Nuclear translocation of calcium/calmodulin-dependent protein kinase IIδ3 promoted by protein phosphatase-1 enhances brain-derived neurotrophic factor expression in dopaminergic neurons.*

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**Background:** The physiological function of CaMKIIδ3 phosphorylation at Ser332 remains unclear.

**Results:** CaMKIIδ3 dephosphorylation at Ser332 promotes its nuclear localization and stimulates BDNF expression.

**Conclusion:** Dopamine D2 receptor stimulation triggers CaMKIIδ3 dephosphorylation at Ser332 by PP1.

**Significance:** CaMKIIδ3 (Ser332) dephosphorylation is critical for neurite extension and survival of dopaminergic neurons.

**SUMMARY**

We previously reported that dopamine D2 receptor (D2R) stimulation activates calcium/calmodulin-dependent protein kinase II (CaMKII)δ3, a CaMKII nuclear isoform, increasing brain-derived neurotrophic factor (BDNF) gene expression. However, mechanisms underlying that activity remained unclear. Here, we report that CaMKIIδ3 is dephosphorylated at Ser332 by protein phosphatase-1 (PP1), promoting CaMKIIδ3 nuclear translocation. Neuro-2a cells transfected with CaMKIIδ3 showed cytoplasmic and nuclear staining, but staining was predominantly nuclear when CaMKIIδ3 was co-expressed with PP1. Indeed, PP1 and CaMKIIδ3 co-expression significantly increased nuclear CaMKII activity and enhanced BDNF expression. In support of this idea, chronic administration of the dopamine D2R partial agonist aripiprazole (APZ) increased PP1 activity and promoted nuclear CaMKIIδ3 translocation and BDNF expression in the rat brain substantia nigra. Moreover, APZ 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 Ser332 by PP1 likely accounts for BDNF expression and subsequent neurite extension and survival of dopaminergic neurons.
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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 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 Ser332 residue immediately C-terminal to the NLS (KKRK332) can be phosphorylated by the CaMK family members CaMKI or CaMKIV, blocking association of CaMKII with the NLS receptor m-pendulin and thus prohibiting nuclear localization (11).

Nuclear CaMKII likely functions in transcriptional regulation of the neurotrophin brain-derived neurotrophic factor (BDNF) (12, 13) through phosphorylation of diverse nuclear proteins, including cAMP response element binding protein (CREB) (14, 15), activating transcription factor (ATF) (16, 17), CCAAT/enhancer-binding protein (C/EBP) (18, 19), serum response factor (SRF) (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 previously demonstrated that CaMKIIδ3 is highly expressed in dopaminergic rat substantia nigra (SN) neurons (24), and that stimulation of the dopamine D2 receptor (D2R) promotes CaMKIIδ3 activation, thereby inducing BDNF gene expression in NG108-15 cells (25). This work strongly suggests that D2R/CaMKIIδ3 signaling is important for survival and/or differentiation of dopaminergic neurons. However, precise mechanisms linking BDNF expression with CaMKIIδ3 activation have remained unclear.

In the present study, we show that CaMKIIδ3 Ser332 site is directly dephosphorylated by protein phosphatase (PP)-1, promoting CaMKIIδ3 nuclear translocation, and that aripiprazole (APZ), a dopamine D2R 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 Ser332-phosphopeptide antibody- A rabbit polyclonal antibody was raised against phosphorylated CaMKIIδ3 (Ser332) using a phosphopeptide (CDGVKKRK-phosphoS-SS-(NH2)) (MBL, Nagoya, Japan). In vitro CaMKII autophosphorylation was carried out in a reaction containing purified rat brain CaMKII in 40mM Tris-HCl (pH 7.5), 1mM MnCl₂, 10mM MgCl₂, 50μM ATP and 0.2mg/mL BSA. When indicated, 1mM CaCl₂ plus 0.15μM CaM was added to the incubation mixture to detect Ca²⁺/CaM-dependent phosphorylation. The reaction was initiated by addition of purified CaMKII, carried out for 10min at 30°C, and terminated by addition of SDS sample buffer and boiling for 3min. A dephosphorylation reaction was carried out in the same buffer for 10min 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 (Ser332) (1:1000) or pCaMKII (Thr286/Thr287) (1:5000) (27).

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

Cell culture and transfection- Neuro-2a cells were grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 units/100 μg/ml) in a 5% CO2 incubator at 37°C. Neuro-2a cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and experiments were performed 48h later as described (29). Primary cultures of mesencephalic neurons were established using previously described methods 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, St. Louis, MO, USA). 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 (31). Briefly, neuronal cultures at DIV10 were transferred from Neurobasal medium to extracellular solution (ECS) containing 140 mM NaCl, 1.3 mM CaCl2, 5 mM KCl, 25 mM HEPES (pH 7.4), 33 mM glucose, 0.5 mM tetrodotoxin, 1 μM strychnine, and 20 μM bicusculine methiodide. After 10 min in ECS, cells were treated with 200 μM glycine in ECS for 3 min and then incubated in ECS without glycine for indicated amounts of time. For APZ stimulation, cells were incubated in Krebs-Ringer-HEPES (KRH) solution containing 128 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM glucose, 2.7 mM CaCl2, 20 mM HEPES for 30 min and then stimulated with 10 μM APZ for indicated times. Some cells were pre-treated with 0.5 μM okadaic acid (Calbiochem, San Diego, CA, USA) 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 i.p. daily for 7 days. 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., Rockford, IL, USA) according to the manufacturer’s protocol. The kit allows separation of cytoplasmic, membrane, nuclear soluble and chromatin-bound protein extracts. 2×10⁶ cells were washed with PBS and collected by incubating with trypsin-EDTA at 37°C for 10 min. The 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. The membrane extraction buffer containing protease inhibitors was added to precipitants, followed by vortex and incubation at 4°C for 30 min. The nuclear extraction buffer containing protease inhibitors was added to cell pellets. Cells were incubated at 4°C for 10 min. Then the homogenates were centrifuged (3000×g) for 5 min, and the supernatants (membrane extracts) were transferred to new tubes. The nuclear extraction buffer containing protease inhibitors was added to cell pellets, followed by vortex and incubation at 4°C for 30 min. The homogenates were centrifuged (5000×g) for 5 min, supernatants (soluble nuclear extracts) were transferred to new tubes. The nuclear extraction buffer containing protease
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Inhibitors, 5mM CaCl\(_2\) and Micrococcal Nuclease (300 units) was added to cell pellets, followed by vortex and incubation at room temperature for 15min. The homogenates were centrifuged (15,000 \(\times\) g) for 5min, and the supernatants (chromatin-bound nuclear extract) were transferred to new tubes. Protein concentrations were estimated by the 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 0.15M NaCl, 50mM Tris-HCl (pH 7.5), 0.5% Triton-X 100, 4mM EDTA, 4mM EGTA, 1mM Na\(_3\)VO\(_4\), 50mM NaF, 1mM DTT and protease inhibitors (trypsin inhibitor, pepstatin A and leupeptin) and centrifuged at 20,000 \(\times\) g for 10min. Supernatants (the cytosol fraction) were transferred to a fresh tube, while pellet crude nuclei were resuspended in ice-cold high salt buffer containing 0.5M NaCl, 50mM Tris-HCl (pH 7.5), 0.5% Triton-X 100, 4mM EDTA, 4mM EGTA, 1mM Na\(_3\)VO\(_4\), 50mM NaF, 1mM DTT and protease inhibitors. After centrifugation of the latter at 20,000 \(\times\) g for 10min, the supernatant was transferred to a fresh tube (nuclear fraction).

**Immunoprecipitation and immunoblotting** - Immunoprecipitation and immunoblotting were performed as described (29). Antibodies included rabbit polyclonal antibodies against pCaMKII (Ser332) (1:1000), pCaMKII (Thr286/Thr287) (1:5000) (27); CaMKII\(\alpha/\beta\) (1:5000) (27); CaMKII\(\delta\) (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 PP1\(\gamma-1\) and -2) (33); Histone H3 (1:1000, Cell Signaling Technology) and GFP (1:1000, Clontech, Mountain View, CA, USA). Mouse monoclonal antibodies used included \(\beta\)-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 (34). PP1 activity was assessed using previously described methods with slight modifications (33). Briefly, frozen SN samples were homogenized using a handheld homogenizer in 200\(\mu\)l of homogenizing buffer containing 0.5% Triton X-100, 50mM Tris-HCl (pH 7.4), 0.5M NaCl, 4mM EDTA, 4mM EGTA, 1mM Na\(_3\)VO\(_4\), 50mM NaF, 1mM DTT, 2\(\mu\)g/ml pepstatin A, and 1\(\mu\)g/ml leupeptin. Insoluble material was removed by centrifugation at 20,000 \(\times\) g for 5min. 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\(\mu\)l buffer containing Tris-HCl (40mM, pH 7.5), BSA (1mg/ml), EDTA (1mM), and 1\(\mu\)g supernatant from SN slices in the presence of okadaic acid (1nM) to inhibit PP2A activity or in the presence of calyculin A (100nM) to inhibit PP1/PP2A. The reaction was initiated by adding \(^{32}\)P-casein (1\(\mu\)g). After 15min incubation at 30°C, the reaction was terminated by adding 30\(\mu\)l 40% trichloroacetic acid plus 20\(\mu\)l 25mg/ml BSA. After vortexing, the mixture was kept on ice for 10min and then centrifuged at 20,000 \(\times\) g for 10min. An aliquot (20\(\mu\)l) 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 Camui or Camui\(\delta\)3 plasmids. Two days later, cells were exposed to externally applied 60mM high-KCl in KRH buffer and imaged. Wavelengths used for FRET imaging were 438/24nm (excitation), 483/32nm (CFP emission) and 542/27nm (YFP emission) separated by a 458nm dichroic mirror and analysis was performed every 3s. 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 CCD camera (ORCA-ER; Hamamatsu, Japan). Captured images were analyzed using the Metafluor imaging system (Molecular Devices, Sunnyvale, CA, USA).

**Immunohistochemistry** - Immunohistochemistry of Neuro-2a cells was performed as described (29). Cells were fixed in 4% paraformaldehyde in phosphate buffer for 30min at room temperature and washed in.
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PBS, treated with 0.1% Triton-X for 15min, blocked with 3% bovine serum albumin in PBS for 1h, and then incubated with first antibodies in blocking solution at 4°C for 24h. First antibodies included mouse monoclonal antibodies against FLAG (1:1000; Sigma-Aldrich) and rabbit polyclonal antibody against tyrosine hydroxylase (TH) (1:1000; Millipore). After PBS washing, sections were incubated with secondary antibodies in blocking solution at 20°C for 3h. Antibodies included Alexa 594-labeled anti-mouse IgG and Alexa 448-labeled anti-rabbit IgG (1:500; Invitrogen). For nuclear staining, sections were incubated with DAPI (Vector Laboratories, Burlingame, CA, USA). Immunohistochemistry of brain slices was performed as described (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 594-labeled anti-mouse IgG and Alexa 448-labeled anti-rabbit IgG (1:500, Invitrogen). After several PBS washes, sections were mounted on slides with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Immunofluorescent images were analyzed using a confocal laser scanning microscope (LSM700; Zeiss, Thornwood, NY, USA). Quantification of neurite sprouting and cell survival in primary dopaminergic neurons- Neurite sprouting was quantified as described (36). Briefly, primary dopaminergic neurons were stained with anti-TH antibody at DIV10, and immunofluorescent 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 10μM APZ for 48h. To assess survival, at DIV10 1-methyl-4-phenylpyridinium (MPP⁺, Sigma-Aldrich) was added at a final concentration of 500μM for 24h. Surviving cells were determined by the appearance of TH and DAPI staining. Six fields (10 cells/field) in each condition were chosen randomly and photographed.

Real Time PCR quantification of Bdnf mRNA- Real time PCR analysis was performed as described (29) in 48-well plates (Mini Opticon Real Time PCR System, Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix 2× (Bio-Rad). Mouse Bdnf exon 4 primer sequences (sense, 5’-CAGAGCAGCTGCTTGGATGTT-3’, and antisense, 5’-GCCTTGTTCGTGACGTCTTA-3’); and mouse Gapdh primer sequences (sense, 5’-TGTTCCGTCGTGGATCTGA-3’, and antisense, 5’-CACACCTCTTTGATGTCATCATA-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 template and performing a standard melting curve analysis.

Statistical analysis- All values were expressed as means ± S.E.M. Comparison between two experimental groups was made using the unpaired Student's-t test. Statistical significance for differences among groups was tested by One-way ANOVA with post-hoc Tukey tests. P<0.05 was considered significant.

RESULTS
CaMKII Ser332 is dephosphorylated by PP1 in vitro- Isoforms of 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 Ser332 blocks nuclear localization (11) (Fig 1A). To investigate function of CaMKII phosphorylation, we first tested the specificity of an antibody against phospho-(p)CaMKII (Ser332). To do so, we performed an in vitro phosphorylation assay using purified rat brain CaMKII. Conventional CaMKIIα/β antibodies recognized 50 and 60kDa immunoreactive bands, corresponding to the α and β subtypes, respectively (Fig 1B, lane 1). Likewise, a conventional CaMKIIδ antibody detected a 57kDa immunoreactive band, corresponding to the δ isoform (Fig 1B, lane 2). CaMKII
phosphorylation at α-Thr286 and β, γ and δ-Thr287 corresponds to the activated form of the protein (37). A pCaMKII (Thr286/Thr287) antibody recognized three bands with molecular masses corresponding to α, β and δ subtypes (Fig 1B, upper panel, lane 5), and these bands disappeared upon treatment with PP1, a major Ser/Thr phosphatase expressed in eukaryotic cells (Fig 1B, upper panel, lane 6). The pCaMKII (Ser332) antibody recognized two immunoreactive bands, CaMKIIαB and CaMKIIδ3 (Fig 1B, lower panel). Importantly, the Ser332 antibody did not recognize CaMKIIβ, which lacks the Ser332 site. The two bands detected by pCaMKII (Ser332) antibody were detected in the presence of EGTA (Fig 1B, lower panel, lane 4), but staining intensity was markedly increased in the presence of Ca2+/CaM, which stimulates autophosphorylation (Fig 1B, lower panel, lane 5). PP1 treatment decreased pSer332 immunoreactivity of these bands to basal levels (Fig 1B, lower panel, lane 6). Detection of a basal level of phosphorylation in purified CaMKII supports the idea that CaMKII (Ser332) phosphorylation is in part resistant to PP1 dephosphorylation. The pCaMKII (Ser332) antibody specifically recognized phosphorylated Ser332, because pre-absorption the antibody with a 100-fold (100 μg/ml) excess amount of phospho-peptide antigen totally eliminated the immunoreactivity on the blots (Fig 1C). Taken together, we conclude that the pSer332 antibody specifically detects CaMKII autophosphorylation at Ser332 of CaMKIIαB and CaMKIIδ3, a site dephosphorylated in part by PP1 in vitro.

PP1α and PP1γ1 predominantly regulate CaMKIIδ3 nuclear translocation- PP1 forms a heterodimer comprised of a catalytic (PP1c) and regulatory subunit. PP1c can form a complex with over 50 regulatory or scaffolding proteins, which regulate substrate specificity and PP1c subcellular distribution (38). PP1c itself is found as four isoforms (α, β or δ, γ1 and γ2) in mammalian cells (39, 40, 41, 42, 43), 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 enhanced green fluorescent protein (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 2A). Nuclear inhibitor of PP1 (NIP1) is a ubiquitously expressed protein that blocks PP1 activity (45). Co-expression of mCherry-tagged NIP1 with different PP1 isoforms in Neuro-2a cells had no effect on PP1 isoform localization (Fig 2B). In agreement, treatment of cells with okadaic acid, an inhibitor of protein phosphatases 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 2C). We next determined whether these PP1 isoforms could alter CaMKIIδ3 localization. To do so, co-transfected Neuro-2a cells with FLAG-tagged CaMKIIδ3 plus PP1 isoforms and determined CaMKIIδ3 localization by immunoblot 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 co-transfected with PP1α or PP1γ1, nuclear CaMKIIδ3 levels significantly increased, particularly in the presence of PP1γ1. We observed no effect on CaMKIIδ3 localization when PP1β was co-transfected (Fig 2D and 2E, n=3 each).

Immunohistochemistry also indicated the presence of FLAG-tagged CaMKIIδ3 in both
cytoplasmic and nuclear compartments. In most cells co-transfected with FLAG-CaMKIIδ3 and PP1γ1-eGFP, CaMKIIδ3 was detected in the nucleus (Fig 2F). In similar experiments, co-expression with PP1γ1-eGFP did not promote nuclear localization of FLAG-tagged CaMKIIα or CaMKIIβ1 (data not shown). To investigate potential CaMKIIδ3/PP1γ1 interaction, we performed immunoprecipitation of FLAG-CaMKIIδ3 from extracts of cell cells co-transfected 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 2G, upper panel). Conversely, FLAG-CaMKIIδ3/PP1γ1-eGFP complexes were observed after GFP immunoprecipitation and immunoblotting with an anti-FLAG antibody (Fig 2G, lower panel).

PP1-dependent CaMKII (Ser332) 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 (Ser332) antibody showed significantly decreased levels of phosphorylated CaMKIIδ3 in cells co-transfected with PP1γ1 (49.0±1.9%) relative to CaMKIIδ3 alone in cytoplasmic (p=0.026, n=3 each) compared to nuclear fractions (Fig 3A). When we measured CaMKII activity in comparable lysates it was significantly elevated in the nuclear fraction of cells co-transfected with PP1γ1 compared to 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 co-transfection (p=0.005, n=3), while the CaMKIIδ3 (S332D) phosphomimic mutant showed minimal activity with or without PP1γ1 co-transfection (Fig 3B).

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 Camuiδ transfected Neuro-2a cells, the FRET signal, an indicator of CaMKIIα activation, was significantly increased in cytoplasmic areas but low in nuclear areas in response to high KCl stimulation (Fig 3C). On the other hand, in Camuiδ3, the FRET signal in nuclei following KCl stimulation markedly increased compared to Camui (p<0.001, n=6-8), (Figs 3D, 3F). Similar analysis in Camuiδ3 cells co-transfected with PP1γ1 demonstrated significantly increased FRET signals in nuclei compared to Camuiδ3 alone (p<0.001, n=8-10), (Figs 3E, 3F). Taken together, these analyses indicate that nuclear CaMKIIδ3 activation is enhanced by PP1-dependent Ser332 dephosphorylation.

Treatment of cultured dopaminergic neurons with the D2R agonist APZ promotes CaMKIIδ3 (Ser332) dephosphorylation- We previously showed that CaMKIIδ3 is highly expressed in rat SN dopaminergic neurons (24) and that dopamine D2R stimulation of NG108-15 cells activates CaMKIIδ3 with concomitantly increased BDNF gene expression (25). Here, we used primary cultured (DIV10) mesencephalic dopaminergic neurons to ask whether CaMKIIδ phosphorylation status changed following chemically induced LTP (c-LTP) or APZ treatment. Because we found Ser332 was markedly increased in the presence of Ca2+/CaM in vitro, we confirmed in situ whether the phosphorylation is occurred on cultured mesencephalic neurons in depolarization condition such as c-LTP. Following c-LTP, levels of phosphorylated CaMKIIδ (Thr287) and CaMKIIδ (Ser332) significantly increased, lasting until 60min after stimulation (Figs 4A, 4B). APZ treatment, on the other hand, significantly decreased CaMKIIδ (Ser332) phosphorylation without altering CaMKIIδ (Thr287) phosphorylation (Figs 4A, 4C).

Treatment of cultured neurons with the phosphatase inhibitor okadaic acid completely blocked CaMKIIδ (Ser332) dephosphorylation following APZ treatment. These findings suggest that PP1/PP2A activation by APZ underlies CaMKIIδ (Ser332) dephosphorylation.

APZ treatment enhances CaMKIIδ nuclear translocation in rat substantia nigra in vivo- Next, we asked whether in vivo
activation of dopamine D_{2R} with APZ altered CaMKII\(\delta\) localization or phosphorylation status. Consistent with our previous study (24), CaMKII\(\delta\) was expressed in both the cytosol and nuclei of rat SN dopaminergic neurons (Fig 5A). Immunoblot analysis demonstrated significantly decreased CaMKII\(\delta\) (Ser332) phosphorylation following chronic APZ treatment compared to vehicle-treated animals (\(p=0.017, n=4\)). APZ treatment, however, did not alter levels of phosphorylated CaMKII\(\delta\) (Thr287) (Fig 5B). Next, we asked whether PP1 activity could account for changes in CaMKII\(\delta\) phosphorylation following APZ treatment. PP1 activity was measured using \(^{32}\)P-casein as substrate by subtracting activity in the presence of 100nM calyculin A to inhibit PP1 and PP2A from activity in the presence of 1nM okadaic acid to inhibit PP2A as previously described (33). Interestingly, we observed increased PP1 activity in SN lysates from APZ-treated rats (\(p=0.04, n=4\), (Fig 5C). Immunoblot analysis showing an increased ratio of nuclear to cytoplasmic CaMKII\(\delta\) immunoreactivity after APZ treatment confirmed CaMKII\(\delta\) nuclear translocation (\(p=0.04, n=3\) (Fig 5D). Moreover, BDNF protein levels also significantly increased following APZ treatment (\(p=0.01, n=4\), (Fig 5E).

Nuclear CaMKII\(\delta\) activation enhances neurite extension and survival in dopaminergic neurons. We next asked whether CaMKII\(\delta\) dephosphorylation at Ser332 regulates BDNF expression and neuronal survival. Bdnf mRNA levels significantly increased following co-transfection of Neuro-2a cells with CaMKII\(\delta\) with PP1\(\gamma\) compared to transfection with CaMKII\(\delta\) alone (\(p=0.027, n=3\)). We confirmed that expression of the CaMKII\(\delta\) alanine mutant (S332A) significantly increased Bdnf mRNA levels without PP1\(\gamma\) transfection and that transfection of the phosphomimic CaMKII\(\delta\) (S332D) had little effect on Bdnf mRNA levels, with or without PP1\(\gamma\) (Fig 6A). Immunoblot analysis confirmed that CaMKII\(\delta\) co-transfection with PP1\(\gamma\) or transfection with CaMKII\(\delta\) (S332A) alone significantly increased BDNF protein expression compared to transfection with wild-type (WT) CaMKII\(\delta\) alone (Fig 6B). Overall, these findings suggest that CaMKII\(\delta\) (Ser332) dephosphorylation by PP1 positively regulates BDNF expression.

Finally, we addressed whether nuclear CaMKII\(\delta\) activation stimulates neurite extension or survival of cultured primary mesencephalic neurons at DIV10. Morphological analysis of dopaminergic (TH-positive) cells co-transfected with CaMKII\(\delta\) and PP1\(\gamma\)1 indicated significantly enhanced neurite extension compared with cells transfected with WT CaMKII\(\delta\) alone. Similarly, CaMKII\(\delta\) (S332A) overexpression significantly stimulated neurite extension relative to WT CaMKII\(\delta\)-transfected cells not treated with siPP1\(\gamma\). Importantly, following APZ treatment, neurite extension significantly increased in TH-positive neurons, and PP1\(\gamma\) knockdown blocked this effect [CaMKII\(\delta\) (WT), 107.3±8.6; CaMKII\(\delta\) (S332A), 132.3±6.1; CaMKII\(\delta\) (WT) + PP1\(\gamma\), 140.3±9.1; CaMKII\(\delta\) (WT) + siPP1\(\gamma\), 80.3±8.8; CaMKII\(\delta\) (WT) + APZ, 141±8.5; CaMKII\(\delta\) (WT) + APZ + siPP1\(\gamma\), 87.3±11.8; CaMKII\(\delta\) (S332A) + APZ, 151.6±14.3; CaMKII\(\delta\) (S332A) + APZ + siPP1\(\gamma\), 134.4±17.6. Values represent average neurite length (\(\mu\m), n=12 each), (Figs 6D, 6E).

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 (MTPP), is preferentially taken up by dopaminergic neurons through dopamine transporter, accumulated in the mitochondria and in turn inhibits complex I of the respiratory chain. Thereby MPP\(^+\) induces oxidative stress leading to mitochondrial dysfunction and causes dopaminergic cell death (46). TH-positive neurons transfected with WT CaMKII\(\delta\) underwent cell death in response to MPP\(^+\), while APZ treatment
significant rescued this effect. Treatment of these cells with siPP1γ partially blocked APZ-dependent neuroprotective effects. In cells transfected with CaMKIIδ3 (S323A), 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 (S323A), 101±5.7%; CaMKIIδ3 (S332A) + MPP⁺, 50.7±7.6%; CaMKIIδ3 (S332A) + MPP⁺ + APZ, 59.1±10.5%; CaMKIIδ3 (S323A) + MPP⁺ + APZ + siPP1γ, 52.2±8.9% of TH-positive neurons in WT CaMKIIδ3 transfected cells (Figs 6F, 6G). Taken together, our analysis indicates that CaMKIIδ3 (Ser332) dephosphorylation in part mediates the neuroprotective effect of APZ in dopaminergic neurons.

**DISCUSSION**

Our study documents a novel mechanism underlying nuclear translocation of CaMKIIδ3 through PP1-dependent dephosphorylation following dopamine D₂R 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 D₂R agonist APZ increases PP1 activity through inactivation of the cAMP/protein kinase A (PKA)/inhibitor-1 (I-1) pathway (47) and, in turn, PP1 dephosphorylates CaMKIIδ3 (Ser332) in the cytoplasm, enabling CaMKIIδ3 nuclear translocation. Based on the idea that under basal conditions CaMKII is autonomously active in part due to spontaneous neuronal activity (48), CaMKIIδ3 is autophosphorylated in the cytoplasm and D₂R-mediated PP1 activation mediates its dephosphorylation at Ser332. Thereafter, nuclear CaMKIIδ3 phosphorylates transcription factors, including MeCP2 and C/EBPα, increasing BDNF expression. Depolarization causes Ca²⁺ entry through N-methyl-D-aspartate receptors (NMDA-Rs) or voltage-dependent calcium channels (VDCCs) and promotes CaMKIIδ3 autophosphorylation at Thr287 and Ser332 in the cytosol. Conversely, nuclear CaMKI or CaMKIV activity may account for CaMKIIδ3 nuclear export through phosphorylation at Ser332, as reported previously (11).

In an in vitro phosphorylation assay using purified rat brain CaMKII, CaMKIIδ was dephosphorylated by PP1 at both Ser332 and Thr287. However, in experiments using primary cultured mesencephalic dopamine neurons and in chronic APZ-treated rats, CaMKIIδ was dephosphorylated only at Ser332 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 (PSD) sites (49). CaMKII autophosphorylation at Thr286/Thr287 stabilizes CaMKII localization at the PSD (50), and the PSD-associated CaMKII holoenzyme is resistant to PP1 dephosphorylation (51). Indeed, the in vitro experimental conditions used here resemble the cytosolic microenvironment, in where 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 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 slightly increased in the nuclear region in response to stimulation with high KCl. Purified Camuiα, the molecular mass of 110kDa, is eluted as a single peak of molecular mass of >1000kDa by gel filtration (28), indicating that Camuiα expressed in Neuro-2a cells oligomerizes with other endogenous isoforms within cells. 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 (54). Nuclear isoforms...
PP1 promotes CaMKIIδ3 nuclear localization

containing an NLS (CaMKIIαB, CaMKIIδ3 and CaMKIIγA) could co-assemble with cytoplasmic subunits, including PSD-associated CaMKIIα (55) and/or F-actin-associated CaMKIIβ (56). Thus, our results support with the idea that assembly of CaMKII isoforms possibly affects nuclear translocation and activation.

Other studies have reported nuclear activity of other CaMKII isoforms, namely 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) also reported that CaMKIIγA functions as a transporter of Ca²⁺/CaM to the nucleus following depolarization of cultured superior cervical ganglion neurons, the Ca²⁺/CaM-CaMKIIγ complex is dephosphorylated at Ser334 by calcineurin, shuttling it to the nucleus. Nuclear delivery of Ca²⁺/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 Ser332 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 increases 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 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's disease patients (62, 63). BDNF also reportedly promotes survival of cultured mesencephalic dopaminergic neurons (64) and in vivo protects dopaminergic neurons from damage by the neurotoxins MPTP and 6-hydroxydopamine (6-ODHA) (65). In addition, D₂R agonists have neuroprotective effects on various neurons in situ (66, 67, 68). For example, cabergoline blocks oxygen/glucose deprivation-induced cell death in SH-SY5Y neuroblastoma cells (66). In vivo, chronic cabergoline treatment antagonizes death of dopaminergic neurons in 6-ODHA-treated mice (67). Since APZ is clinically used 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 whether quinpirole, another D₂R agonist has APZ-like effects on CaMKIIδ3 dephosphorylation and nuclear translocation, quinpirole elicits neuroprotection against glutamate-induced neurotoxicity in cultured rat mesencephalic neurons (68). In addition, we previously documented that stimulation with quinpirole in D₂R-expressed NG108-15 cells activate the nuclear isoform of CaMKII (25). This evidence and our data suggest a critical role for BDNF in supporting survival of midbrain dopaminergic neurons, an activity likely supported by the D₂R-mediated CaMKIIδ3/PP1 pathway.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. CaMKII is dephosphorylated at Ser332 by PP1 in vitro. (A), Comparison of amino acid sequences of CaMKII subunit variable domains. CaMKIIαB and CaMKIIδ3 (δB) have an 11-amino acid insertion containing an NLS (boxed), while CaMKIIα, CaMKIIδ1 and CaMKIIδ2...
PP1 promotes CaMKIIδ3 nuclear localization.

(δC) lack a variable domain. Asterisk denotes phosphorylated Ser332 (black background). (B), Phosphorylation assay of CaMKII purified from rat brain under various conditions in vitro, and western blot with various antibodies to CaMKIIα/β, CaMKIIδ, CaMKII(pThr286/287) and CaMKII(pSer332). (C) Western blot analysis of CaMKII purified from rat brain using CaMKII(pSer332) antibody (left) or the same antibody preincubated with CaMKII(pSer332) antibody blocking phospho-peptide (right).

Figure 2. PP1α and PP1γ1 predominantly regulate CaMKIIδ3 nuclear translocation. (A, B), Localization of PP1 isoforms in Neuro-2a cells. Confocal images show localization of eGFP-tagged PP1 isoforms (green), mCherry-tagged NIP1 (red) and the nuclear marker DAPI (blue). Right panels are single color images of PP1 isoforms (grey, shown at left). Scale bars, 10µm. (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 indicated antibodies. (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 indicated fractions. Each bar represents the mean ± S.E.M. *, p < 0.05 relative to CaMKIIδ3-expressing cells. (F), Confocal images showing co-localization of FLAG-tagged CaMKIIδ3 with eGFP-tagged PP1γ1. Scale bars, 10µm. (G), CaMKIIδ3 and PP1γ1 co-immunoprecipitation in extracts of cells co-transfected with FLAG-CaMKIIδ3 and PP1γ1-eGFP. Extracts were IP’d with anti-FLAG or anti-GFP antibody, and immunoprecipitates were immunoblotted (WB) with anti-GFP (top) or with anti-FLAG (bottom) antibody.

Figure 3. PP1-dependent dephosphorylation of CaMKIIδ3 at Ser332 regulates its nuclear activity. (A), (top) Representative immunoblots probed with pCaMKII (Ser332) antibody in cells transfected with constructs indicated at left. C, cytoplasmic fraction; N, nuclear fraction; S332A, CaMKIIδ3 (S332A). (bottom) Quantitative densitometry analyses of data shown above. * p < 0.05, versus CaMKIIδ3-expressing cells. (B), Ca2+-dependent CaMKII activity in Neuro-2a cells. Bars represent mean ± S.E.M. *, p < 0.05, **, p < 0.01 versus activity in the nuclear fraction of CaMKIIδ3-expressing cells. (C, D, E) Real-time measurement of Camui-FRET signals. Relative fluorescence intensity changes following treatment of Neuro-2a cells with 60mM high KCl after transfection with Camuiα (C), Camuiδ3 (D), or Camuiδ3 and PP1γ1 (E). CFP/YFP ratio values were derived by averaging fluorescence intensity from the entire cytosolic or nuclear area. (F), Shown is the peak ratio value of the release phase relative to the preceding baseline. Each bar represents the mean ± S.E.M. **, p < 0.01.

Figure 4. APZ treatment of cultured mesencephalic dopaminergic neurons promotes CaMKIIδ3 dephosphorylation at Ser332. (A), Representative images of immunoblots probed with antibodies against phosphorylated CaMKII (Thr286/287), phosphorylated CaMKII (Ser332) and β-tubulin in lysates from cultured mesencephalic dopaminergic neurons treated at DIV10 with chemically induced LTP (c-LTP), APZ, or APZ plus okadaic acid (OA). Arrows indicate 57kDa immunoreactive band corresponding to CaMKIIδ. (B, C), Quantitative analyses of phosphorylated CaMKIIδ (Thr287) (B) and phosphorylated CaMKIIδ (Ser332) (C) as analyzed by densitometry. Each bar represents the mean ± S.E.M. *, p < 0.05 versus untreated cells. #, p < 0.05 versus APZ-treated cells.

Figure 5. APZ treatment enhances CaMKIIδ nuclear translocation in rat substantia nigra. (A), Confocal images showing co-localization of CaMKIIδ (green) and dopaminergic TH-positive (red) cells in rat substantia nigra (SN) pars compacta. (B), (left), Representative images of immunoblots of rat SN lysates probed with antibodies against phosphorylated CaMKII (Thr286/287), CaMKIIα/β, phosphorylated CaMKII (Ser332) and CaMKIIδ. Arrows indicate 57kDa immunoreactive band corresponding to CaMKIIδ. (right), Quantitative analyses
of phosphorylated CaMKIIδ (Ser332) and phosphorylated CaMKIIδ (Thr287) as analyzed by densitometry. (C), Increased PP1 activity in the SN of APZ-treated rats. Data are expressed as percentage of activity seen in vehicle-treated rats. (D), Nuclear translocation of CaMKIIδ after APZ treatment. (left), 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), Quantitative analysis of CaMKIIδ as a ratio of nuclear to cytosolic expression. (E), (top), Representative immunoblots of rat SN lysates probed with BDNF and β-tubulin antibodies. (bottom), Quantitative densitometry analyses are shown. Each bar represents the mean ± S.E.M. *, p <0.05 versus vehicle-treated rats.

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. Bars represent the mean ± S.E.M. *, p < 0.05. versus wild-type cells transfected with CaMKIIδ3. (B), (top), Representative immunoblots of Neuro-2a cell lysates probed with BDNF and β-tubulin antibodies. (bottom), 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) and densitometric quantification (bottom) of protein expression. **, p < 0.01 versus cells transfected with control siRNA (siCon.). (D, F), Representative images of TH-positive cultured mesencephalic neurons. (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 co-transfected cells plus APZ. NS, not significant. (G), The number of surviving cells among TH-positive neurons transfected with 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 co-transfected 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⁺.

Figure 7. Schematic representation showing mechanism of CaMKIIδ3 activation and nuclear translocation in dopaminergic neurons. Treatment with the dopamine D2R agonist APZ increases PP1 activity by inactivating the cAMP/protein kinase A (PKA)/inhibitor-1 (I-1) pathway. PP1 then dephosphorylates CaMKIIδ at Ser332 in the cytoplasm, resulting in nuclear translocation. Nuclear CaMKIIδ3 phosphorylates a subset of transcription factors, including MeCP2 and C/EBP, increasing BDNF expression. Depolarization promotes Ca²⁺ entry through N-methyl-D-aspartate receptors (NMDA-Rs) or voltage-dependent calcium channels (VDCCs) and binding to CaM, resulting in CaMKIIδ3 autophosphorylation at Thr287 and Ser332 in the cytoplasm. Nuclear CaMKIIδ3 may also be phosphorylated at Ser332 by active CaMKI or CaMKIV, resulting in export to the cytosol.
Fig. 1
Fig. 4

(A) Western Blot (WB) analysis showing the levels of pThr286/287, pSer332, and β-tubulin in c-LTP, APZ, and APZ+OA conditions over time (0, 5, 30, 60 minutes).

(B) Bar graph showing the fold change in levels of CaMKIIδ (pThr287) over time.

(C) Bar graph showing the fold change in levels of CaMKIIδ (pSer332) over time.
Fig. 5
Fig. 6
Nuclear translocation of calcium/calmodulin-dependent protein kinase IIδ3 promoted by protein phosphatase-1 enhances brain-derived neurotrophic factor expression in dopaminergic neurons.

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