocation of MAP2B-PAGFP. Because of barely detectable PAGFP intensity in the nonactivated state, we randomly selected secondary dendrites of mCherry-expressing pyramidal neurons and delivered photoactivating light using 405-nm laser ($\sim 5 \text{ mW}$, 0.125 seconds/frame, 1 loop; ROI of 50 μm by 30 μm) at 3 minutes after glycine-mediated cLTP induction. For time-lapse imaging, neuronal dendrites were imaged every 20 seconds for 16 minutes following cLTP induction. Individual time-lapse images (512 $\times$ 512 pixel) were obtained by acquiring single plane (NIS-perfect focusing system mode) for GFP and mCherry. The fluorescence MAP2 intensity in spines before and after MAP2 translocation was measured using Image J software (NIH, Bethesda, MD, USA).

2.8 | Stereotaxic injection

C57BL/6 male mice at 6 weeks of age were anesthetized with isoflurane and injected with 1 μL (at a rate of 0.2 $\mu\text{L}/\text{min}$) of control or shMAP2^{HMW} expressing viral solution bilaterally into the hippocampal CA1 region according to the stereotaxic coordinates (anterior-posterior -2.0 mm , lateral $\pm 1.5 \text{ mm}$, dorsal-ventral -1.4 mm from bregma). Glass pipettes used for microinjection were kept in place for 5 minutes after the end of the injection to ensure complete absorption of the viruses. After recovery in a heated chamber, mice were returned to their cages. LTP experiments using electrical stimulation were performed 3 weeks after viral injection.

2.9 | Electrophysiological LTP induction in hippocampal slices

Mice were deeply anesthetized with isoflurane and acute 400- μ m-thick coronal slices were prepared using a VF-200-OZ Compresstome (Precisionary Instruments, Boston, MA, USA) in ice-cold sucrose-based slicing artificial cerebral spinal fluid (ACSF) containing [in mM] 5 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 0.5 CaCl_2 , 10 MgCl_2 , 10 D-glucose and 212.5 Sucrose (pH 7.3). The slices were recovered at 27°C in standard ACSF prepared with [in mM] 124 NaCl, 5 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 2.5 CaCl_2 , 1.5 MgCl_2 , and 10 glucose (pH 7.3) for 1 hour before electrophysiological recording. Slices were placed in a recording chamber, submerged, and continuously perfused (2–3 mL/min) with oxygenated standard ACSF at 27°C. To isolate glutamatergic synaptic responses, 10 μ M bicuculline-methiodide (GABA_A receptor antagonist; Tocris) was added to standard ACSF. Extracellular potentials were recorded through low impedance (2.5–3.5 M Ω) glass micropipettes filled with ACSF and placed onto the CA1 *stratum radiatum* area. For LTP experiments using electrical stimulation, a tungsten bipolar electrode (World Precision Instruments, Sarasota, FL, USA) was placed on the CA3 region. For field potential recordings, test stimuli were applied at low-frequency stimulation (single pulses delivered at 0.05 Hz). Stimulus intensity was determined by constructing an input-output relationship; plotting the field EPSP (fEPSP) slopes against stimulus intensities, and then, adjusting to 50%–60% of the maximum fEPSP slope. After acquiring at least 20 minutes of stable fEPSP responses, high-frequency LTP stimulation (HFS; 100 Hz, 1 seconds) was delivered. For control group, 50 μ M APV (NMDAR antagonist; Tocris Bioscience) was added to standard ACSF 10–15 minutes before HFS.

2.10 | Immunoelectron microscopy

For postembedding immunogold labeling of MAP2, the hippocampal slices subjected to HFS-induced LTP were immersed in fixative (pH 7.4) composed of 2% of glutaraldehyde and 2% of paraformaldehyde in 0.15 M cacodylate buffer overnight at 4°C, followed by postfixation with 2% of osmium tetroxide for 2 hours. The samples were dehydrated with a graded ethanol series and embedded in Spurr medium (Electron Microscopy Sciences, Hatfield, PA, USA). About 70-nm-thick ultrathin sections of area CA1 *stratum radiatum* were obtained using an ultramicrotome (Leica Microsystems, Wetzlar, Germany), and then, mounted on nickel grids. The sections were quenched for free aldehyde groups using 0.05 M glycine in PBS buffer for 30 minutes, and then, treated with 10% of BSA blocking solution for 30 minutes. The grids were rinsed twice with 0.1% of BSA/PBS followed

by incubation with mouse anti-MAP2 antibody (1:70 in 1% of BSA/PBS) for 2 hours. After rinsing with 0.1% of BSA/PBS, the grids were incubated with anti-mouse IgG secondary antibody conjugated to 10 nm of gold particles (1:30; Sigma) for 2 hours, and double-stained with 2% of uranyl acetate for 20 minutes and lead citrate for 15 minutes. The sections were viewed under a Tecnai G2 transmission electron microscope (ThermoFisher Scientific, Hillsboro, OR, USA) at an acceleration voltage of 200 kV. We obtained electron microscopic images of asymmetric synapses in CA1 *stratum radiatum* labeled for MAP2. The gold particles in individual spine heads were counted using ImageJ software. Distances between gold particles and the postsynaptic membrane were measured from the center of the particle to the outer edge of the postsynaptic membrane as described previously.³⁴ Postsynaptic density (PSD) area was defined by the postsynaptic membrane and two parallel lines from the edge of the PSD extending 100 nm into the cytoplasm.

2.11 | Statistical analysis

All data represent mean $\pm$ SEM unless otherwise indicated. For two-sample comparisons, the two-tailed unpaired Student's *t* test was used, except where specified. One-way ANOVA and post hoc Tukey Honest Significant Difference (HSD) tests were used for significance calculations in multiple group comparisons. For comparing cumulative distributions, the Kolmogorov-Smirnov (KS) test was used. *P* < .05 was considered a statistically significant difference.

3 | RESULTS

3.1 | High-molecular-weight MAP2 is required for LTP induction in CA1 neurons

MAP2 is a cytoskeletal protein enriched in the somatodendritic compartment of neurons.³⁵ Among three isoforms of MAP2 in the rodent brain, MAP2C expression is abundant during early development while MAP2A and 2B are enriched in mature stages.²¹ Indeed, western blot analysis showed high levels of HMW MAP2 compared to very low levels of MAP2C in mature hippocampal cultures (days in vitro [DIV] 20–24) (Supplemental Figure 1A).

Because a role for HMW MAP2 in mature hippocampal networks has not been determined, we first tested whether HMW MAP2 is required for activity-dependent synaptic plasticity such as LTP, the best-studied cellular mechanism of synaptic plasticity.³ We designed, generated, and validated lentiviral particles expressing shRNA specifically against HMW MAP2 (shMAP2^{HMW}) (Supplemental Figure 2). We then conducted stereotaxic delivery of the lentivirus

expressing shMAP2^{HMW} into the mouse hippocampal CA1 area and subsequently induced LTP at Schaffer collateral-to-CA1 synapses (Figure 1A).

The neurons expressing shMAP2^{HMW} showed no significant difference in the input/output response curve (afferent stimulation vs fEPSP slope) compared to controls (Figure 1B), indicating that basal synaptic transmission is not affected by shMAP2^{HMW} expression. Surprisingly, LTP induction elicited by HFS (100 Hz, 1 second) was significantly reduced in the hippocampal slices expressing shMAP2^{HMW} as compared to controls (Figure 1C,D) ($P < .0001$, t test vs control). These results demonstrate that HMW MAP2 is essential for LTP induction in the hippocampal CA1 pyramidal neurons.

To corroborate above findings, we asked whether silencing of HMW MAP2 affects LTP-induced synaptic insertion of AMPARs in mature (DIV 21) hippocampal neurons, using live-cell labeling of surface GluA1. We employed bath application of glycine (200 μ M, 3 minutes; with 1 μ M of strychnine to block glycine receptors), a well-established chemical LTP (cLTP) protocol that shares biochemical, morphological, and functional synaptic responses with electrophysiologically induced LTP.⁴

In basal conditions, the hippocampal neurons transduced with lentivirus expressing shMAP2^{HMW} displayed comparable levels of surface GluA1 to controls (Figure 1E,F), consistent with the normal basal synaptic transmission in shMAP2^{HMW} expressing brain slices (Figure 1B). In contrast, neurons expressing shMAP2^{HMW} failed to show an increase in surface GluA1 levels after cLTP stimulation (Figure 1E,F) (relative integrated intensity: control 1.00 ± 0.03 , control + cLTP 1.15 ± 0.03 , $P = .007$; shMAP2^{HMW} 1.00 ± 0.04 , shMAP2^{HMW} + cLTP 0.95 ± 0.03 , $P = .648$; one-way ANOVA and post hoc Tukey HSD), supporting our observation that HMW MAP2 is necessary for LTP induction in the hippocampal CA1 neurons.

We next examined the effect of MAP2 depletion on LTP-induced spine enlargement. Under basal conditions, silencing of MAP2 in mature hippocampal neurons (transfection at DIV17 and fixation at DIV 21) did not change the dendritic complexity, spine density and size (Figure 1G-J and Supplemental Figure 3). At 30 minutes after cLTP stimulation, we observed a significant increase in spine size in control neurons, while this spine enlargement was occluded in MAP2 depleted neurons (Figure 1H) (spine size [μ m²]: control 2.54 ± 0.08 , control + cLTP 3.24 ± 0.14 , $P = .0004$; shMAP2^{HMW} 2.50 ± 0.08 , shMAP2^{HMW} + cLTP 2.49 ± 0.10 ; one-way ANOVA and post hoc Tukey HSD). The cumulative distribution of the individual spine size also showed a significant rightward shift in control group after cLTP stimulation, but not in MAP2 depleted group (Figure 1J) ($P < .001$ for control + cLTP; Kolmogorov-Smirnov test). Additionally, we did not detect a change in spine density in any condition (Figure 1I) (spine density [per 10 μ m]: control 9.26 ± 0.29 ,

control + cLTP 9.44 ± 0.18 , shMAP2^{HMW} 9.19 ± 0.34 , shMAP2^{HMW} + cLTP 9.17 ± 0.45 ; one-way ANOVA and post hoc Tukey HSD). It is possible that the relatively brief duration of the study (30 minutes after LTP stimulation) may not be enough to induce a net change in spine number. Together, these results indicate that MAP2 translocation affects LTP-induced spine enlargement in mature hippocampal neurons.

3.2 | LTP induces translocation of MAP2 to dendritic spines

In basal conditions, MAP2 immunoreactivity is primarily confined to dendritic shafts. If so, how MAP2 can mediate LTP induction at postsynaptic sites? Previous studies have suggested that MAP2 is also localized in dendritic spines and interacts with actin filaments in vitro.²⁵⁻²⁸ These pieces of evidence led us to hypothesize that MAP2 may act as a mobile element in response to extracellular stimulation.

To test this idea, we examined the effect of acute or chronic changes in neuronal activity on MAP2 immunoreactivity. We manipulated the activity state of mature hippocampal neurons, followed by immunolabeling against MAP2 (clone HM2 antibody) together with the spine-associated postsynaptic marker SPAR.³³ Chronic hyperactivity induced by the GABA_A receptor antagonist picrotoxin (PTX, 100 μ M, 24 hours) or acute membrane depolarization with KCl (40 mM, 40 minutes) did not affect the spatial distribution of MAP2 immunoreactivity (Figure 2A,B). Chronic inactivity induced by the reversible sodium channel blocker tetrodotoxin (TTX, 2 μ M, 24 hours) or the NMDAR antagonist 2-amino-5-phosphonopentanoic acid (APV, 200 μ M, 24 hours) was also ineffective in causing appreciable redistribution of MAP2 immunoreactivity (Figure 2A,B). In contrast, cLTP stimulation with glycine (200 μ M, 3 minutes; including 1 μ M of strychnine) produced punctate MAP2 signals that robustly colocalized with SPAR in dendritic spines (Figure 2A,B) (intensity ratio of synaptic MAP2 to dendritic MAP2: control 0.07 ± 0.02 , PTX 0.11 ± 0.03 , TTX 0.05 ± 0.01 , KCl 0.06 ± 0.03 , APV 0.03 ± 0.01 , cLTP 0.65 ± 0.03 ; $P < .001$, one-way ANOVA and post hoc Tukey HSD test).

Time-course experiments showed that synaptic translocation of MAP2 peaked within 5 min post-cLTP and returned to basal levels within 2 hours after stimulation (Figure 2C,D) (percentage of neurons with synaptic MAP2 signals [time after cLTP]: 0 minutes 0.20 ± 0.20 , 3 minutes 3.80 ± 0.76 , 5 minutes 11.78 ± 0.74 , 10 minutes 8.60 ± 0.60 , 20 minutes 6.60 ± 0.95 , 40 minutes 4.89 ± 0.71 , 60 minutes 2.75 ± 0.33 , 120 minutes 0.40 ± 0.27 , 210 minutes 0.25 ± 0.25 , 300 minutes 0.20 ± 0.20 ; $P < .001$, one-way ANOVA and post hoc Tukey HSD test). Prolonged cLTP stimulation with glycine (200 μ M, 6 minutes; including 1 μ M of strychnine) markedly

increased the percentage of neurons with synaptic MAP2 signals (Figure 2E) (percentage of neurons with synaptic MAP2 signals: glycine for 3 minutes 12.80 ± 1.24 , glycine for 6 minutes 58.00 ± 3.27 ; $P < .001$, two-tailed unpaired t test).

Due to extremely low MAP2 levels in dendritic spines under basal conditions, MAP2 fluorescence intensity

remarkably increased in spines with glycine-induced LTP (Figure 2F) (relative integrated intensity of synaptic MAP2: control 1.0 ± 0.37 , cLTP 76.2 ± 13.41 ; $P < .001$, two-tailed unpaired t test). Concomitantly, MAP2 immunoreactivity remaining in the dendritic shafts was reduced by 20% with cLTP and was no longer uniform in appearance, but often

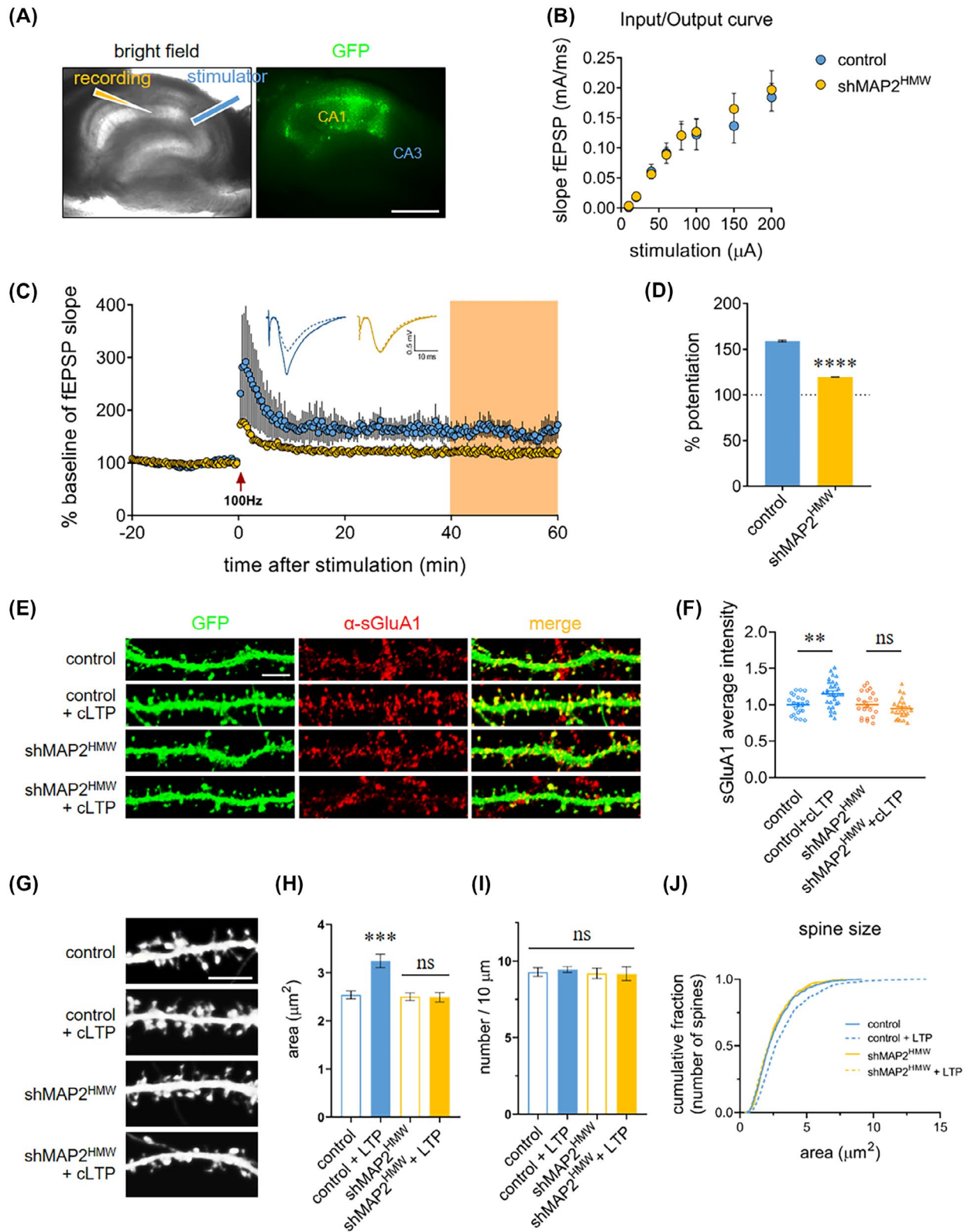


FIGURE 1 High-molecular-weight (HMW) MAP2 is required for LTP induction in the hippocampal neurons. A, Representative experimental setup of field excitatory postsynaptic potential (fEPSP) stimulation and recording at Schaffer collateral-CA1 synapses. GFP fluorescence indicates CA1 neurons transduced with lentivirus. Scale bar, 500 μ m. B, Input/output response curve (afferent stimulation vs fEPSP slope) in the hippocampal slices from mice transduced with either lentivirus expressing shRNA against HMW MAP2 (shMAP2^{HMW}) or control lentivirus (control, $n = 12$ slices in 3 mice; shMAP2^{HMW}, $n = 8$ slices in 3 mice). $P > .05$ (t test vs control). C, fEPSP slope before and after high-frequency stimulation (HFS, 100 Hz, 1 second) of the Schaffer collateral pathway in the hippocampal slices transduced with shMAP2^{HMW} or control lentivirus (control, $n = 7$ slices in 3 mice; shMAP2^{HMW}, $n = 8$ slices in 3 mice). Inset: representative fEPSP traces from the two groups. D, Percentage of potentiation in the hippocampal slices between the two groups. **** $P < .0001$ (t test vs control). E, Representative images of cultured hippocampal neurons transfected with either shMAP2^{HMW} or control vector in the presence or absence of 200 μ M of glycine (cLTP, with 1 μ M of strychnine for 3 minutes), followed by immunostaining for MAP2 and endogenous surface GluA1 (sGluA1). Scale bar, 5 μ m. F, Quantification of average intensity of sGluA1 levels in neuronal dendrites ($n = 21$ –29 neurons/condition). ** $P = .007$; ns, not significant (one-way ANOVA with post hoc Tukey HSD). G, Representative dendritic segments of hippocampal neurons transfected with either shMAP2^{HMW} or control vector at DIV 17, fixed at DIV 21 with or without cLTP stimulation (200 μ M of glycine with 1 μ M of strychnine for 3 minutes), and immunolabeled with anti-GFP antibody. Scale bar, 5 μ m. H, Quantification of average spine size in each condition ($n = 6$ –7 neurons/condition). *** $P = .0004$; ns, not significant (one-way ANOVA with post hoc Tukey HSD). I, Quantification of average spine density in each condition ($n = 6$ –7 neurons/condition). J, Cumulative distribution of dendritic spine size in each condition ($n = 753$ –1092 spines/condition). ** $P < .001$ for control + cLTP (Kolmogorov-Smirnov test). Data are presented as mean $\pm$ SEM

displayed grooved regions seemingly depleted of MAP2 (Figure 2G,H) (relative integrated intensity of dendritic shaft MAP2: control 100 ± 1.84 , cLTP 82.1 ± 3.16 ; $P < .001$, t test vs control). Together with the rapid appearance of the synaptic MAP2, these results suggest that a subpopulation of preexisting MAP2 molecules moved from the dendritic shafts to neighboring spines.

To verify these observations, we tested two additional independent MAP2 antibodies (clone AP20 and chicken polyclonal) and different postsynaptic markers (PSD95 as well as the F-actin stain phalloidin), which yielded essentially identical results (Supplemental Figure 4).

We next evaluated whether the spine translocation of MAP2 can be reproduced by other cLTP protocols such as forskolin/rolipram treatment³¹ or APV withdrawal.^{32,36} Elevation of cAMP levels by forskolin/rolipram treatment (10 μ M and 100 nM, respectively) for 15 minutes was sufficient for MAP2 translocation to postsynaptic spines (Figure 2I,J). MAP2 spine translocation was also induced with NMDAR activation by APV withdrawal (Figure 2I,J). A similar response from three different cLTP protocols reflects a reproducible and likely shared underlying mechanism.

As expected for NMDAR-dependent LTP, MAP2 spine translocation induced by cLTP stimulation was abolished with APV treatment (200 μ M) or with the addition of an extracellular calcium chelator, EGTA (1 mM) (Figure 2J). In addition, neuronal depolarization with KCl for 40 minutes (40 mM) or chemical long-term depression³⁷ with NMDA for 3 minutes (50 μ M) failed to induce significant synaptic translocation of MAP2 (Figure 2J), indicating that the process does not respond efficiently to generalized synaptic activity and is selective for cLTP stimulation. Notably, the effect of APV withdrawal on MAP2 spine translocation

was abolished in the presence of a microtubule-stabilizing agent, Taxol (10 nM) (Figure 2J), suggesting that intact MT dynamics may be essential for this process (percentage of neurons with synaptic MAP2 signals: control 0.20 ± 0.20 , glycine 11.40 ± 0.67 , glycine + APV 0.25 ± 0.25 , glycine + EGTA 0.00 ± 0.00 , forskolin + rolipram 11.00 ± 1.36 , APV withdrawal 10.50 ± 1.24 , APV withdrawal + Taxol 0.22 ± 0.22 , NMDA 1.25 ± 0.75 , KCl 2.00 ± 0.47 ; $P < .001$, one-way ANOVA and post hoc Tukey HSD test).

3.3 | MAP2 spine translocation is coupled with LTP-induced spine enlargement and AMPAR insertion

Previous studies established that LTP induction triggers enlargement of dendritic spines.^{38,39} To examine the relationship between spine enlargement and MAP2 synaptic translocation, mature hippocampal neurons (DIV 18–19) were cotransfected with MAP2B-GFP and RFP to visualize spine morphology. At 3 days after transfection, time-lapse live-cell imaging was performed using confocal microscopy. Consistent with previous reports,^{38,39} spine size was found to be enlarged after glycine-mediated cLTP induction (Figure 3A,B) (normalized RFP intensity: before glycine 1.03 ± 0.05 , 30 minutes after glycine 1.24 ± 0.23 , $P < .0001$, one-way ANOVA). Spine head expansion occurred within 5 minutes of glycine application. The increase in spine volume was long-lasting, persisting for at least 30 minutes after glycine addition.

Under this LTP paradigm, we observed an increase in MAP2B fluorescence intensity in the spines normalized to fluorescence in the adjacent dendritic shaft area (Figure 3C)

(normalized MAP2 intensity [glycine-LTP]: before glycine 1.00 ± 0.07 , 30 minutes after glycine 1.17 ± 0.15 , $P < .0001$, paired t test). The increase in the intensity of MAP2B-GFP in spines mirrored the time course of spine growth (RFP intensity), indicating that synaptic translocation of MAP2 is accompanied by cLTP-induced spine enlargement. The change

in MAP2 concentration within the spine during LTP was measured by dividing MAP2B-GFP intensity by RFP intensity (Figure 3D), showing the persistent spine translocation of MAP2 for at least 30 minutes after LTP induction (Figure 3E).

To further provide direct evidence for MAP2 translocation, we created a photoactivatable GFP-tagged MAP2 probe

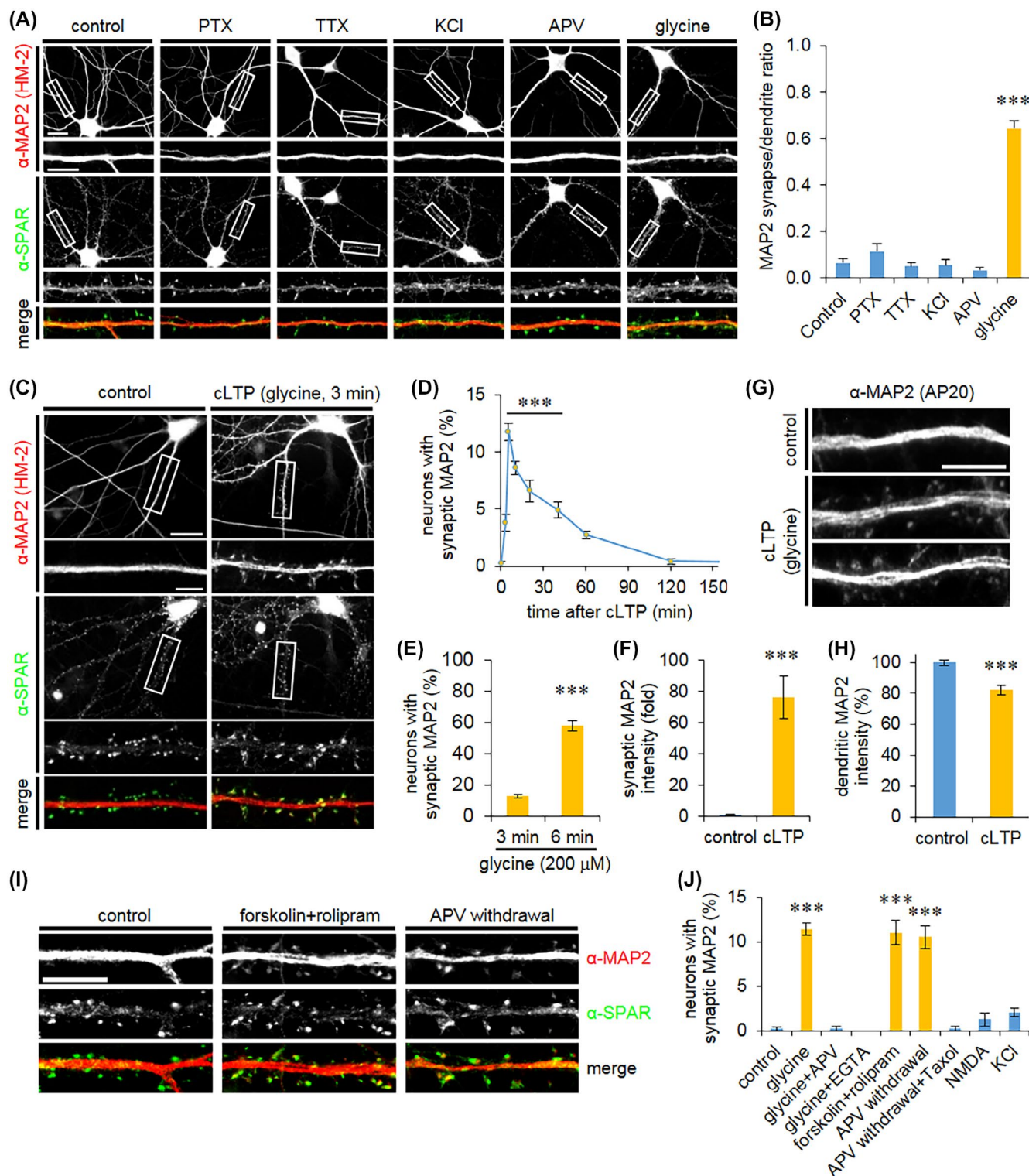


FIGURE 2 Synaptic translocation of MAP2 induced by cLTP. A, Representative images of cultured hippocampal neurons (DIV 20-21) treated with vehicle (control), 100 μ M of PTX (24 hours), 2 μ M of TTX (24 hours), 40 mM of KCl (40 minutes), 200 μ M of APV (24 hours), or 200 μ M of glycine (cLTP, with 1 μ M of strychnine for 3 minutes) before co-immunostaining for MAP2 (clone HM-2, red) and the postsynaptic marker SPAR (green). Scale bar, 20 μ m. Inset scale bar, 10 μ m. B, Quantification of the synaptic MAP2 intensity compared to dendritic shaft signals ($n = 10$ neurons/each condition). *** $P < .001$ (t test vs control). C, Cultured hippocampal neurons (DIV 21) treated with vehicle (control) or 200 μ M of glycine (cLTP, with 1 μ M of strychnine for 3 minutes) before co-immunostaining for the dendritic spine marker SPAR (green) and MAP2 (clone HM-2, red). Scale bar, 20 μ m. Inset scale bar, 5 μ m. D, Quantification of the percentage of neurons with MAP2-positive spines after cLTP induction ($n = 8$ -10 sets of neurons/time-point, 50 neurons/set). *** $P < .001$ (one-way ANOVA with post hoc Tukey HSD). E, Quantification of the percentage of neurons with MAP2-positive spines after glycine stimulation for 3 and 6 minutes ($n = 10$ sets of neurons, 10-50 neurons/set). *** $P < .001$ (t test vs glycine for 3 minutes). F, Quantification of synaptic MAP2 immunoreactive intensity ($n = 21$ -23 neurons/condition). *** $P < .001$ (t test vs control). G, Representative images of dendritic MAP2 (clone AP20) immunoreactivity with or without cLTP induction. Scale bar, 10 μ m. H, Quantification of MAP2 immunoreactive intensity in dendritic shafts ($n = 15$ neurons/condition). *** $P < .001$ (t test vs control). I, Representative images of the hippocampal neurons treated with vehicle (control), a combination of forskolin and rolipram, or APV withdrawal for cLTP induction before immunostaining against MAP2 (red) and the postsynaptic marker SPAR (green). Scale bar, 10 μ m. J, Quantification of the percentage of neurons with MAP2-positive spines induced by cLTP protocols in combination with target drugs ($n = 8$ -10 sets of neurons/ time-point, 40-50 neurons/set). Combination details are as follows: vehicle (control), glycine for 3 minutes, glycine for 3 minutes in the presence of either APV or EGTA, forskolin and rolipram, APV withdrawal alone or in the presence of Taxol, NMDA alone, and KCl alone. *** $P < .001$ (one-way ANOVA with post hoc Tukey HSD). Data are presented as mean $\pm$ SEM

(MAP2-PAGFP) and examined cLTP-induced MAP2 spine translocation using this construct. Photoactivation at 3 minutes after cLTP stimulation revealed that a subset of activated MAP2 in the dendritic shaft entered into neighboring spines (Figure 3F). Quantification confirmed the marked increase of MAP2-PAGFP intensity in individual spines after translocation (Figure 3G) (PAGFP intensity: before translocation 34.29 ± 5.25 , after translocation 150.9 ± 12.28 , $P < .0001$, paired t test).

Because LTP stimulation also promotes the synaptic insertion of AMPARs,^{4,5} we next tested whether LTP-induced MAP2 spine translocation is related to synaptic transport of AMPARs. Live-cell labeling of endogenous surface-expressed GluA1 under nonpermeabilizing conditions showed that only neurons with MAP2-positive spines after cLTP had significantly increased levels of surface GluA1, compared to either control neurons or neurons with MAP2-negative spines after cLTP induction (Figure 3H,I) (relative integrated intensity: control 1.00 ± 0.16 , synaptic MAP2-positive neurons after glycine 2.54 ± 0.57 , synaptic MAP2-negative neurons after glycine 1.39 ± 0.37 , glycine + APV 1.17 ± 0.08 , glycine + FTS 0.86 ± 0.29 , glycine + PD98059 1.01 ± 0.31 , glycine + Taxol 1.24 ± 0.24 ; $P = .024$, one-way ANOVA). As expected, cLTP-induced insertion of surface GluA1 was prevented in the presence of pharmacological inhibitors against Ras and MEK (farnesyl thiosalicylic acid and PD98059, respectively), which are involved in the LTP-induced AMPAR insertion.^{40,41} Again, application of Taxol that abolished MAP2 spine translocation (Figure 2J), also blocked synaptic AMPAR delivery following LTP stimulation (Figure 3I). Thus, these findings indicate that MAP2 spine translocation is closely correlated with surface AMPAR delivery and spine enlargement during LTP.

3.4 | Ultrastructural localization of increased synaptic MAP2 following LTP induction

To test whether spine translocation of MAP2 can occur in more physiologically relevant systems with an electrical LTP induction protocol, we next delivered high-frequency stimulation (HFS; 100 Hz, 1 seconds) to Shaffer collateral-CA1 synapses in the mouse hippocampal slices,⁴² a preparation that retains more intact synaptic networks than the dissociated neuron cultures. Postembedding immunogold labeling was used to study the ultrastructural distribution of endogenous MAP2 in the dendritic spines of CA1 *stratum radiatum*.

Immunostaining in the absence of primary antibody did not show nonspecific gold particles (Figure 4A1), indicating the specificity of MAP2 antibody used in this experiment. MAP2-immunogold signals were rarely observed within the CA1 spine heads in control slices stimulated with HFS protocol in the presence of the NMDA receptor antagonist APV (50 μ M) (Figure 4A2). In contrast, LTP stimulation led to a significant increase in the number of MAP2-immunogold particles within the CA1 spine heads (Figure 4A3,A4,B,C) (average gold particles per spine head: control 1.61 ± 0.14 , LTP 4.49 ± 0.25 ; $P < .001$, t test versus control; the Kolmogorov-Smirnov test for comparing cumulative fractions, $P < .001$), as compared to control slices. A more detailed analysis on the distributions of MAP2 in the individual spine heads revealed that the average distance of gold particles from the postsynaptic membrane was slightly but significantly increased following electrical LTP induction (Figure 4D,E) (in nm: control 36.6 ± 3.40 , LTP 46.6 ± 1.95 ; $P = .015$, t test vs control), further supporting the concept that more MAP2 protein is

accumulated to the spine heads upon LTP induction. The distribution of gold particles showed a significantly higher particle concentration over the range of 0 ~ 120 nm after HFS (Figure 4F) ($P < .001$, t test versus control).

Additionally, some gold particles were detected along the “tracks” that appeared to traverse the spine neck (Figure 4A5), indicating a snapshot of MAP2 subpopulation being transported to the spine heads. These results demonstrated that

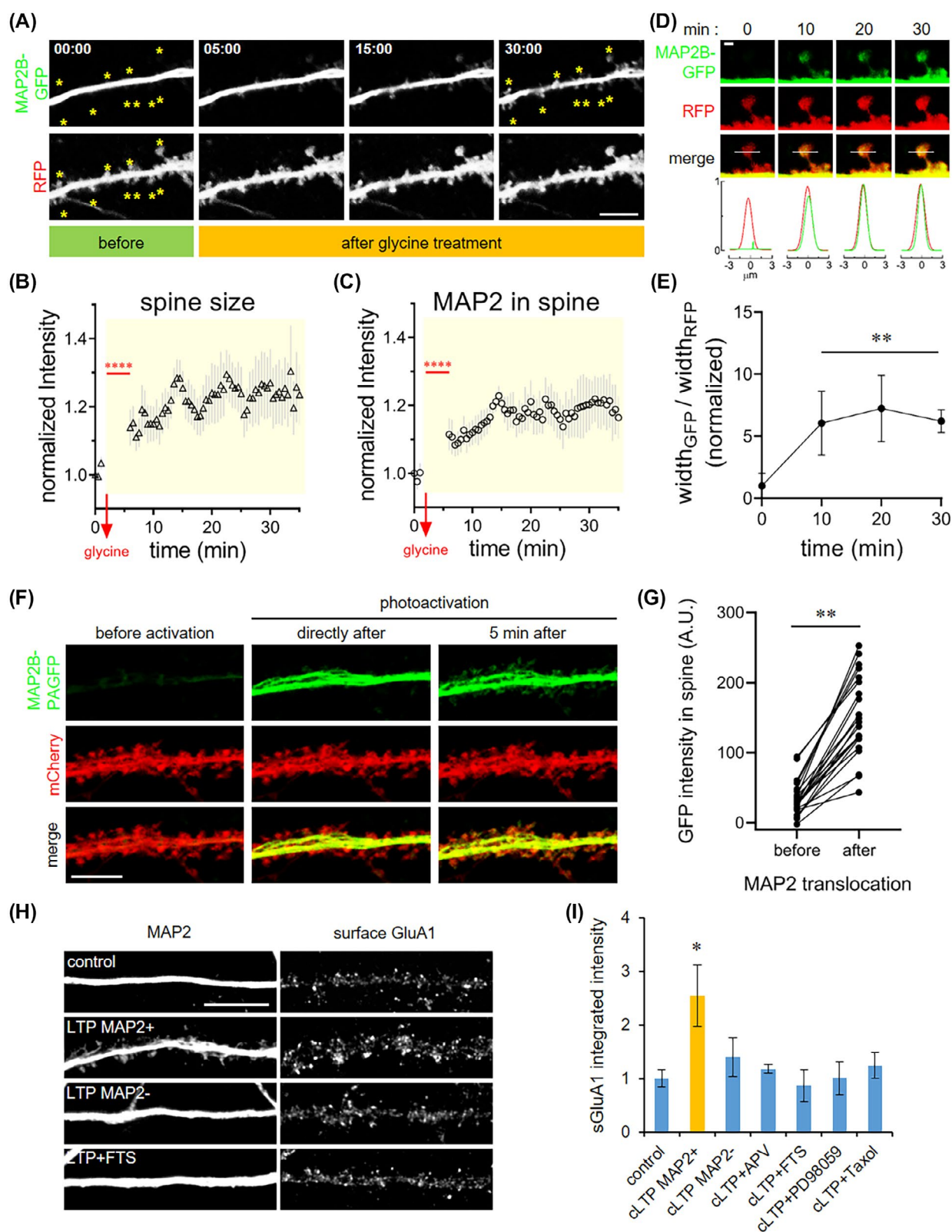


FIGURE 3 MAP2 spine translocation is coupled with LTP-induced spine enlargement and surface insertion of AMPARs. A, Representative live images of cultured hippocampal neurons expressing RFP and MAP2B-GFP in a time-course following glycine-mediated cLTP induction. The amount of MAP2 (upper panels, yellow asterisks) and the spine volume (lower panels, yellow asterisks), both increased after cLTP induction. Scale bar, 5 μ m. B and C, Quantification of the extent of dendritic spine enlargement by normalized RFP intensity (B) and MAP2 translocation into dendritic spines by normalized GFP intensity (C) ($n = 70$ spines from 7 neurons). **** $P < .0001$ (one-way ANOVA). D, Distribution of MAP2 within the spine head during LTP. Green and red fluorescence signals from a line across the spine head (white line) were normalized to the peak value. Width was calculated as the full-width at half-height from a Gaussian fitting curve. Scale bar, 1 μ m. E, Time course of change in MAP2 distribution within the spine volume (the ratio of green width and red width) ($n = 7$ spines). ** $P < .01$ (one-way ANOVA with post hoc Tukey HSD). F, Representative time-course (before, directly after, and 5 minutes after photoactivation) images showing a dendritic segment expressing MAP2-PAGFP following glycine-mediated cLTP induction. Scale bar, 5 μ m. G, Quantification of the MAP2 intensity in spines before and after MAP2 translocation ($n = 22$ spines). **** $P < .0001$ (paired t test). H, Representative images of cultured hippocampal neurons stimulated with glycine-mediated cLTP induction protocol. Neurons were treated at DIV 21 with vehicle (control) or glycine (200 μ M, 3 minutes with 1 μ M of strychnine) in the presence or absence of each drug (APV, Ras inhibitor FTS, MEK inhibitor PD98059, or Taxol), followed by co-immunostaining for MAP2 and endogenous surface-expressed GluA1. I, Quantification of the integrated intensity of surface GluA1 levels in dendrites ($n = 9$ -10 cells/condition). * $P = .024$ (one-way ANOVA with post hoc Tukey HSD). Scale bar, 10 μ m. Data are presented as mean $\pm$ SEM

electrical LTP stimulation is sufficient to induce MAP2 spine translocation in the hippocampal slices, in which much of the complex cellular integrity and intact circuitry is preserved, thus, supporting the physiological relevance of these events.

3.5 | Isoform-specific translocation of MAP2

To examine whether LTP-induced MAP2 spine translocation is isoform-specific, we next transfected mature hippocampal neurons (DIV 21) with plasmids expressing MAP2A, B, and C tagged with GFP. At 3 days after transfection, we induced cLTP with glycine, and then, immunostained for GFP (Figure 5A). Under basal conditions, both HMW isoforms, MAP2A- and MAP2B-GFP, were observed primarily in the dendritic shafts. After cLTP stimulation, MAP2A- and MAP2B-GFP signals were detected in dendritic spines (Figure 5A,C). Interestingly, the overexpressed MAP2C-GFP was observed in dendritic spines of mature hippocampal neurons under basal conditions prior to cLTP induction, and did not further enrich in spines following cLTP (Figure 5A,C) (percentage of neurons with synaptic MAP2-GFP signals at DIV 24: MAP2A control 13.13 ± 4.51 , MAP2A glycine 42.98 ± 7.30 , $P = .014$; MAP2B control 21.67 ± 3.97 , MAP2B glycine 63.81 ± 3.23 , $P = .0002$; MAP2C control 75.83 ± 5.34 , MAP2C glycine 80.00 ± 8.16 , $P = .68$; t test vs control). Considering that endogenous MAP2C expression occurs dominantly during early dendritic development⁴³ (Supplemental Figure 1), the synaptic MAP2C-GFP signals under basal conditions may reflect ectopic expression (or misexpression) of MAP2C.

To address these possibilities, we next transfected young hippocampal neurons (at DIV 10) with a plasmid overexpressing MAP2C-GFP (Figure 5B). After 2 days, we induced cLTP as before, and immunostained for GFP and endogenous HMW MAP2 (clone AP20; specific for

MAP2A/B). In young neurons, MAP2C-GFP and endogenous MAP2A/B immunoreactivities were confined to the dendritic shaft under basal conditions (Figure 5B,C). Upon cLTP induction, endogenous HMW MAP2 was translocated to the dendritic protrusions, while MAP2C-GFP remained largely in the dendritic shafts of the same neurons (Figure 5B,C) (percentage of neurons with synaptic MAP2C-GFP signals at DIV 12: MAP2C control 5.00 ± 5.00 , MAP2C glycine 11.25 ± 6.57 ; $P = .48$, t test vs control). These data indicated that HMW isoforms of MAP2 are likely to selectively mobilize from the dendritic shaft to postsynaptic sites in response to cLTP stimulation, while MAP2C may be dedicated to the early development of dendritic arbors.

3.6 | Ras-MAPK pathway is involved in MAP2 spine translocation

MAP2 binds to MTs in a phosphorylation-dependent manner,⁴⁴ and depolarizing stimulation induced a transient bidirectional change in the phosphorylation of MAP2 in the hippocampal slices.^{45,46}

Because MAP2 is phosphorylated by multiple protein kinases involved in synaptic plasticity, including PKA, CaMKII, PKC, and ERK,⁴⁷ we tested whether LTP-induced MAP2 spine translocation could be blocked by various pharmacological inhibitors of kinase-associated pathways (Figure 6): farnesyl thiosalicylic acid (FTS) for Ras, PD98059 for MEK, FR180204 for ERK, KN62 for the CaMKII family, H89 and KT5720 for PKA, staurosporine (STS) and bisindolylmaleimide (BIS) for PKC, and JNK inhibitor II. Importantly, only inhibitors of Ras, MEK, or ERK significantly reduced the number of neurons with synaptic MAP2 signals in response to cLTP induction (Figure 6A,B), indicating that the Ras- and

mitogen-activated protein kinase (MAPK) pathway is closely related to cLTP-induced synaptic translocation of MAP2. In contrast, the CaMKII inhibitor KN62 and other kinase inhibitors of PKA, PKC, and JNK did not block cLTP-dependent spine translocation of MAP2 (Figure 6B).

Notably, the rapid synaptic translocation of MAP2 was not affected by the protein synthesis inhibitor cycloheximide (Figure 6B), further indicating that preexisting dendritic MAP2 mobilized to neighboring synapses (percentage of neurons with synaptic MAP2 signals: control 0.13 ± 0.13 ,

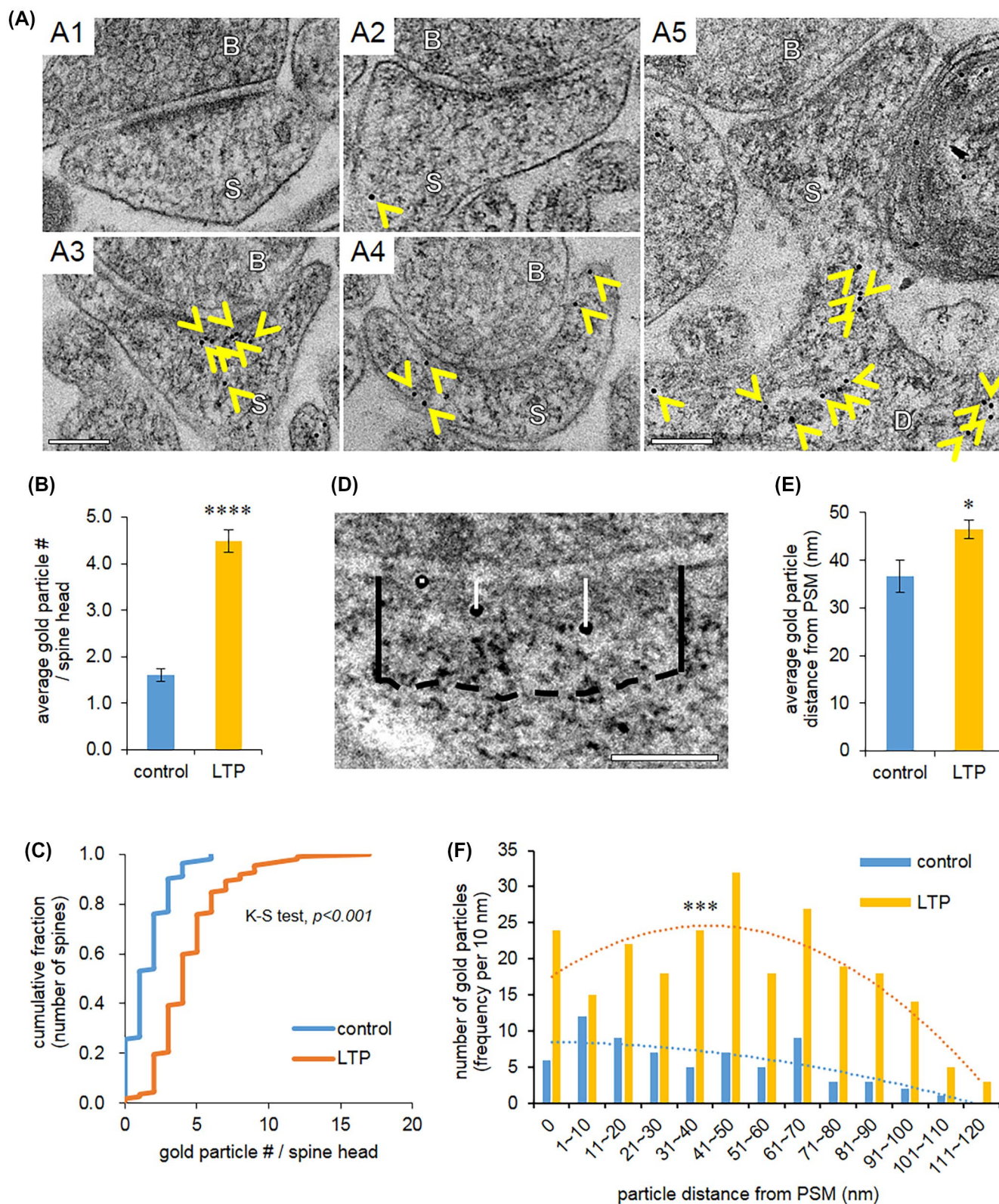


FIGURE 4 Ultrastructural localization of MAP2 in the spine heads of CA1 neurons after LTP induction. A, Representative images of the redistribution of endogenous MAP2 in CA1 synapses of the hippocampal slices stimulated by electrical LTP. High-frequency stimulation (HFS, 100 Hz, 1 second) was delivered to mouse brain slices in the presence or absence of 50 μ M of APV (termed control and LTP, respectively). Ultrathin (70-nm-thick) serial sections of CA1 *stratum radiatum* mounted on nickel grids were subjected to immunogold labeling against endogenous MAP2 (yellow arrow heads). Sections, labeled without primary antibodies (A1, *negative control*), show no gold particles, reflecting the specificity of binding of the primary antibody to the antigen. Compared to HFS stimulation in the presence of APV (A2, control), HFS stimulation alone (A3–5, LTP) significantly increased the localization of immunogold particles to MAP2 in the spine heads. Note the MAP2 particles along the tracks through the spine neck in A5. B, Quantification of average number of gold particles in individual spine heads after LTP ($n = 112$ spine heads) compared to the control ($n = 113$ spine heads). **** $P < .0001$ (t test vs control). C, Cumulative distribution of number of gold particles in individual spine heads after LTP. *** $P < .001$ (Kolmogorov-Smirnov test). D, Representative electron micrograph of CA1 spine head labeled for MAP2 with 10 nm gold particles (black dots). Distance of gold particles from the postsynaptic membrane is indicated by the white lines. Black lines depict the boundaries of the defined PSD area. E, Average distance of gold particles from the postsynaptic membrane. (control, $n = 69$ gold particles from 43 spine heads; LTP, $n = 240$ gold particles from 82 spine heads; * $P < .05$, t test vs control). F, Gold particle distribution as a function of distance from the postsynaptic membrane. *** $P < .001$ (t test vs control). Scale bar, 100 nm. Data are presented as mean $\pm$ SEM. B, presynaptic bouton; D, dendritic shaft; PSM, postsynaptic membrane; S, dendritic spine

glycine 12.80 ± 1.24 , glycine + farnesyl thiosalicylic acid 3.38 ± 0.75 , glycine + PD98059 2.67 ± 0.82 , glycine + FR180204 3.67 ± 0.66 , glycine + KN62 12.75 ± 1.00 , glycine + H89 12.29 ± 1.02 , glycine + KT5720 12.25 ± 0.88 , glycine + staurosporine 14.00 ± 1.25 , glycine + bisindolylmaleimide 14.25 ± 1.10 , glycine + JNK inhibitor II 14.50 ± 0.98 , glycine + Cycloheximide 12.75 ± 1.06 ; $P < .001$, one-way ANOVA and post hoc Tukey HSD test).

Recent live-cell imaging studies have shown that MTs transiently invade the dendritic spines to regulate synaptic plasticity.^{48,49} Thus, we finally tested whether MAP2 in postsynaptic sites may colocalize with dynamic MTs entering dendritic spines. However, we found that neither tyrosinated (active) tubulin nor total beta-3 tubulin translocated into the synaptic areas upon cLTP stimulation (Figure 6C,D).

4 | DISCUSSION

Activity-dependent synapse remodeling is a cellular mechanism widely associated with information storage. However, the molecular mechanisms that coordinate crosstalk between dendritic shafts and activated spines during synaptic plasticity are not well understood. In this study, we found that HMW MAP2 is one such molecule that mediates LTP induction via translocation to dendritic spines in mature hippocampal network.

Specific silencing of HMW MAP2 completely prevents LTP induction and synaptic AMPAR insertion in mature hippocampal CA1 neurons (Figure 1), demonstrating that HMW MAP2 is essential for LTP induction. Then, how can MAP2 in dendritic shafts mediate LTP induction at postsynaptic sites? Intriguingly, we observed that HMW MAP2 may act as a mobile element in response to LTP stimulation. Both various chemical and electrophysiological LTP induction protocols consistently promoted spine translocation of MAP2 that persisted for tens of minutes (Figure 2). MAP2 spine translocation

peaked within 5 minutes and returned to basal levels within 2 hours after cLTP. This time course is reminiscent of previous reports regarding “synaptic tag” lifetimes,⁵⁰ and we speculate that MAP2 could function in a synaptic tagging capacity.

A number of studies have shown that LTP induction promotes spine enlargement and surface insertion of AMPARs.^{51,52} Consistent with this concept, our imaging studies showed an increase in spine volume following cLTP induction (Figure 3A–E). Under this condition, MAP2 spine translocation occurred concomitantly with spine enlargement (Figure 3A–G). Furthermore, live cell-surface receptor staining revealed increased GluA1 levels only in the neurons showing MAP2 spine translocation after cLTP induction (Figure 3H,I). Importantly, depletion of HMW MAP2 in mature hippocampal neurons prevented LTP-induced spine enlargement and surface AMPAR insertion (Figure 1G–J). This indicates that MAP2 synaptic transport is closely coupled with LTP-associated increases in spine size and AMPAR content.

Consistent with light microscopic data of mature hippocampal cultures (Figures 2 and 3), ultrastructural localization of MAP2 using immunogold electron microscopy revealed a significant increase in MAP2-positive gold particles in the vicinity of PSD (35 ~ 50 nm away from the postsynaptic membrane) after HFS at Shaffer collateral-CA1 synapses of the hippocampal slices (Figure 4). This region of the dendritic spine contains various scaffolding proteins, such as Shank2 and GKAP,^{34,53} as well as actin-binding proteins, such as α -actinin and neurabin,^{54,55} all of which are involved in the regulation of spine morphology. While screening experiments to find molecular partners binding to synaptic MAP2 are ongoing, this observation hints at possible functions of MAP2 within the spines.

It is likely that only HMW MAP2 exhibits activity-dependent synaptic translocation in mature hippocampal neurons transfected with GFP-tagged MAP2 isoforms (Figure 5). Using an antibody specific for HMW MAP2,

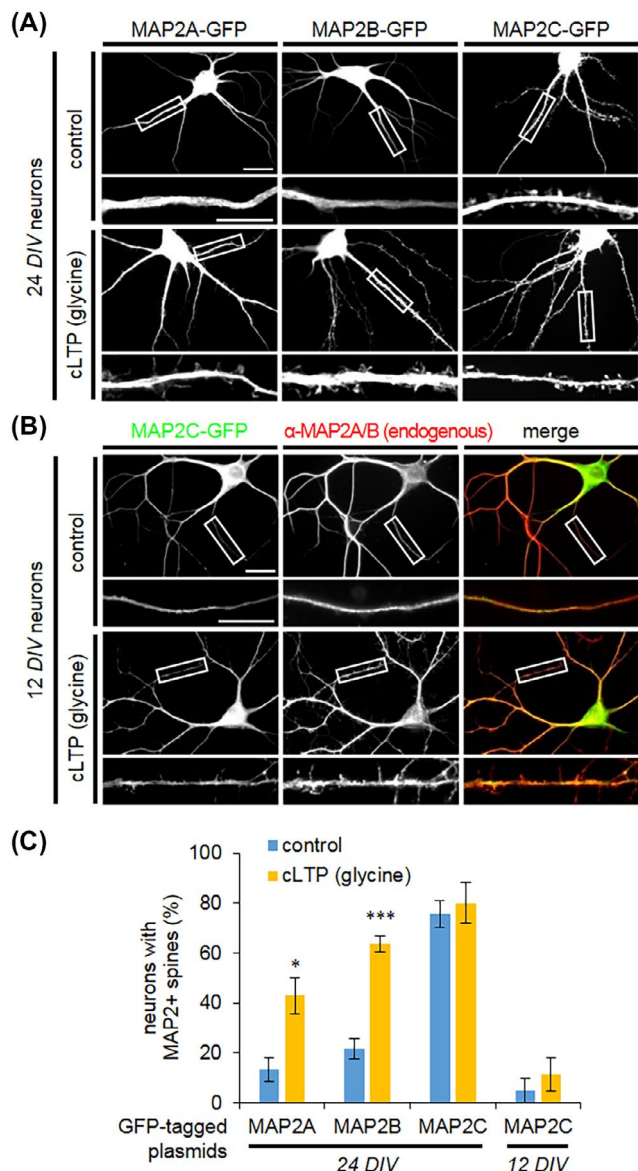


FIGURE 5 Isoform-specific translocation of MAP2 in the hippocampal neurons. A, Representative images of cultured hippocampal neurons (days in vitro [DIV] 21) transfected with plasmids expressing GFP-tagged MAP2A, B, or C for 3 days, and treated with vehicle (control), or 200 μ M of glycine (cLTP, with 1 μ M of strychnine for 3 minutes) before immunostaining for GFP. B, Young hippocampal neurons (DIV 10) transfected with a plasmid expressing MAP2C-GFP for 2 days and treated with vehicle (control), or 200 μ M of glycine (cLTP, with 1 μ M of strychnine for 3 minutes) before co-immunostaining for GFP and endogenous high-molecular-weight MAP2 (MAP2A/B, clone AP20 antibody). C, Quantification of the number of neurons with MAP2-positive spines induced by vehicle (control) or glycine-mediated cLTP (n = 4 sets of neurons/group, 4-7 transfected neurons/set). Scale bar, 20 μ m. Inset scale bar, 10 μ m. * P < .05, *** P < .001 (t test vs control). Data are presented as mean $\pm$ SEM.

we showed that cLTP stimulation in young neurons induced the translocation of endogenous HMW MAP2 to dendritic protrusions, while retaining the overexpressed

MAP2C-GFP in the dendritic shafts. This isoform selectivity indicates that nonspecific passive diffusion cannot explain the movement of MAP2 into the neighboring spines, and may further explain why dynamic translocation of MAP2 was not detected in a previous study using transgenic mice overexpressing LMW MAP2C.⁵⁶

For cLTP induction, we applied three independent protocols commonly used to biochemical study of synaptic plasticity: glycine-induced LTP,⁴ APV withdrawal,³² and treatment with forskolin/rolipram.³¹ Both glycine stimulation and APV withdrawal, involving NMDAR activation, were sufficient to produce postsynaptic MAP2 translocation (Figures 2, 3, and 6). The synaptic transport of MAP2 was also shown by an increase in cAMP levels after forskolin/rolipram treatment, but it was not prevented by pharmacological inhibitors against PKA, a major downstream target of cAMP (Figure 6B). These results may suggest that MAP2 mobilization to the spines is mediated by other cAMP-sensitive pathways, such as Epac. This is supported by previous findings that the activation of Epac helps in maintaining LTP in the CA1 area of the mouse hippocampal slices.⁵⁷ Moreover, Epac/Rap1/AF-6 signaling pathways regulate spine morphogenesis and synaptic AMPAR content in response to cLTP induction.³² Importantly, we showed that cLTP-induced MAP2 spine translocation was significantly impaired by inhibitors of the Ras-MAPK signaling cascade, which is a pathway that has been implicated in LTP, synaptic insertion of AMPARs, and memory formation.⁵⁸ Therefore, MAP2 spine transport may serve as a missing molecular link between MAPK activation and the surface delivery of AMPARs. It would be of interest to identify MAPK-directed phosphorylation sites on MAP2 and perform the synaptic translocation experiments using MAP2 phospho-mimic or phospho-mutant constructs for the detailed molecular mechanisms of MAP2 in synaptic plasticity.

On the contrary, a variety of inhibitors for other key kinases involved in synaptic plasticity, including CaMKII, PKA, and PKC⁵⁹⁻⁶¹ did not affect MAP2 spine translocation upon LTP induction, indicating that multiple pathways act in parallel to perform distinct subsets of plasticity-related functions. Additionally, MAP2 spine translocation was not affected by a protein synthesis inhibitor, suggesting that pre-existing MAP2 in the dendritic shafts can be transported to the spines (Figure 6B).

Our results indicate that the synaptic trafficking of MAP2 requires intact MT dynamics, because MAP2 translocation was abolished in the presence of low-dose Taxol (MT-stabilizing agent, 10 nM). It is currently unclear how Taxol treatment prevented cLTP-dependent MAP2 spine translocation. However, Taxol has been reported to induce a substantial change in microtubular structure by binding to β -tubulin subunit,^{62,63} which may interfere with MAP2 phosphorylation or dissociation from MTs.

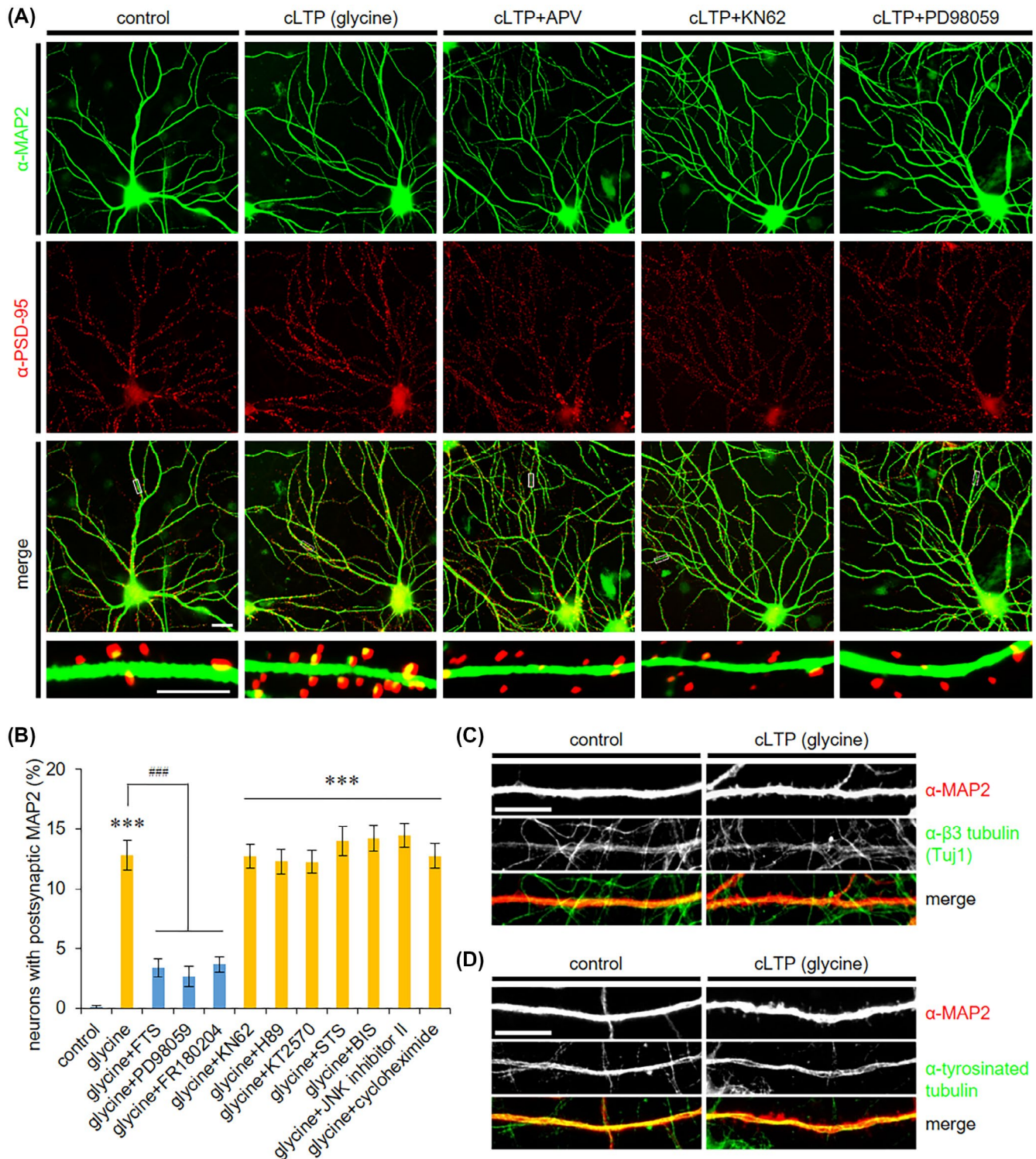


FIGURE 6 Signaling pathways involved in MAP2 synaptic translocation. A, Representative images of cultured hippocampal neurons at DIV 20–21 treated with vehicle (control), or 200 μ M of glycine (cLTP, with 1 μ M of strychnine for 3 min) in the presence or absence of APV, KN62, and PD98059 before co-immunostaining for MAP2 (chicken polyclonal, green) and postsynaptic marker PSD95 (red). Scale bar, 10 μ m. Inset scale bar, 5 μ m. B, Quantification of the number of neurons with MAP2-positive spines induced by vehicle (control), glycine-mediated cLTP alone, or cLTP in the presence of indicated target drugs ($n = 8$ –10 sets of neurons/time-point, 40–50 neurons/set). Target drug details are as follows: farnesyl thiosalicylic acid (FTS), PD98059, FR180204, KN62, H89, KT5720, staurosporine (STS), bisindolylmaleimide (BIS), JNK inhibitor II, or cycloheximide. *** $P < .001$ vs control; ### $P < .001$ vs glycine (one-way ANOVA with post hoc Tukey HSD). C and D, Representative images of cultured hippocampal neurons treated at DIV 20–21 with vehicle (control), or 200 μ M of glycine (cLTP, with 1 μ M of strychnine for 3 minutes) before co-immunostaining for MAP2 (chicken polyclonal, red) and neuron-specific β -tubulin III (Tuj1, C) or dynamic microtubules (tyrosinated tubulin, D) (green). Scale bar, 10 μ m.

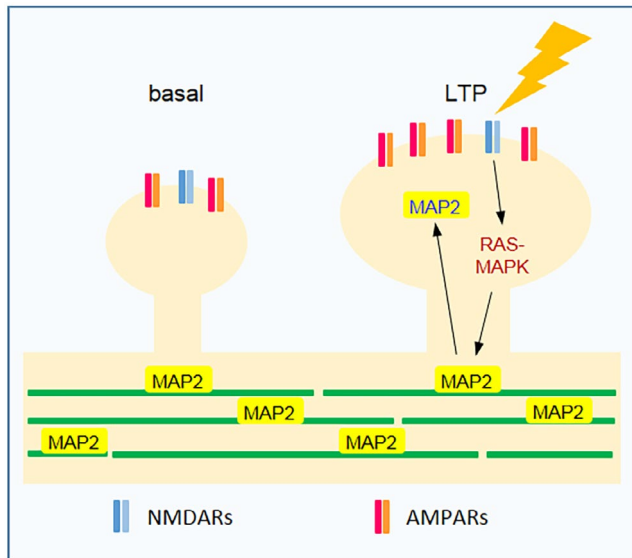


FIGURE 7 A schematic model for MAP2 spine translocation during LTP. Upon LTP stimulation, the activation of NMDA receptors triggers the Ras-MAPK signaling pathway. The activated MAPK induces translocation of MAP2 to the neighboring spines. It is plausible that MAP2 in spines may associate with actin, promoting spine enlargement and synaptic expression of AMPARs

Recent imaging studies have shown that MTs transiently invade the dendritic spines to regulate synaptic plasticity.^{48,49} Thus, we reasoned that MAP2 in spine heads may colocalize with dynamic MTs entering the dendritic spines. However, neither tyrosinated (active) tubulin nor total beta-3 tubulin colocalized with synaptic MAP2 upon cLTP stimulation (Figure 6C,D). Although we did not find localization of dynamic and stable MTs in MAP2-translocated spines after LTP stimulation using immunocytochemistry, we should not rule out the possibility of cotrafficking of MAP2 and dynamic MTs to spines because low doses of Taxol prevented LTP-induced MAP2 spine translocation (Figure 2J). It is also possible that a subset of dynamic MTs invading into spines could be very labile and not typically resistant to standard chemical fixation. Therefore, cotrafficking of MAP2 and dynamic MTs will await further detailed live imaging studies.

Based on our findings in this study and previously established evidence, we propose the following model for MAP2 spine translocation (Figure 7): activation of NMDARs during LTP stimulation triggers the Ras-MAPK signaling pathway. The activated MAPK then triggers translocation of MAP2 to the neighboring spines. It is plausible that MAP2 in spines may associate with actin (Supplemental Figure 4), promoting spine enlargement. This signaling cascade seems to be an additional early obligatory step in LTP induction because lentivirus-mediated knockdown of the HMW MAP2 completely abolished LTP induction at Schaffer collateral-CA1 synapses (Figure 1). Notably, it is possible that different MAPs function in parallel to

participate in distinct and sequential plasticity-related processes, as exemplified in a recent study showing that MAP6 in dendritic spines plays a role in the stabilization of actin filaments during the maintenance of LTP.²²

Taken together, our findings demonstrate a novel dynamic role of MAP2 in LTP induction, although further studies are warranted to elucidate exact molecular details of MAP2 spine translocation. It would be of great interest to examine physiological roles of HMW MAP2 in regulation of hippocampus-dependent cognitive behaviors.

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AUTHOR CONTRIBUTIONS

K. J. Lee and D. T. Pak designed the experiments, wrote the manuscript, and supervised the project; Y. Kim, Y. -N. Jang, J. -Y. Kim, N. Kim, S. Noh, H. Kim, B. N. Queenan, R. Bellmore, J. Y. Mun, H. Park, J. C. Rah, and K. J. Lee performed experiments and analyzed data. Y. Kim wrote the initial draft of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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