THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 35, pp. 21663–21675, August 28, 2015

Nuclear Translocation of Calcium/Calmodulin-dependent Protein Kinase IIδ3 Promoted by Protein Phosphatase-1 Enhances Brain-derived Neurotrophic Factor Expression in Dopaminergic Neurons*

Received for publication, May 13, 2015, and in revised form, June 25, 2015 Published, JBC Papers in Press, July 10, 2015, DOI 10.1074/jbc.M115.664920

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Background: The physiological function of CaMKIIδ3 phosphorylation at Ser^{332} remains unclear.
Results: CaMKIIδ3 dephosphorylation at Ser^{332} promotes its nuclear localization and stimulates BDNF expression.
Conclusion: Dopamine D_2 receptor stimulation triggers CaMKIIδ3 dephosphorylation at Ser^{332} by PP1.
Significance: CaMKIIδ3 (Ser^{332}) dephosphorylation is critical for neurite extension and survival of dopaminergic neurons.

We have reported previously that dopamine D_2 receptor stimulation activates calcium/calmodulin-dependent protein kinase II (CaMKII) δ3, a CaMKII nuclear isoform, increasing BDNF gene expression. However, the mechanisms underlying that activity remained unclear. Here we report that CaMKIIδ3 is dephosphorylated at Ser^{332} by protein phosphatase 1 (PP1), promoting CaMKIIδ3 nuclear translocation. Neuro-2a cells transfected with CaMKIIδ3 showed cytoplasmic and nuclear staining, but the staining was predominantly nuclear when CaMKIIδ3 was coexpressed with PP1. Indeed, PP1 and CaMKIIδ3 coexpression significantly increased nuclear CaMKII activity and enhanced BDNF expression. In support of this idea, chronic administration of the dopamine D_2 receptor partial agonist aripiprazole increased PP1 activity and promoted nuclear CaMKIIδ3 translocation and BDNF expression in the rat brain substantia nigra. Moreover, aripiprazole treatment enhanced neurite extension and inhibited cell death in cultured dopaminergic neurons, effects blocked by PP1γ knockdown. Taken together, nuclear translocation of CaMKIIδ3 following dephosphorylation at Ser^{332} by PP1 likely accounts for BDNF expression and subsequent neurite extension and survival of dopaminergic neurons.

Calcium/calmodulin (Ca^{2+}/CaM)-dependent[3] protein kinase II (CaMKII), a multifunctional serine/threonine protein kinase (1), regulates diverse Ca^{2+}-mediated neuronal activities in the brain, including neurotransmitter release, gene expression, and synaptic plasticity (2, 3). Four CaMKII subunits (α, β, γ, and δ) are encoded by distinct genes in eukaryotes (4), and their mRNAs undergo differential splicing at the so-called variable domain (5). Among those isoforms, CaMKIIαB (6), CaMKIIγA (7), and CaMKIIδ3 (also called CaMKIIδB) (8) display consensus (KKRK) sequences in the variable domain that resemble a nuclear localization signal (NLS) homologous to the SV40 large T antigen NLS (9).

In the rat brain, CaMKIIαB and CaMKIIδ3 are expressed in nuclei of neurons (6, 10). The activity of both is reportedly regulated by the NLS motif, which, when phosphorylated, prevents nuclear localization. For example, the CaMKIIδ3 Ser^{332} residue immediately C-terminal to the NLS (^{328}KKRKS^{332}) can be phosphorylated by the CaMK family members CaMKI or CaMKIV, blocking association of CaMKII with the NLS receptor m-pendulin, therefore prohibiting nuclear localization (11).

Nuclear CaMKII likely functions in transcriptional regulation of the neurotrophin BDNF (12, 13) through phosphorylation of diverse nuclear proteins, including cAMP response element-binding protein (CREB) (14, 15), activating transcription factor (16, 17), CCAAT/enhancer-binding protein (18, 19), serum response factor (20), and methyl CpG binding protein 2 (MeCP2) (21). BDNF promotes neuronal survival and axonal and dendritic growth, thereby triggering changes in synaptic plasticity (22, 23). We have demonstrated previously that CaMKIIδ3 is highly expressed in dopaminergic rat substantia nigra (SN) neurons (24) and that stimulation of the dopamine D_2 receptor (D_2R) promotes CaMKIIδ3 activation, thereby inducing BDNF gene expression in NG108-15 cells (25). This work strongly suggests that D_2R/CaMKIIδ3 signaling is important for survival and/or differentiation of dopaminergic neu-

* This work was supported by Grant-in-Aids 25110705 and 25460090 (to N. S.) and 24102505 and 25293124 (to K. F.) for Scientific Research in the Innovative Area "Foundation of Synapse and Neurocircuit Pathology" from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The authors declare that they have no conflicts of interest with the contents of this article.

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[3] The abbreviations used are: CaM, calmodulin; CaMKII, calmodulin-dependent protein kinase II; NLS, nuclear localization signal; CREB, cAMP response element-binding protein; SN, substantia nigra; D_2R, dopamine D_2 receptor; APZ, aripiprazole; DIV, day(s) *in vitro*; c-LTP, chemically induced long-term potentiation; TH, tyrosine hydroxylase; MPP^+, 1-methyl-4-phenylpyridinium; eGFP, enhanced GFP.

rons. However, the precise mechanisms linking BDNF expression with CaMKIIδ3 activation have remained unclear.

In this study, we show that the CaMKIIδ3 Ser^{332} site is directly dephosphorylated by protein phosphatase 1 (PP1), promoting CaMKIIδ3 nuclear translocation, and that aripiprazole (APZ), a dopamine D_2R partial agonist, promotes CaMKIIδ3 nuclear translocation and enhances BDNF expression. Overall, our findings demonstrate a critical function for CaMKIIδ3 nuclear translocation in promoting survival and enhancing neurite extension of dopaminergic neurons.

Experimental Procedures

Characterization of a Ser^{332}-Phosphopeptide Antibody—A rabbit polyclonal antibody was raised against phosphorylated CaMKIIδ3 (Ser^{332}) using a phosphopeptide (CDGVKKRK-phosphoS-SS-(NH_2)) (MBL, Nagoya, Japan). *In vitro* CaMKII autophosphorylation was carried out in a reaction containing purified rat brain CaMKII (26) in 40 mM Tris-HCl (pH 7.5), 1 mM $MnCl_2$, 10 mM $MgCl_2$, 50 μM ATP, and 0.2 mg/ml BSA. When indicated, 1 mM $CaCl_2$ and 0.15 μM CaM were added to the incubation mixture to detect Ca^{2+}/CaM-dependent phosphorylation. The reaction was initiated by addition of purified CaMKII, carried out for 10 min at 30 °C, and terminated by addition of SDS sample buffer and boiling for 3 min. A dephosphorylation reaction was carried out in the same buffer for 10 min at 30 °C following addition of 0.9 units PP1 (Millipore, Bedford, MA). Phosphorylated CaMKII was detected by immunoblotting with rabbit polyclonal antibodies against pCaMKII (Ser^{332}) (1:1000) or pCaMKII (Thr^{286}/Thr^{287}) (1:5000) (27).

Plasmid Constructs and siRNA—The CaMKIIδ3 plasmid was prepared as described previously (25). CaMKIIδ3 (S332A) and CaMKIIδ3 (S332D) mutants were generated using the KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan) according to the protocol of the manufacturer. The Camuiα plasmid, a FRET-based reporter of CaMKIIα activity (28), was provided by Dr. Yasunori Hayashi (RIKEN Brain Science Institute, Wako City, Japan). The Camuiδ3 plasmid was generated by replacing the CaMKIIα coding sequence in the Camuiα plasmid with CaMKIIδ3 cDNA. The PP1α, PP1β, PP1γ1, and NIPP1 plasmids were provided by Dr. Laura Trinkle-Mulcahy (University of Ottawa, Ottawa, ON, Canada). PP1γ siRNA (sense, 5′-CAUUCAGAAAGCUUCAAAUdTdT-3′; antisense, 5′-AUUUGAAGCUUUCUGAAUGdTdT-3′) and negative control siRNA were purchased from Sigma-Aldrich. Transfections were performed using 100 nM PP1γ siRNA according to published methods (29).

Cell Culture and Transfection—Neuro-2a cells were grown in DMEM supplemented with 10% heat-inactivated FBS and penicillin/streptomycin (100 units/100 μg/ml) in a 5% CO_2 incubator at 37 °C. Neuro-2a cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen), and experiments were performed 48 h later as described previously (29). Primary cultures of mesencephalic neurons were established using methods described previously with slight modifications (30). Briefly, SN tissue was dissected from embryonic day 18 Wistar rats and dissociated by trypsin treatment and trituration through a Pasteur pipette. Neurons were plated on coverslips coated with poly-L-lysine in minimum essential medium (Invitrogen) supplemented with 10% FBS, 0.6% glucose (Wako, Osaka, Japan), and 1 mM pyruvate (Sigma-Aldrich). After cell attachment, coverslips were transferred to dishes containing a glial cell monolayer and maintained in Neurobasal medium (Invitrogen) containing 2% B27 supplement (Invitrogen) and 1% GlutaMax (Invitrogen). 5 μM cytosine β-D-arabinofuranoside (Sigma-Aldrich) was added to cultures at DIV3 (3 days *in vitro*) after plating to inhibit glial proliferation. Primary mesencephalic neurons were transfected with expression vectors and siRNAs using electroporation (NEPA21, Nepagene Co. Ltd., Chiba, Japan) at DIV0.

Chemically Induced Long-term Potentiation or APZ Stimulation of Primary Mesencephalic Neurons—Chemically induced long-term potentiation (c-LTP) was induced as described previously (31). Briefly, neuronal cultures at DIV10 were transferred from Neurobasal medium to extracellular solution containing 140 mM NaCl, 1.3 mM $CaCl_2$, 5 mM KCl, 25 mM HEPES (pH 7.4), 33 mM glucose, 0.5 μM tetrodotoxin, 1 μM strychnine, and 20 μM bicuculline methiodide. After 10 min in extracellular solution, cells were treated with 200 μM glycine in extracellular solution for 3 min and then incubated in extracellular solution without glycine for the indicated amounts of time. For APZ stimulation, cells were incubated in Krebs-Ringer-HEPES solution containing 128 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 2.7 mM $CaCl_2$, and 20 mM HEPES for 30 min and then stimulated with 10 μM APZ for the indicated times. Some cells were pretreated with 0.5 μM okadaic acid (Calbiochem, San Diego, CA) for 30 min before c-LTP or APZ treatment.

In Vivo APZ Treatment—Adult 8-week-old male Wistar rats (180–220 g) were housed under climate-controlled conditions with a 12-h light/dark cycle and provided standard food and water *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee at Tohoku University. APZ (Wako, 0.3 mg/kg dissolved in 0.5% carboxymethylcellulose) or vehicle was administered to rats intraperitoneal daily for 7 days. The animals were then sacrificed, and the brain was removed and perfused with ice-cold buffer for 3 min (0.32 M sucrose, 20 mM Tris-HCl (pH 7.4)). The SN region was then dissected and subjected to immunoblotting.

Cell Fractionation—Fractionation of Neuro-2a cells was performed using the subcellular protein fractionation kit for cultured cells (Pierce, Thermo Fisher Scientific Inc.) according to the protocol of the manufacturer. The kit allows separation of cytoplasmic, membrane, nuclear soluble, and chromatin-bound protein extracts. 2×10^6 cells were washed with PBS and collected by incubation with trypsin-EDTA at 37 °C for 3 min. Extraction buffer for cytoplasmic isolation containing protease inhibitors was added to cell pellets. Cells were incubated at 4 °C for 10 min. Then the homogenates were centrifuged (500 × *g*) for 5 min. The supernatants (cytoplasmic extracts) were transferred to new tubes. Membrane extraction buffer containing protease inhibitors was added to precipitants, followed by vortexing and incubation at 4 °C for 10 min. The homogenates were centrifuged (3000 × *g*) for 5 min, and the supernatants (membrane extracts) were transferred to new tubes. Nuclear extraction buffer containing protease inhibitors was added to cell pellets, followed by vortexing and incubation at 4 °C for 30 min. The homogenates were centrifuged (5000 × *g*) for 5 min,

and supernatants (soluble nuclear extracts) were transferred to new tubes. Nuclear extraction buffer containing protease inhibitors, 5 mM $CaCl_2$, and micrococcal nuclease (300 units) was added to cell pellets, followed by vortexing and incubation at room temperature for 15 min. The homogenates were centrifuged (15,000 × *g*) for 5 min, and the supernatants (chromatin-bound nuclear extracts) were transferred to new tubes. Protein concentrations were estimated by Bradford assay. Rat SN tissues were fractionated into cytosolic and nuclear extracts as follows. SN samples were extracted with ice-cold low-salt buffer containing 0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 4 mM EDTA, 4 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM DTT, and protease inhibitors (trypsin inhibitor, pepstatin A, and leupeptin) and centrifuged at 20,000 × *g* for 10 min. Supernatants (the cytosol fractions) were transferred to a fresh tube, whereas pelleted crude nuclei were resuspended in ice-cold high-salt buffer containing 0.5 M NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 4 mM EDTA, 4 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM DTT, and protease inhibitors. After centrifugation of the latter at 20,000 × *g* for 10 min, the supernatant was transferred to a fresh tube (nuclear fraction).

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as described previously (29). Antibodies included rabbit polyclonal antibodies against pCaMKII (Ser^{332}) (1:1000), pCaMKII (Thr^{286}/Thr^{287}, 1:5000) (27), CaMKIIα/β (1:5000) (27), CaMKIIδ (1:1000, Trans Genic Inc., Kobe, Japan), BDNF (1:500, Millipore), calcineurin (1:1000) (32), MeCP2 (1:1000; Cell Signaling Technology, Beverly, MA), CREB (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), PP1 (1:1000, the catalytic subunit of PP1γ-1 and PP1γ-2) (33), histone H3 (1:1000, Cell Signaling Technology), and GFP (1:1000, Clontech, Mountain View, CA). Mouse monoclonal antibodies used included β-tubulin (1:10000, Sigma-Aldrich) and FLAG (1:1000, Sigma-Aldrich).

CaMKII and PP1 Activity Assays—A Ca^{2+}/CaM-dependent CaMKII activity assay was performed as described previously (34). PP1 activity was assessed using methods described previously with slight modifications (33). Briefly, frozen SN samples were homogenized using a handheld homogenizer in 200 μl of homogenizing buffer containing 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 4 mM EDTA, 4 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM DTT, 2 μg/ml pepstatin A, and 1 μg/ml leupeptin. Insoluble material was removed by centrifugation at 20,000 × *g* for 5 min. Calyculin A- or okadaic acid-sensitive protein phosphatase activities were measured using [^{32}P]casein as substrate. The phosphatase assay was carried out in 45 μl of buffer containing Tris-HCl (40 mM (pH 7.5)), BSA (1 mg/ml), EDTA (1 mM), and 1 μg supernatant from SN slices in the presence of okadaic acid (1 nM) to inhibit PP2A activity or in the presence of calyculin A (100 nM) to inhibit PP1/PP2A. The reaction was initiated by adding [^{32}P]casein (1 μg). After 15-min incubation at 30 °C, the reaction was terminated by adding 30 μl of 40% trichloroacetic acid plus 20 μl of 25 mg/ml BSA. After vortexing, the mixture was kept on ice for 10 min and then centrifuged at 20,000 × *g* for 10 min. An aliquot (20 μl) of supernatant was counted for ^{32}P radioactivity released during the incubation. PP1 activity was determined by subtracting activity in the presence of calyculin A from activity in the presence of okadaic acid.

Camui-FRET Analysis—Neuro-2a cells were grown on 0.01% poly-L-lysine (Sigma-Aldrich)-coated glass bottom dishes. To monitor CaMKII activation, cells were transfected with the Camuiα or Camuiδ3 plasmids. Two days later, cells were exposed to externally applied 60 mM high-KCl in Krebs-Ringer-HEPES buffer and imaged. The wavelengths used for FRET imaging were 438/24 nm (excitation), 483/32 nm (cyan fluorescent protein emission), and 542/27 nm (YFP emission) separated by a 458-nm dichroic mirror, and analysis was performed every 3 s. Ratio values were calculated by averaging fluorescence intensity from the entire cytosolic or nuclear area. FRET images were monitored using an inverted microscope (Leica DM IRB, Japan) equipped with a charge-coupled device camera (ORCA-ER, Hamamatsu, Japan). Captured images were analyzed using the Metafluor imaging system (Molecular Devices, Sunnyvale, CA).

Immunohistochemistry—Immunohistochemistry of Neuro-2a cells was performed as described previously (29). Cells were fixed in 4% paraformaldehyde in phosphate buffer for 30 min at room temperature, washed in PBS, treated with 0.1% Triton-X for 15 min, blocked with 3% bovine serum albumin in PBS for 1 h, and then incubated with first antibodies in blocking solution at 4 °C for 24 h. First antibodies included mouse monoclonal antibodies against FLAG (1:1000, Sigma-Aldrich) and rabbit polyclonal antibody against tyrosine hydroxylase (TH, 1:000, Millipore). After washing with PBS, sections were incubated with secondary antibodies in blocking solution at 20 °C for 3 h. Antibodies included Alexa Fluor 594-labeled anti-mouse IgG and Alexa Fluor 448-labeled anti-rabbit IgG (1:500, Invitrogen). For nuclear staining, sections were incubated with DAPI (Vector Laboratories, Burlingame, CA). Immunohistochemistry of brain slices was performed as described previously (35). Primary antibodies included mouse monoclonal anti-TH (1:1000, Immunostar, Hudson, WI) and rabbit polyclonal anti-CaMKIIδ (1:1000, Trans Genic Inc.). Secondary antibodies included Alexa Fluor 594-labeled anti-mouse IgG and Alexa Fluor 448-labeled anti-rabbit IgG (1:500, Invitrogen). After several PBS washes, sections were mounted on slides with Vectashield (Vector Laboratories Inc.). Immunofluorescence images were analyzed using a confocal laser-scanning microscope (LSM700, Zeiss, Thornwood, NY).

Quantification of Neurite Sprouting and Cell Survival in Primary Dopaminergic Neurons—Neurite sprouting was quantified as described previously (36). Briefly, primary dopaminergic neurons were stained with anti-TH antibody at DIV10, and immunofluorescence images were analyzed using a confocal laser-scanning microscope. A neurite was defined as a process arising from the soma and neurite length as the distance from the soma to the tip of the longest branch. For APZ treatment, DIV8 neurons were treated with 10 μM APZ for 48 h. To assess survival, at DIV10, 1-methyl-4-phenylpyridinium (MPP^+, Sigma-Aldrich) was added at a final concentration of 500 μM for 24 h. Surviving cells were determined by the appearance of TH and DAPI staining. Six fields (10 cells/field) under each condition were chosen randomly and photographed.

Real-time PCR Quantification of Bdnf mRNA—Real-time PCR analysis was performed as described previously (29) in 48-well plates (Mini Opticon real-time PCR system, Bio-Rad) using iQ SYBR Green Supermix 2× (Bio-Rad). Mouse *Bdnf* exon 4 primer sequences (sense, 5′-CAGAGCAGCTGCCTTGATGTT-3′; antisense, 5′-GCCTTGTTCGTGGACGTTTA-3′) and mouse *Gapdh* primer sequences (sense, 5′-TGTGTCCGTCGTGGATCTGA-3′; antisense, 5′-CACCACCTTCTTGATGTCATCATAC-3′) were purchased from FASMAC (Tokyo, Japan). Relative quantities of target mRNAs were determined by the comparative threshold cycle (ΔCT) method and normalized to *Gapdh* quantity. Product purity and specificity were confirmed by omitting the template and performing a standard melting curve analysis.

Statistical Analysis—All values are expressed as mean ± S.E. Comparison between two experimental groups was made using unpaired Student's *t* test. Statistical significance for differences among groups was tested by one-way analysis of variety with post hoc Tukey tests. $p < 0.05$ was considered significant.

Results

CaMKII Ser^{332} Is Dephosphorylated by PP1 in Vitro—Isoforms of the CaMKII α, β, γ, and δ subunits can be distinguished in part by an 11-amino acid KRKSSSSVQMM sequence in the variable region between the regulatory and association domains. CaMKIIαB and CaMKIIδ3 display this motif, and phosphorylation of CaMKIIαB and CaMKIIδ3 Ser^{332} blocks nuclear localization (11) (Fig. 1*A*). To investigate the function of CaMKII phosphorylation, we first tested the specificity of an antibody against phospho-CaMKII (Ser^{332}). To do so, we performed an *in vitro* phosphorylation assay using purified rat brain CaMKII. Conventional CaMKIIα/β antibodies recognized 50- and 60-kDa immunoreactive bands corresponding to the α and β subtypes, respectively (Fig. 1*B*, *lane 1*). Likewise, a conventional CaMKIIδ antibody detected a 57-kDa immunoreactive band corresponding to the δ isoform (Fig. 1*B*, *lane 2*). CaMKII phosphorylation at α-Thr^{286} and β-, γ-, and δ-Thr^{287} corresponds to the activated form of the protein (37). A pCaMKII (Thr^{286}/Thr^{287}) antibody recognized three bands with molecular masses corresponding to the α, β, and δ subtypes (Fig. 1*B*, *top panel*, *lane 5*), and these bands disappeared upon treatment with PP1, a major Ser/Thr phosphatase expressed in eukaryotic cells (Fig. 1*B*, *top panel*, *lane 6*). The pCaMKII (Ser^{332}) antibody recognized two immunoreactive bands, CaMKIIαB and CaMKIIδ3 (Fig. 1*B*, *bottom panel*). Importantly, the Ser^{332} antibody did not recognize CaMKIIβ, which lacks the Ser^{332} site. The two bands detected by pCaMKII (Ser^{332}) antibody were detected in the presence of EGTA (Fig. 1*B*, *bottom panel*, *lane 4*), but staining intensity was increased markedly in the presence of Ca^{2+}/CaM, which stimulates autophosphorylation (Fig. 1*B*, *bottom panel*, *lane 5*). PP1 treatment decreased Ser(P)^{332} immunoreactivity of these bands to basal levels (Fig. 1*B*, *bottom panel, lane 6*). Detection of a basal level of phosphorylation in purified CaMKII supports the idea that CaMKII (Ser^{332}) phosphorylation is in part resistant to PP1 dephosphorylation. The pCaMKII (Ser^{332}) antibody specifically recognized phosphorylated Ser^{332} because preabsorption of the antibody with a 100-fold (100 μg/ml) excess amount of phosphopeptide antigen totally eliminated the immunoreactivity on the blots (Fig. 1*C*). Taken together, we conclude that the Ser(P)^{332} antibody specifically detects CaMKII autophosphorylation at Ser^{332} of CaMKIIαB and CaMKIIδ3, a site dephosphorylated in part by PP1 *in vitro*.

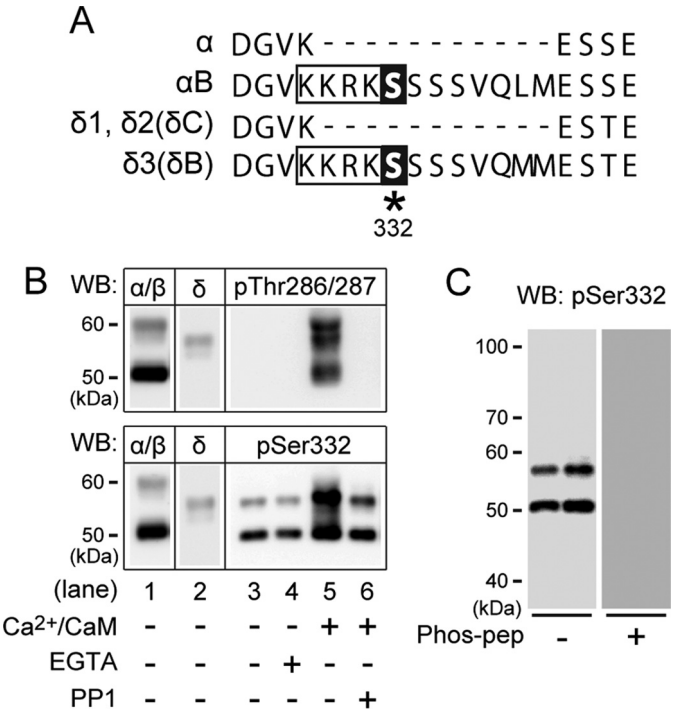


FIGURE 1. **CaMKII is dephosphorylated at Ser^{332} by PP1 *in vitro*.** *A*, comparison of amino acid sequences of the CaMKII subunit variable domains. CaMKIIαB and CaMKIIδ3 (*δB*) have an 11-amino acid insertion containing an NLS (*boxed*), whereas CaMKIIα, CaMKIIδ1, and CaMKIIδ2 (*δC*) lack a variable domain. The *asterisk* denotes phosphorylated Ser^{332} (*black background*). *B*, phosphorylation assay of CaMKII purified from rat brain under various conditions *in vitro* and Western blot (*WB*) with various antibodies to CaMKIIα/β, CaMKIIδ, CaMKII(Thr(P)$^{286/287}$) and CaMKII(Ser(P)^{332}). *C*, Western blot analysis of CaMKII purified from rat brain using CaMKII(Ser(P)^{332}) antibody (*left panel*) or the same antibody preincubated with a CaMKII(Ser(P)^{332}) antibody-blocking phosphopeptide (*Phos-pep*, *right panel*).

PP1α and PP1γ1 Predominantly Regulate CaMKIIδ3 Nuclear Translocation—PP1 forms a heterodimer comprised of a catalytic (PP1c) and a regulatory subunit. PP1c can form a complex with over 50 regulatory or scaffolding proteins that regulate substrate specificity and PP1c subcellular distribution (38). PP1c itself is found as four isoforms (α, β, or δ and γ1 and γ2) in mammalian cells (39–43) and three (PP1α, PP1β, and PP1γ1) highly expressed in the brain (44). All isoforms show nearly 90% amino acid homology and are most divergent at the N and C termini. To determine subcellular localization of these proteins in neurons, we employed confocal microscopy of the enhanced GFP (eGFP)-tagged PP1 isoforms α, β, and γ1 in Neuro-2a cells. PP1α-eGFP and eGFP-PP1β were primarily cytoplasmic but showed low levels of nuclear fluorescence. However, PP1γ1-eGFP signals were diffuse in the cytoplasm and nucleoplasm and accumulated in unidentified nuclear bodies (Fig. 2*A*). Nuclear inhibitor of PP1 (NIPP1) is a ubiquitously expressed protein that blocks PP1 activity (45). Coexpression of mCherry-tagged NIPP1 with different PP1 isoforms in Neuro-2a cells had no effect on PP1 isoform localization (Fig. 2*B*). In agreement, treatment of cells with okadaic acid, an

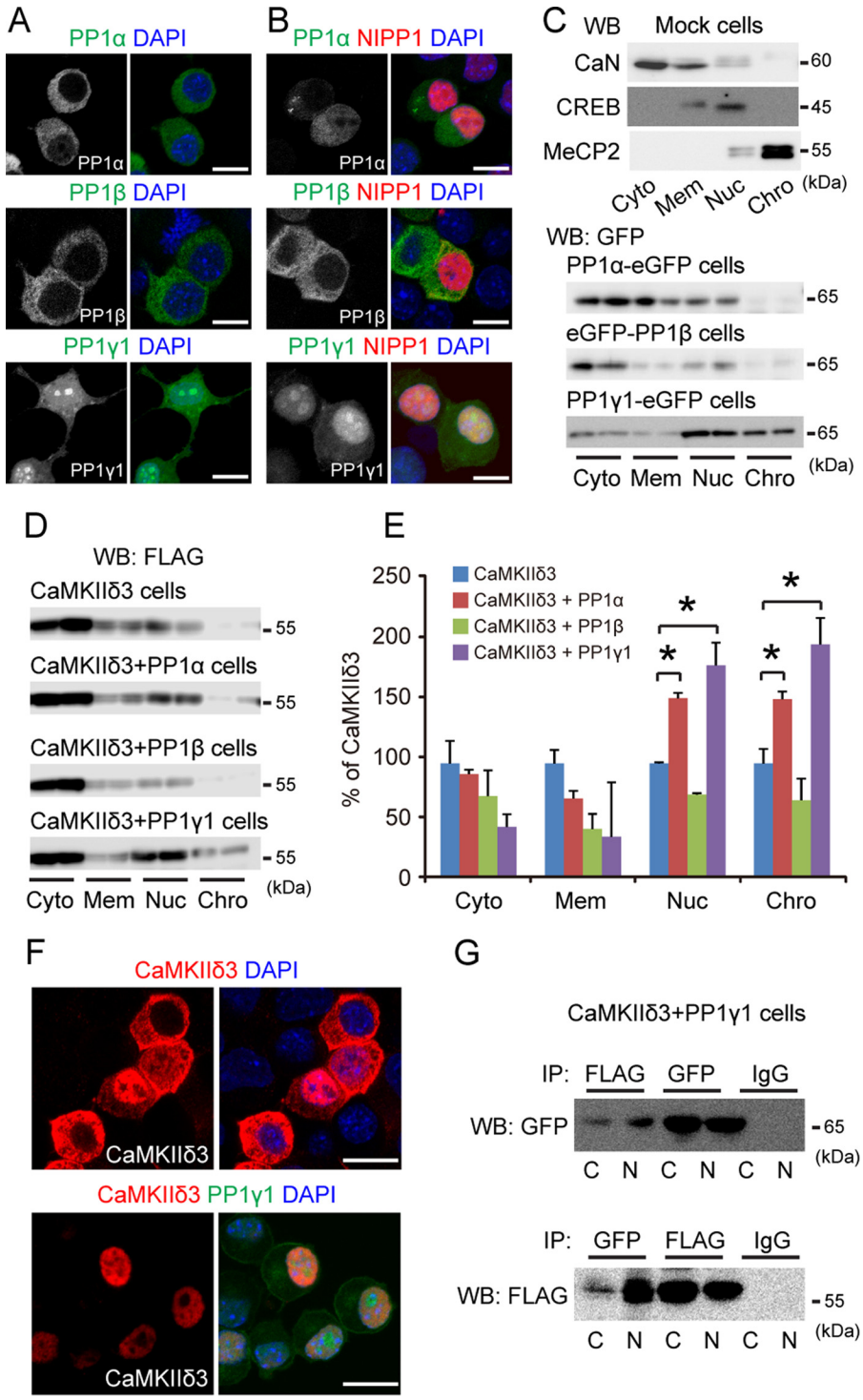


FIGURE 2. **PP1α and PP1γ1 predominantly regulate CaMKIIδ3 nuclear translocation.** *A* and *B*, localization of PP1 isoforms in Neuro-2a cells. Confocal images show the localization of eGFP-tagged PP1 isoforms *(green)*, mCherry-tagged NIPP1 (*red*), and the nuclear marker DAPI (*blue*). *Right panels*, single color images of PP1 isoforms (*gray*, *left*). *Scale bars* = 10 μm. *C*, Neuro-2a cells expressing eGFP-tagged PP1 isoforms separated into cytoplasmic (*Cyto*), membrane (*Mem*), nuclear soluble (*Nuc*), and chromatin-bound (*Chro*) fractions. Equal volumes of each fraction were separated by SDS-PAGE, followed by immunoblotting with the indicated antibodies. *WB*, Western blot. *D*, lysates of Neuro-2a cells expressing FLAG-tagged CaMKIIδ3 and eGFP-tagged PP1 isoforms were fractionated as indicated, and equal volumes of each were separated by SDS-PAGE and immunoblotted with FLAG antibody. *E*, quantitative densitometry analyses of FLAG-CaMKIIδ3 in the indicated fractions. Data are mean ± S.E. *, $p < 0.05$ relative to CaMKIIδ3-expressing cells. *F*, confocal images showing colocalization of FLAG-tagged CaMKIIδ3 with eGFP-tagged PP1γ1. *Scale bars* = 10 μm. *G*, CaMKIIδ3 and PP1γ1 coimmunoprecipitation in extracts of cells cotransfected with FLAG-CaMKIIδ3 and PP1γ1-eGFP. Extracts were immunoprecipitated (*IP*) with anti-FLAG or anti-GFP antibody, and immunoprecipitates were immunoblotted with anti-GFP *(top panel)* or with anti-FLAG (*bottom panel*) antibody. *C*, cytoplasmic fractions; *N*, nucleoplasm fractions.

inhibitor of PP1 and PP2A, also had no effect on PP1 isoform localization (data not shown), suggesting overall that, in Neuro-2a cells, PP1 localization is not altered by changes in its activity.

To further investigate differences in PP1 subcellular localization, we fractionated lysates of Neuro-2a cells transfected with eGFP-tagged PP1 isoforms into cytoplasmic, membrane, nuclear soluble, and chromatin-bound fractions. We confirmed the quality of these fractions with antibodies against calcineurin (CaN, a cytosolic protein marker), CREB (a nuclear protein marker), and MeCP2 (a chromatin-bound protein marker). High levels of both PP1α-eGFP and eGFP-PP1β were seen in the cytoplasmic fraction and relatively low in other fractions. However, PP1γ1-eGFP was mainly expressed in nuclei and in chromatin fractions (Fig. 2*C*). We next determined whether these PP1 isoforms could alter CaMKIIδ3 localization. To do so, we cotransfected Neuro-2a cells with FLAG-tagged CaMKIIδ3 plus PP1 isoforms and determined CaMKIIδ3 localization by immunoblotting with an anti-FLAG antibody. In cells expressing CaMKIIδ3 alone, CaMKIIδ3 was primarily cytoplasmic, with relatively low levels in membrane and nuclear fractions. However, in cotransfected with PP1α or PP1γ1, nuclear CaMKIIδ3 levels increased significantly, particularly in the presence of PP1γ1. We observed no effect on CaMKIIδ3 localization when PP1β was cotransfected (Fig. 2, *D* and *E*; $n = 3$ each).

Immunohistochemistry also indicated the presence of FLAG-tagged CaMKIIδ3 in both cytoplasmic and nuclear compartments. In most cells cotransfected with FLAG-CaMKIIδ3 and PP1γ1-eGFP, CaMKIIδ3 was detected in the nucleus (Fig. 2*F*). In similar experiments, coexpression with PP1γ1-eGFP did not promote nuclear localization of FLAG-tagged CaMKIIα or CaMKIIδ1 (data not shown). To investigate the potential CaMKIIδ3/PP1γ1 interaction, we performed immunoprecipitation of FLAG-CaMKIIδ3 from extracts of cell cells cotransfected with FLAG-CaMKIIδ3 and PP1γ1-eGFP using anti-FLAG antibody, followed by immunoblotting with a GFP antibody. A CaMKIIδ3-PP1γ1 complex was detected in both cytoplasmic and nuclear fractions, and GFP staining was more robust in the latter (Fig. 2*G*, *top panel*). Conversely, FLAG-CaMKIIδ3·PP1γ1-eGFP complexes were observed after GFP immunoprecipitation and immunoblotting with an anti-FLAG antibody (Fig. 2*G*, *bottom panel*).

PP1-dependent CaMKII (Ser^{332}) Dephosphorylation Alters Nuclear CaMKII Activity—We next asked whether CaMKIIδ3 is dephosphorylated by PP1 *in situ*. To do so, we fractionated extracts of Neuro-2a cells transfected with CaMKIIδ3 with or without PP1γ1 into cytoplasmic and nucleoplasm fractions. Immunoblot analysis with the pCaMKII (Ser^{332}) antibody showed significantly decreased levels of phosphorylated CaMKIIδ3 in cells cotransfected with PP1γ1 (49.0% ± 1.9%) relative to CaMKIIδ3 alone in cytoplasmic ($p = 0.026$, $n = 3$ each) compared with nuclear fractions (Fig. 3*A*). When we measured CaMKII activity in comparable lysates, it was elevated significantly in the nuclear fraction of cells cotransfected with PP1γ1 compared with CaMKIIδ3 alone ($p = 0.033$, $n = 3$). Lysates of Neuro-2a cells transfected with a phosphorylation-resistant mutant (CaMKIIδ3 (S332A)) showed significantly increased nuclear CaMKII activity in the absence of PP1γ1 cotransfection ($p = 0.005$, $n = 3$), whereas the CaMKIIδ3 (S332D) phosphomimetic mutant showed minimal activity with or without PP1γ1 cotransfection (Fig. 3*B*).

To monitor CaMKII activity in cells, we assessed dynamic real-time CaMKII activation using Camui, a FRET based-biosensor molecule including full-length CaMKII (28). In Camuiα-transfected Neuro-2a cells, the FRET signal, an indicator of CaMKIIα activation, was increased significantly in cytoplasmic areas but low in nuclear areas in response to high KCl stimulation (Fig. 3*C*). On the other hand, in Camuiδ3, the FRET signal in nuclei following KCl stimulation increased markedly compared with Camuiα ($p < 0.001$, $n = 6–8$), (Fig. 3, *D* and *F*). A similar analysis in Camuiδ3 cells cotransfected with PP1γ1 demonstrated significantly increased FRET signals in nuclei compared with Camuiδ3 alone ($p < 0.001$, $n = 8–10$; Fig. 3, *E* and *F*). Taken together, these analyses indicate that nuclear CaMKIIδ3 activation is enhanced by PP1-dependent Ser^{332} dephosphorylation.

Treatment of Cultured Dopaminergic Neurons with the D_2R Agonist APZ Promotes CaMKIIδ3 (Ser^{332}) Dephosphorylation—We have shown previously that CaMKIIδ3 is highly expressed in rat SN dopaminergic neurons (24) and that dopamine D_2R stimulation of NG108-15 cells activates CaMKIIδ3 with concomitantly increased BDNF gene expression (25). Here we used primary cultured (DIV10) mesencephalic dopaminergic neurons to find out whether the CaMKIIδ phosphorylation status changed following c-LTP or APZ treatment. Because we found that Ser^{332} was increased markedly in the presence of Ca^{2+}/CaM *in vitro*, we confirmed *in situ* whether the phosphorylation occurred on cultured mesencephalic neurons under depolarization conditions such as c-LTP. Following c-LTP, levels of phosphorylated CaMKIIδ (Thr^{287}) and CaMKIIδ (Ser^{332}) increased significantly, lasting until 60 min after stimulation (Fig. 4, *A* and *B*). APZ treatment, on the other hand, significantly decreased CaMKIIδ (Ser^{332}) phosphorylation without altering CaMKIIδ (Thr^{287}) phosphorylation (Fig. 4, *A* and *C*). Treatment of cultured neurons with the phosphatase inhibitor okadaic acid completely blocked CaMKIIδ (Ser^{332}) dephosphorylation following APZ treatment. These findings suggest that PP1/PP2A activation by APZ underlies CaMKIIδ (Ser^{332}) dephosphorylation.

APZ Treatment Enhances CaMKIIδ Nuclear Translocation in the Rat Substantia Nigra in Vivo—Next we asked whether *in vivo* activation of dopamine D_2R with APZ altered CaMKIIδ localization or phosphorylation status. Consistent with our previous study (24), CaMKIIδ was expressed in both the cytosol and nuclei of rat SN dopaminergic neurons (Fig. 5*A*). Immunoblot analysis demonstrated significantly decreased CaMKIIδ (Ser^{332}) phosphorylation following chronic APZ treatment compared with vehicle-treated animals ($p = 0.017$, $n = 4$). APZ treatment, however, did not alter the levels of phosphorylated CaMKIIδ (Thr^{287}) (Fig. 5*B*). Next we asked whether PP1 activity could account for changes in CaMKII (Ser^{332}) phosphorylation following APZ treatment. PP1 activity was measured using [^{32}P]casein as substrate by subtracting activity in the presence of 100 nM calyculin A to inhibit PP1 and PP2A from activity in the presence of 1 nM okadaic acid to inhibit PP2A, as described

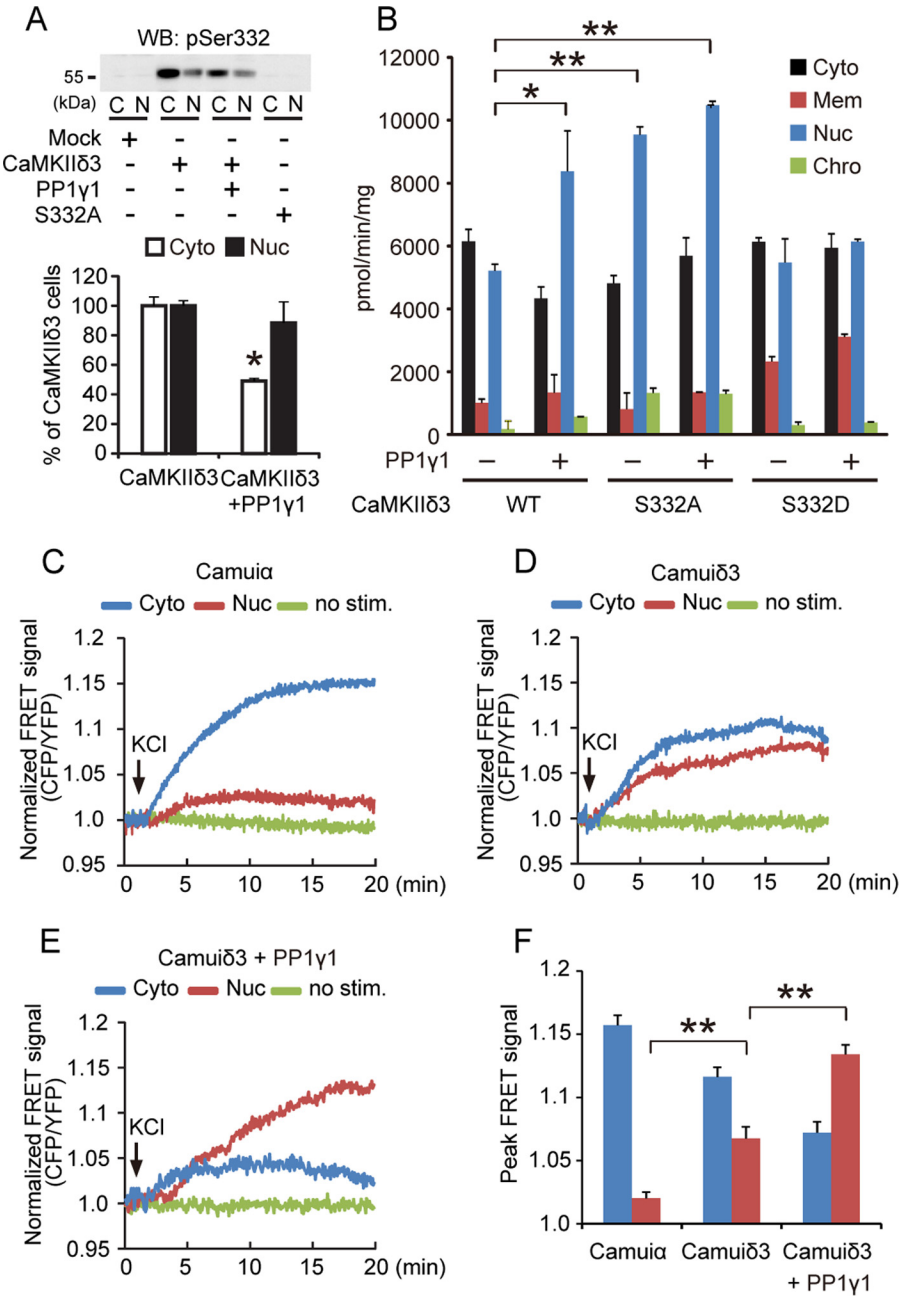


FIGURE 3. **PP1-dependent dephosphorylation of CaMKIIδ3 at Ser^{332} regulates its nuclear activity.** *A*, *top panel*, representative immunoblots probed with pCaMKII (Ser^{332}) antibody in cells transfected with constructs indicated at the *left*. *C*, cytoplasmic fraction; *N*, nuclear fraction; *S332A*, CaMKIIδ3 (S332A); *WB*, Western blot. *Bottom panel*, quantitative densitometry analyses of the data shown above. *, $p < 0.05$. *versus* CaMKIIδ3-expressing cells. *B*, Ca^{2+}-dependent CaMKII activity in Neuro-2a cells. Data are mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* activity in the nuclear fraction of CaMKIIδ3-expressing cells. *Cyto*, cytoplasmic fraction; *Mem*, membrane fraction; *Nuc*, nuclear soluble fraction; *Chro*, chromatin-bound fraction. *C–E*, real-time measurement of Camui-FRET signals. Shown are relative fluorescence intensity changes following treatment of Neuro-2a cells with 60 mM high KCl after transfection with Camuiα (*C*), Camuiδ3 (*D*), or Camuiδ3 and PP1γ1 (*E*). Cyan fluorescent protein (*CFP*)/YFP ratio values were derived by averaging the fluorescence intensity from the entire cytosolic or nuclear area. *No stim*, no stimulation. *F*, the peak ratio value of the release phase relative to the preceding baseline. Data are mean ± S.E. **, $p < 0.01$.

previously (33). Interestingly, we observed increased PP1 activity in SN lysates from APZ-treated rats ($p = 0.04$, $n = 4$; Fig. 5*C*). Immunoblot analysis showing an increased ratio of nuclear to cytoplasmic CaMKIIδ immunoreactivity after APZ treatment confirmed CaMKIIδ nuclear translocation ($p = 0.04$, $n = 3$; Fig. 5*D*). Moreover, BDNF protein levels also increased significantly following APZ treatment ($p = 0.01$, $n = 4$; Fig. 5*E*).

Nuclear CaMKIIδ3 Activation Enhances Neurite Extension and Survival in Dopaminergic Neurons—We next asked whether CaMKIIδ3 dephosphorylation at Ser^{332} regulates BDNF expression and neuronal survival. *Bdnf* mRNA levels increased significantly following cotransfection of Neuro-2a cells with CaMKIIδ3 plus PP1γ1 compared with transfection with CaMKIIδ3 alone ($p = 0.027$, $n = 3$). We confirmed that expression of the CaMKIIδ3 alanine mutant (S332A) significantly increased *Bdnf* mRNA levels without PP1γ1 transfection and that transfection of the phosphomimetic CaMKIIδ3 (S332D) had little effect on *Bdnf* mRNA levels with or without PP1γ1 (Fig. 6*A*). Immunoblot analysis confirmed that CaMKIIδ3 cotransfection with PP1γ1 or transfection with

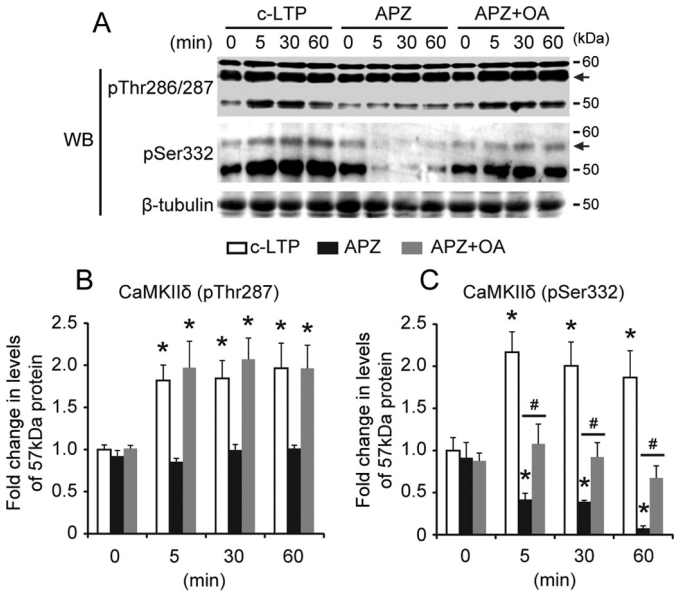


FIGURE 4. **APZ treatment of cultured mesencephalic dopaminergic neurons promotes CaMKIIδ3 dephosphorylation at Ser^{332}.** *A*, representative images of immunoblots probed with antibodies against phosphorylated CaMKII (Thr$^{286/287}$), phosphorylated CaMKII (Ser^{332}), and β-tubulin in lysates from cultured mesencephalic dopaminergic neurons treated at DIV10 with c-LTP, APZ, or APZ plus okadaic acid (*OA*). *Arrows* indicate a 57-kDa immunoreactive band corresponding to CaMKIIδ. *WB*, Western blot. *B* and *C*, quantitative analyses of phosphorylated CaMKIIδ (Thr^{287}) (*B*) and phosphorylated CaMKIIδ (Ser^{332}) (*C*) as analyzed by densitometry. Data are mean ± S.E. *, $p < 0.05$ *versus* untreated cells; #, $p < 0.05$ *versus* APZ-treated cells.

CaMKIIδ3 (S332A) alone significantly increased BDNF protein expression compared with transfection with WT CaMKIIδ3 alone (Fig. 6*B*). Overall, these findings suggest that CaMKIIδ3 (Ser^{332}) dephosphorylation by PP1 positively regulates BDNF expression.

Finally, we addressed whether nuclear CaMKIIδ3 activation stimulates neurite extension or survival of cultured primary mesencephalic neurons at DIV10. Morphological analysis of dopaminergic (TH-positive) cells cotransfected with CaMKIIδ3 and PP1γ1 indicated significantly enhanced neurite extension compared with cells transfected with WT CaMKIIδ3 alone. Similarly, CaMKIIδ3 (S332A) overexpression significantly stimulated neurite extension. We also assessed whether PP1γ knockdown altered neurite extension stimulated by nuclear CaMKIIδ3 in these cells. Therefore, we first tested oligomeric siRNA targeting PP1γ (siPP1γ) and found that knockdown decreased endogenous PP1γ expression to ~30% of the levels seen in cultures of mesencephalic neurons (Fig. 6*C*). Cells transfected with WT CaMKIIδ3 plus siPP1γ showed significantly decreased neurite extension relative to WT CaMKIIδ3-transfected cells not treated with siPP1γ. Importantly, following APZ treatment, neurite extension increased significantly in TH-positive neurons, and PP1γ knockdown blocked this effect (CaMKIIδ3 (WT), 107.3 ± 8.6; CaMKIIδ3 (S332A), 132.3 ± 6.1; CaMKIIδ3 (WT) + PP1γ1, 140.3 ± 9.1; CaMKIIδ3 (WT) + siPP1γ, 80.3 ± 8.8; CaMKIIδ3 (WT) + APZ, 141 ± 8.5; CaMKIIδ3 (WT) + APZ + siPP1γ, 87.3 ± 11.8; CaMKIIδ3 (S332A) + APZ, 151.6 ± 14.3; CaMKIIδ3 (S332A) + APZ + siPP1γ, 134.4 ± 17.6; values represent average neurite length (micrometers), $n = 12$ each) (Fig. 6, *D* and *E*).

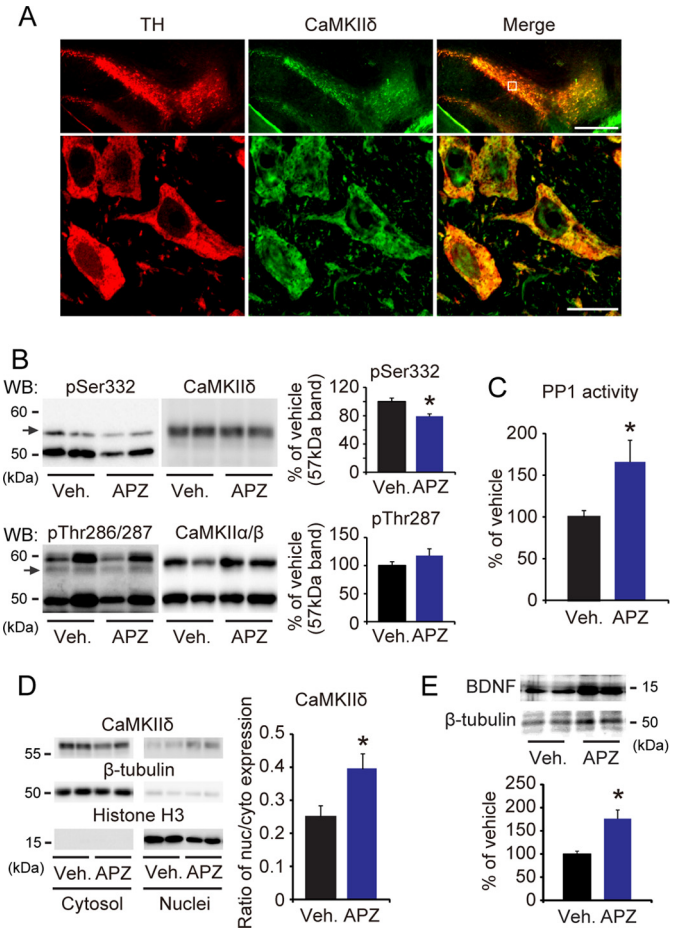
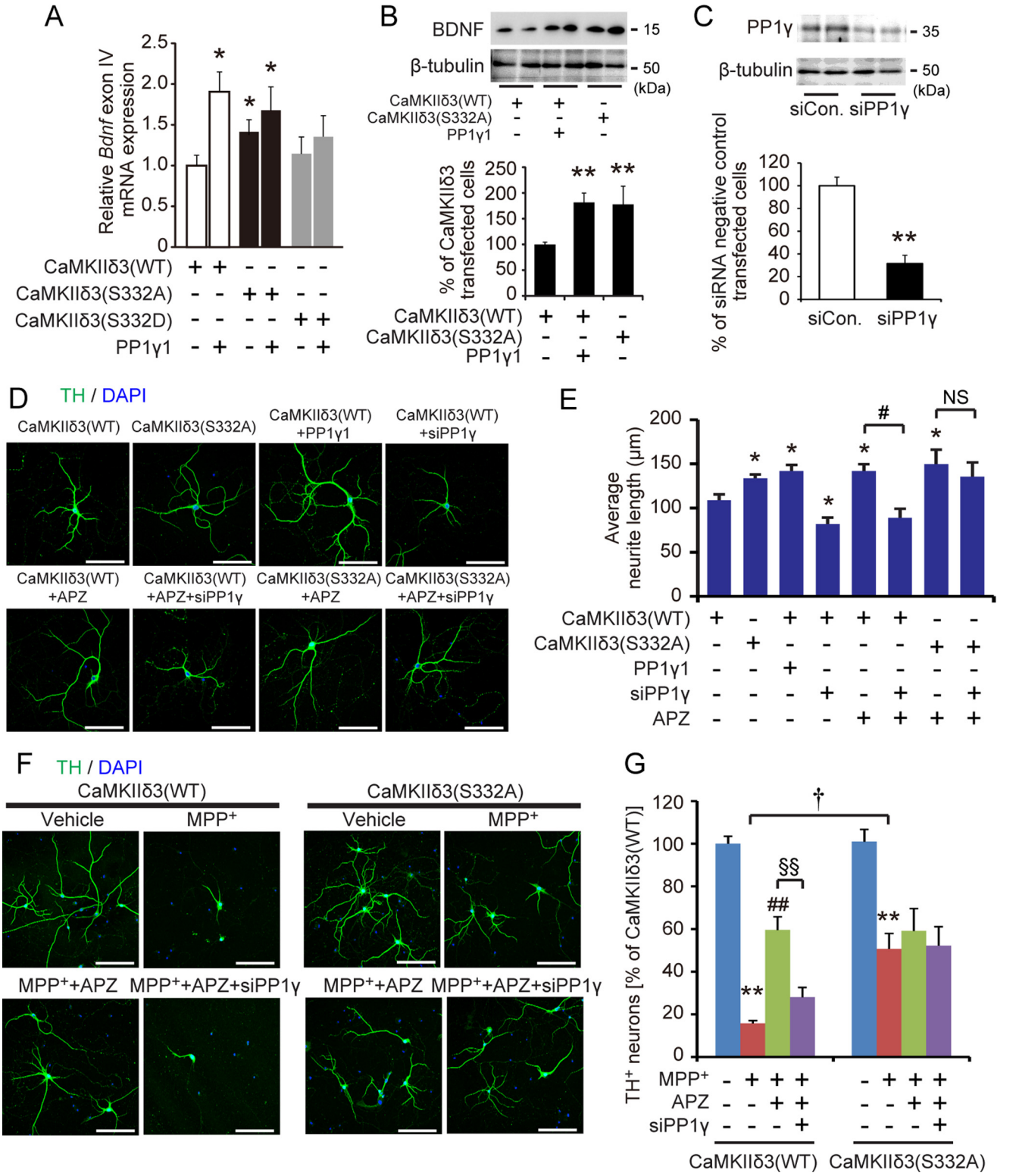


FIGURE 5. **APZ treatment enhances CaMKIIδ nuclear translocation in the rat substantia nigra.** *A*, confocal images showing colocalization of CaMKIIδ (*green*) and dopaminergic TH-positive (*red*) cells in the rat SN pars compacta. *Top panel, scale bar* = 250 μm; *bottom panel, scale bar* = 20 μm. *B*, *left panel*, representative images of immunoblots of rat SN lysates probed with antibodies against phosphorylated CaMKII (Thr$^{286/287}$), CaMKIIα/β, phosphorylated CaMKII (Ser^{332}), and CaMKIIδ. *Arrows* indicate a 57-kDa immunoreactive band corresponding to CaMKIIδ. *Right panel*, quantitative analyses of phosphorylated CaMKIIδ (Ser^{332}) and phosphorylated CaMKIIδ (Thr^{287}) as analyzed by densitometry. *WB*, Western blot; *Veh*, vehicle. *C*, increased PP1 activity in the SN of APZ-treated rats. Data are expressed as the percentage of activity seen in vehicle-treated rats. *D*, nuclear translocation of CaMKIIδ after APZ treatment. *Left panel*, representative images of immunoblots of cytoplasmic or nuclear extracts from rat SN tissues showing CaMKIIδ, β-tubulin (a cytosolic marker), and histone H3 (a nuclear marker) immunoreactivity. *Right panel*, quantitative analysis of CaMKIIδ as a ratio of nuclear to cytosolic expression. *E*, *top panel*, representative immunoblots of rat SN lysates probed with BDNF and β-tubulin antibodies. *Bottom panel*, quantitative densitometry analyses. Data are mean ± S.E. *, $p < 0.05$ *versus* vehicle-treated rats.

To assess cell survival, we assayed cell death induced by MPP^+ in cultured mesencephalic neurons. MPP^+, the metabolite of 1-methyl-1,2,3,6-tetrahydropiridine, is preferentially taken up by dopaminergic neurons through the dopamine transporter and accumulated in the mitochondria, and, in turn, inhibits complex I of the respiratory chain. Thereby, MPP^+ induces oxidative stress, leading to mitochondrial dysfunction and causing dopaminergic cell death (46). TH-positive neurons transfected with WT CaMKIIδ3 underwent cell death in response to MPP^+, whereas APZ treatment significantly rescued this effect. Treatment of these cells with siPP1γ partially blocked the APZ-dependent neuroprotective effects. In cells transfected with CaMKIIδ3 (S332A), TH-positive cell death

in response to MPP^+ was less robust relative to neurons transfected with WT CaMKIIδ3. Unlike cells transfected with WT CaMKIIδ3, CaMKIIδ3 (S332A) cells showed APZ-induced neuroprotective effects in the presence of PP1γ knockdown (CaMKIIδ3 (WT) + MPP^+, 15.8% ± 1.3%; CaMKIIδ3 (WT) + MPP^+ + APZ, 59.6% ± 6.2%; CaMKIIδ3 (WT) + MPP^+ + APZ + siPP1γ, 28.1% ± 4.5%; CaMKIIδ3 (S332A), 101% ± 5.7%; CaMKIIδ3 (S332A) + MPP^+, 50.7% ± 7.6%; CaMKIIδ3 (S332A) + MPP^+ + APZ, 59.1% ± 10.5%; CaMKIIδ3 (S332A) + MPP^+ + APZ + siPP1γ, 52.2% ± 8.9% of TH-positive neurons in WT CaMKIIδ3-transfected cells) (Fig. 6, *F* and *G*). Taken together, our analysis indicates that CaMKIIδ3 (Ser^{332}) dephosphorylation in part mediates the neuroprotective effect of APZ in dopaminergic neurons.



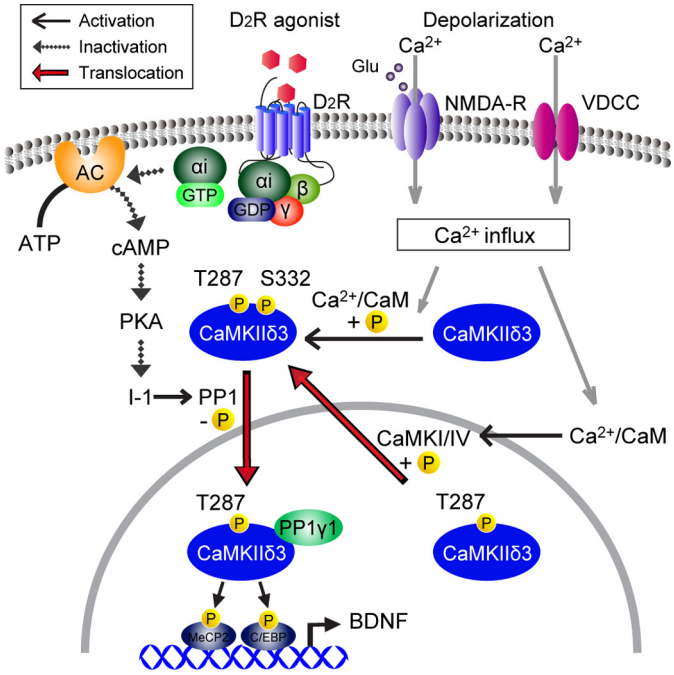


FIGURE 7. **Schematic showing the mechanism of CaMKIIδ3 activation and nuclear translocation in dopaminergic neurons.** Treatment with the dopamine D_2R agonist APZ increases PP1 activity by inactivating the cAMP/PKA/I-1 pathway. PP1 then dephosphorylates CaMKIIδ3 at Ser^{332} in the cytoplasm, resulting in nuclear translocation. Nuclear CaMKIIδ3 phosphorylates a subset of transcription factors, including MeCP2 and CCAAT/enhancer-binding protein, increasing BDNF expression. Depolarization promotes Ca^{2+} entry through NMDA receptors or voltage-dependent calcium channels and binding to CaM, resulting in CaMKIIδ3 autophosphorylation at Thr^{287} and Ser^{332} in the cytoplasm. Nuclear CaMKIIδ3 may also be phosphorylated at Ser^{332} by active CaMKI or CaMKIV, resulting in export to the cytosol.

Discussion

Our study documents a novel mechanism underlying the nuclear translocation of CaMKIIδ3 through PP1-dependent dephosphorylation following dopamine D_2R activation. Furthermore, we report that increased nuclear CaMKIIδ3 activity mediates BDNF expression, which, in turn, may promote neurite extension and neuroprotection in dopaminergic neurons. Our working model is shown in Fig. 7. Stimulation with the dopamine D_2R agonist APZ increases PP1 activity through inactivation of the cAMP/PKA/inhibitor 1 (I-1) pathway (47), and, in turn, PP1 dephosphorylates CaMKIIδ3(Ser^{332}) in the cytoplasm, enabling CaMKIIδ3 nuclear translocation. On the basis of the idea that, under basal conditions, CaMKII is autonomously active in part because of spontaneous neuronal activity (48), CaMKIIδ3 is autophosphorylated in the cytoplasm, and D_2R-mediated PP1 activation mediates its dephosphorylation at Ser^{332}. Thereafter, nuclear CaMKIIδ3 phosphorylates transcription factors, including MeCP2 and CCAAT/enhancer-binding protein, increasing BDNF expression. Depolarization causes Ca^{2+} entry through NMDA receptors or voltage-dependent calcium channels and promotes CaMKIIδ3 autophosphorylation at Thr^{287} and Ser^{332} in the cytosol. Conversely, nuclear CaMKI or CaMKIV activity may account for CaMKIIδ3 nuclear export through phosphorylation at Ser^{332}, as reported previously (11).

In an *in vitro* phosphorylation assay using purified rat brain CaMKII, CaMKIIδ was dephosphorylated by PP1 at both Ser^{332} and Thr^{287}. However, in experiments using primary cultured mesencephalic dopamine neurons and in chronic APZ-treated rats, CaMKIIδ was dephosphorylated only at Ser^{332} by activated PP1. This discrepancy may be explained by the binding of various proteins in the CaMKII-PP1 complex. For example, spinophilin targets PP1 to postsynaptic density sites (49). CaMKII autophosphorylation at Thr^{286}/Thr^{287} stabilizes CaMKII localization at the postsynaptic density site (50), and the postsynaptic density-associated CaMKII holoenzyme is resistant to PP1 dephosphorylation (51). Indeed, the *in vitro* experimental conditions used here resemble the cytosolic microenvironment, in which PP1 directly dephosphorylates cytosolic CaMKIIδ3. We did not define proteins binding to the CaMKIIδ3-PP1 complex *in vivo*. Further studies are required to precisely identify proteins that directly regulate the CaMKIIδ3 phosphorylation status in the nucleus and cytoplasm.

In FRET-based CaMKII activity assays in Neuro-2a cells using the cytoplasmic isoform CaMKIIα probe, the FRET signal in Camuiα-transfected cells was increased slightly in the nuclear region in response to stimulation with high KCl. Purified Camuiα with a molecular mass of 110 kDa was eluted as a single peak of a molecular mass of >1000 kDa by gel filtration (28), indicating that Camuiα expressed in Neuro-2a cells oligomerizes with other endogenous isoforms within cells. The CaMKII holoenzyme is normally a dodecameric complex formed via isoform variable regions, and its composition affects CaMKII localization (52, 53). The ability of CaMKII to translocate to the nucleus is then limited by the presence of nuclear *versus* cytoplasmic isoforms that comprise the holoenzyme (9). Nuclear isoforms containing an NLS (CaMKIIαB, CaMKIIδ3, and CaMKIIγA) could coassemble with cytoplasmic subunits, including postsynaptic density-associated CaMKIIα (55) and/or F-actin-associated CaMKIIβ (56). Therefore, our results support the idea that assembly of CaMKII isoforms possibly affects nuclear translocation and activation.

FIGURE 6. **Nuclear CaMKIIδ3 localization enhances neurite extension and inhibits death of dopaminergic neurons.** *A*, real-time PCR showing *Bdnf* exon IV mRNA expression in Neuro-2a cells. Data are mean ± S.E. *, $p < 0.05$. *versus* wild-type cells transfected with CaMKIIδ3. *B*, *top panel*, representative immunoblots of Neuro-2a cell lysates probed with BDNF and β-tubulin antibodies. *Bottom panel*, quantitative densitometry analyses are shown. **, $p < 0.01$ *versus* wild-type cells transfected with CaMKIIδ3. *C*, effect of siRNA-mediated PP1γ knockdown in DIV10 cultured mesencephalic neurons. Immunoblot analysis (*top panel*) and densitometric quantification (*bottom panel*) of protein expression is shown. **, $p < 0.01$ *versus* cells transfected with control siRNA (*siCon*). *D* and *F*, representative images of TH-positive cultured mesencephalic neurons at DIV10. *Scale bars* in $D = 100$ μm; in $F = 120$ μm. *E*, average neurite length in TH-positive neurons transfected with the indicated constructs and/or treated with APZ. *, $p < 0.05$ *versus* wild-type CaMKIIδ3 transfected cells; #, $p < 0.05$ CaMKIIδ3 transfected cells plus APZ *versus* CaMKIIδ3 and PP1γ siRNA cotransfected cells plus APZ; *NS*, not significant. *G*, the number of surviving cells among TH-positive neurons transfected with the indicated constructs and/or treated with drugs. **, $p < 0.01$ *versus* wild-type CaMKIIδ3 transfected cells; ##, $p < 0.01$ CaMKIIδ3-transfected plus MPP^+ cells *versus* CaMKIIδ3-transfected cells plus MPP^+ and APZ; §§, $p < 0.01$ CaMKIIδ3-transfected plus MPP^+ and APZ cells *versus* cells cotransfected with CaMKIIδ3 and PP1γ siRNA plus MPP^+ and APZ; †, $p < 0.05$ wild-type CaMKIIδ3-transfected cells plus MPP^+ *versus* CaMKIIδ3 (S332A)-transfected cells plus MPP^+.

Other studies have reported nuclear activity of other CaMKII isoforms, CaMKIIαB and CaMKIIγA, in neurons (57, 58). For example, CaMKIIαB expression and nuclear translocation increase via an unknown mechanism following glutamate-induced cell death in rat retinal ganglion cells (57). CaMKIIαB knockdown also decreases neuronal BDNF expression (56). Ma *et al.* (58) have also reported that CaMKIIγA functions as a transporter of Ca^{2+}/CaM to the nucleus following depolarization of cultured superior cervical ganglion neurons and that the Ca^{2+}·CaM-CaMKIIγ complex is dephosphorylated at Ser^{334} by calcineurin, shuttling it to the nucleus. Nuclear delivery of Ca^{2+}/CaM activates nuclear CaM kinases, including CaMKIV and CaMKK, driving CREB phosphorylation and transcription of CRE-regulated target genes (58). Therefore, phosphatases other than PP1, such as calcineurin and/or PP2A, may dephosphorylate at Ser^{332} in other types of neurons.

We report here that chronic APZ treatment significantly increased BDNF protein expression concomitant with nuclear CaMKIIδ3 translocation. APZ treatment also enhanced sprouting and survival of cultured dopaminergic neurons through the CaMKIIδ3/PP1 pathway. Consistent with our results, APZ treatment for 8 weeks reportedly significantly increased plasma BDNF levels in first-episode untreated schizophrenia patients (59). APZ treatment also increases the pool size of long 3′-UTR *Bdnf* transcripts in the rat ventral hippocampus (60). *Bdnf* mRNAs carrying this type of UTR undergo dendritic targeting, and dendritically synthesized BDNF protein functions in dendritic morphogenesis (61). This evidence indicates that enhanced expression of BDNF protein following APZ treatment may represent a means to enhance the availability of *Bdnf* mRNA transcripts in dendrites, not only in nuclei, stimulating neurite extension. BDNF protein expression decreases in the dopamine-deficient substantia nigra of Parkinson disease patients (62, 63). BDNF also reportedly promotes the survival of cultured mesencephalic dopaminergic neurons (64) and, *in vivo*, protects dopaminergic neurons from damage by the neurotoxins 1-methyl-1,2,3,6-tetrahydropiridine and 6-hydroxydopamine (65). In addition, D_2R agonists have neuroprotective effects on various neurons *in situ* (54, 66, 67). For example, cabergoline blocks oxygen/glucose deprivation-induced cell death in SH-SY5Y neuroblastoma cells (66). *In vivo*, chronic cabergoline treatment antagonizes the death of dopaminergic neurons in 6-hydroxydopamine-treated mice (67). Because APZ is used clinically as a common prescription drug in schizophrenia, bipolar disorder, and depression, we selected APZ to define the mechanism underlying its neuroprotective effect. Although we have no data regarding whether quinpirole, another D_2R agonist, has APZ-like effects on CaMKIIδ3 dephosphorylation and nuclear translocation, quinpirole elicits neuroprotection against glutamate-induced neurotoxicity in cultured rat mesencephalic neurons (54). In addition, we have documented previously that stimulation with quinpirole in D_2R-expressed NG108-15 cells activate the nuclear isoform of CaMKII (25). This evidence and our data suggest a critical role for BDNF in supporting the survival of midbrain dopaminergic neurons, an activity likely supported by the D_2R-mediated CaMKIIδ3/PP1 pathway.

Author Contributions—N. S. and K. F. conceived and coordinated the study and wrote the paper. N. S. and M. S. designed, performed, and analyzed the experiments shown in all figures. Y. I. and T. S. provided technical assistance. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Dr. Yasunori Hayashi (RIKEN Brain Science Institute, Wako City, Japan) for the Camuiα plasmid and Dr. Laura Trinkle-Mulcahy (University of Ottawa, Ottawa, ON, Canada) for the PP1α, PP1β, PP1γ1, and NIPP1 plasmids.

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