# Supplemental Data

# Activity-Dependent Modulation of Limbic Dopamine D3 Receptors by CaMKII

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## Supplemental Figures

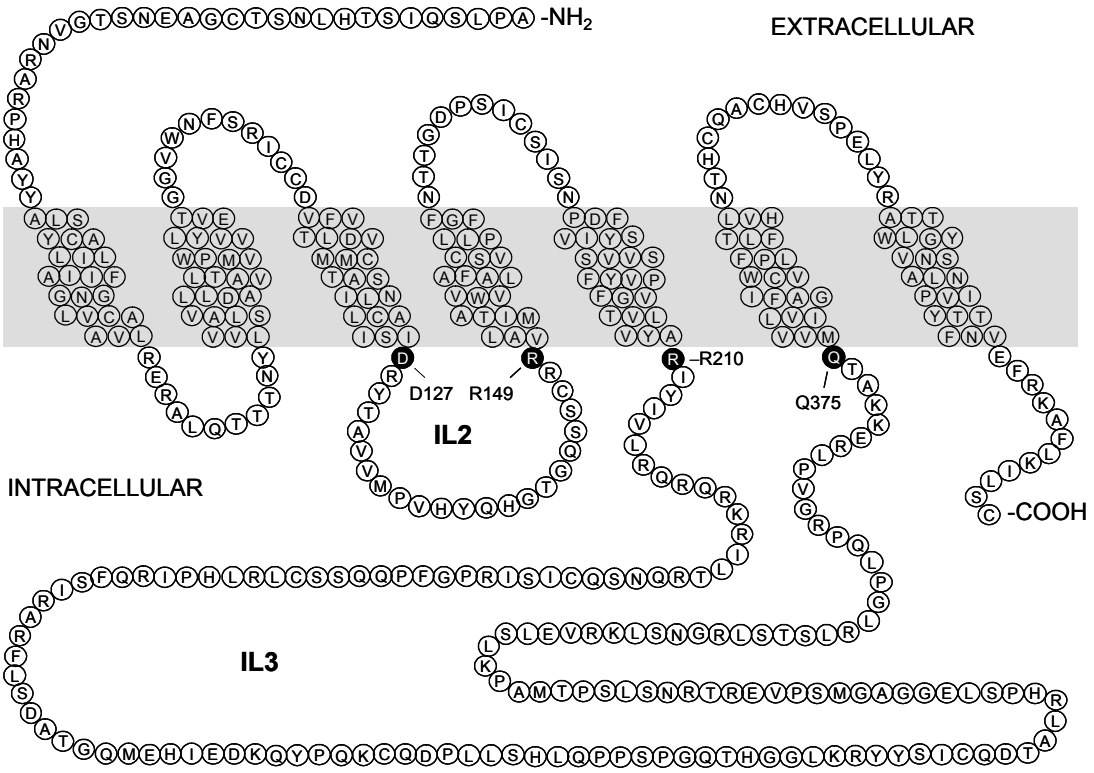


**Figure S1. Diagram of the rat D3R sequence.**

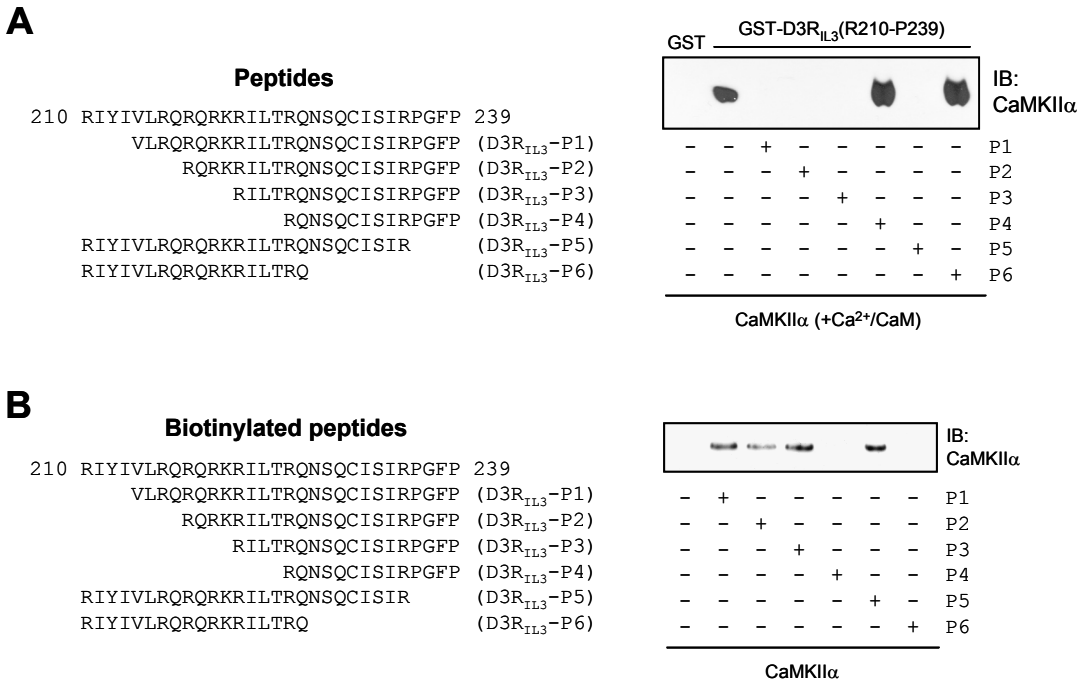


**Figure S2. Analysis of CaMKIIα-D3R$_{IL3}$ interactions with synthetic peptides. (A)** The effect of the peptides (P1-P6) on the $Ca^{2+}$/CaM-regulated binding of CaMKIIα to D3R$_{IL3}$(R210-P239). The primary sequence of the first N-terminal 30 amino acids of D3R$_{IL3}$ (R210-P239) is shown. *In vitro* binding assays were performed in the presence of $CaCl_2$ (0.5 mM) and CaM (1 μM). **(B)** Four biotinylated peptides (P1, P2, P3, and P5) pulled down CaMKIIα. All peptides are biotinylated and utilized in pull-down assays with CaMKIIα. Bound CaMKIIα was visualized by immunoblots (IB) of the eluted proteins using a specific antibody.

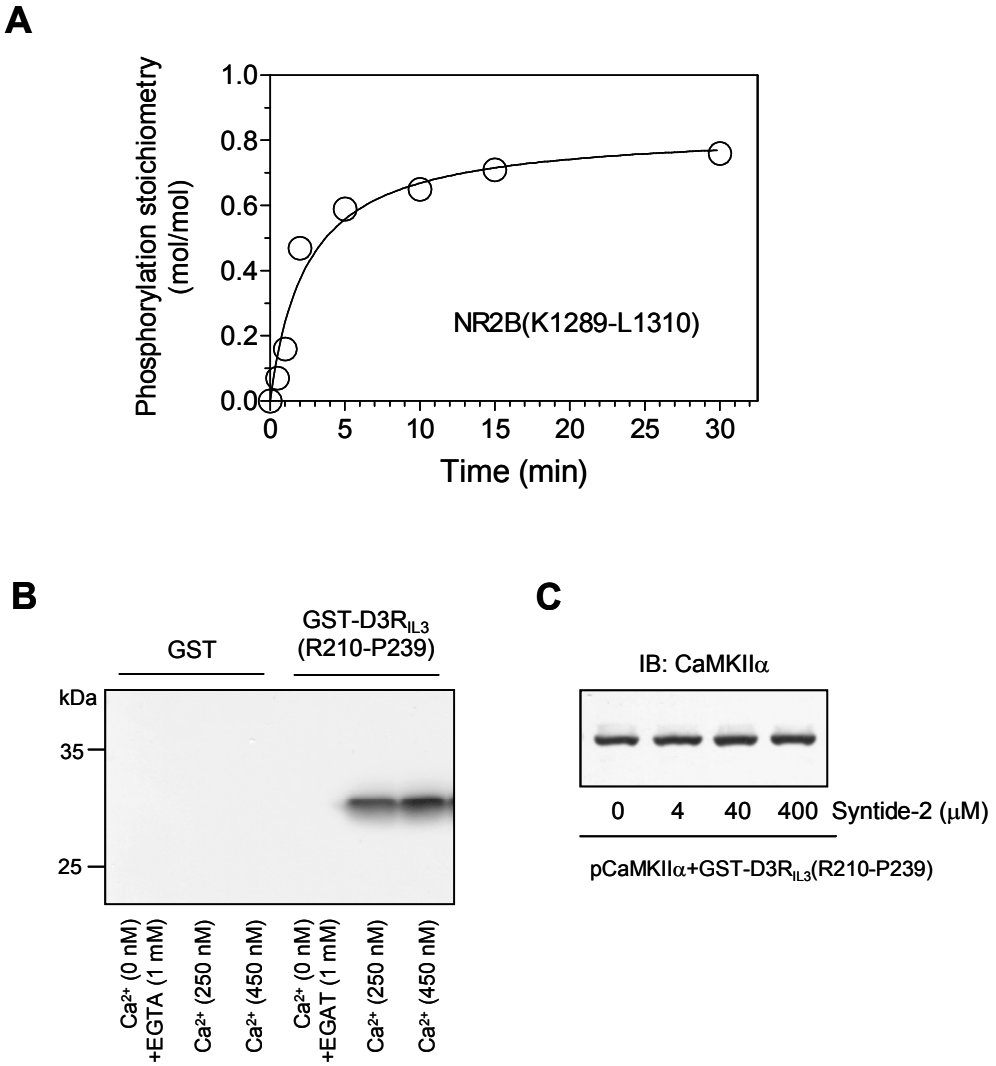


**Figure S3. Phosphorylation of the NR2B and D3R$_{IL3}$ fragments by CaMKIIα *in vitro* as detected by $^{32}$P incorporation.** **(A)** Time course of NR2B(K1289-L1310) phosphorylation. **(B)** Phosphorylation of GST-D3R$_{IL3}$(R210-P239) but not GST at physiological concentrations of $Ca^{2+}$. Substrate proteins at 0.1 μM were incubated at 30°C for 10 min (B) or indicated times (A) with [γ-$^{32}$P]ATP and CaMKIIα in the presence of $Ca^{2+}$ (0.5 mM)/CaM (1 μM) (A) or $Ca^{2+}$ at 0 (1 mM EGTA), 250, or 450 nM (B). **(C)** No competition for the CaMKIIα-D3R$_{IL3}$ interaction by syntide-2. GST-D3R$_{IL3}$(R210-P239) was allowed to bind 100 nM of pCaMKIIα in the presence of the indicated concentrations of syntide-2 peptides (PLARTLSVAGLPGKK). Bound CaMKIIα was visualized by immunoblots (IB) of the eluted proteins using a specific antibody.

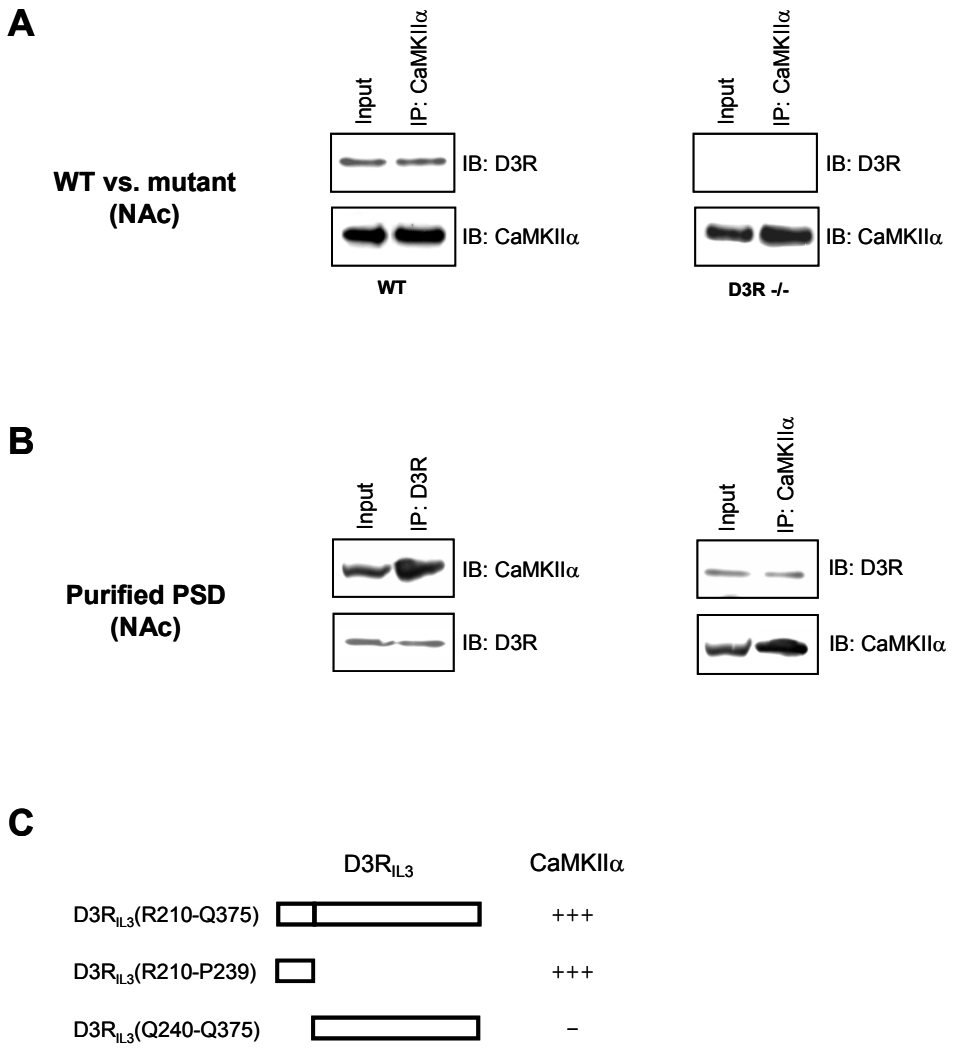


**Figure S4. The interaction between CaMKIIα and D3Rs. (A and B)** The interaction between CaMKIIα and D3Rs detected by coimmunoprecipitation from the synaptosomal fraction of the rat NAc from wild-type (WT) and D3R mutant mice **(A)** or the PSD fraction of rat accumbal neurons **(B)**. After immunoprecipitation (IP), bound CaMKIIα or D3R proteins were visualized by immunoblots (IB) of the eluted proteins using a specific antibody. **(C)** The interaction between the N-terminal CaMKIIα (residues 1-316) and the three D3R$_{IL3}$ fragments detected by yeast two-hybrid assays. Strength of an interaction is indicated by +++ (strong) on the basis of β-galactosidase assays, whereas - denotes no interaction (see Supplemental Experimental Procedures).

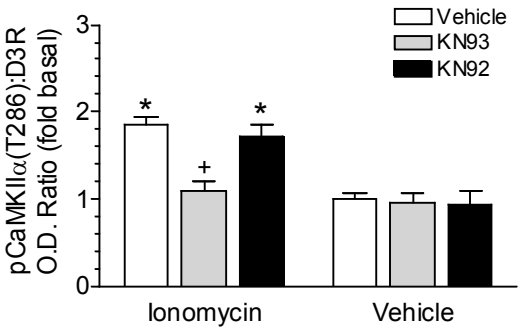


**Figure S5. The effect of the CaMKII inhibitor KN93 on the ionomycin-induced association of pCaMKIIα(T286) with D3Rs.** KN93 but not its inactive analog KN92 blocked the effect of ionomycin. Ionomycin (10 μM) was co-treated with KN93 or KN92 at 20 μM for 15 min. Immunoblots of pCaMKIIα(T286) or D3Rs were performed on D3R precipitates from drug-treated accumbal slices. Data are presented as means ± SEM for 4 experiments per group. *$p < 0.05$ versus vehicle + vehicle. +$p < 0.05$ versus vehicle + ionomycin.

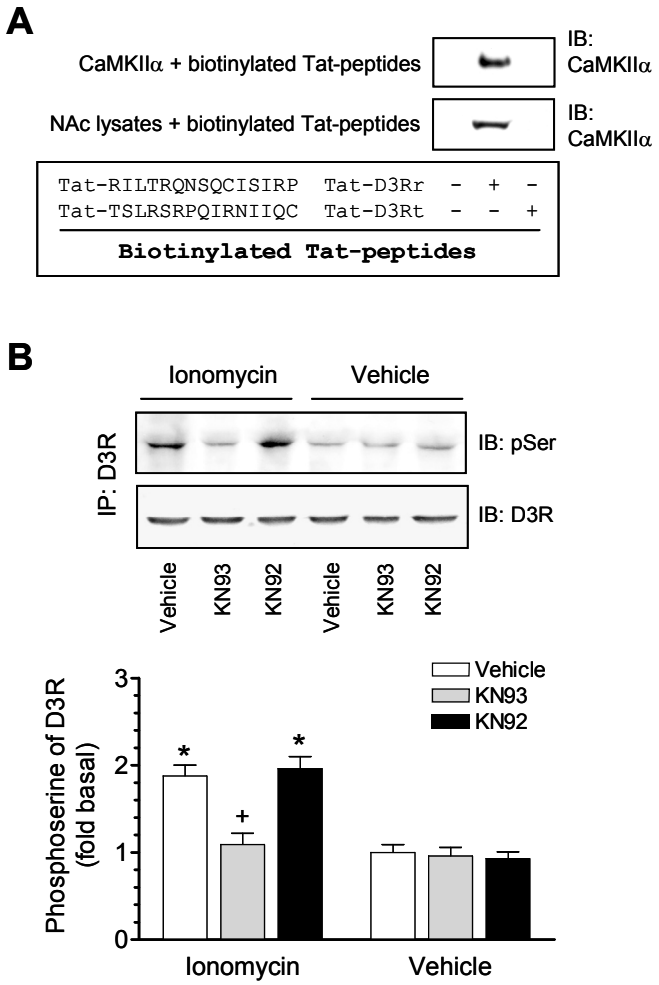


**Figure S6. (A)** The biotinylated Tat-D3Rr peptide pulled down purified CaMKIIα and endogenous CaMKIIα from rat NAc lysates. Bound CaMKIIα was visualized by immunoblots (IB) of the eluted proteins using a specific antibody. **(B)** KN93 blocked the ionomycin-stimulated serine phosphorylation of D3Rs. Rat accumbal slices were treated with ionomycin (10 μM) alone or with KN93 or KN92 (20 μM) for 15 min. After immunoprecipitation (IP) with an anti-D3R antibody, phosphoserine or D3R proteins in D3R precipitates were detected by immunoblots (IB) using a specific antibody. Data are presented as means ± SEM for 4 experiments per group. *$p < 0.05$ versus vehicle + vehicle. +$p < 0.05$ versus vehicle + ionomycin.

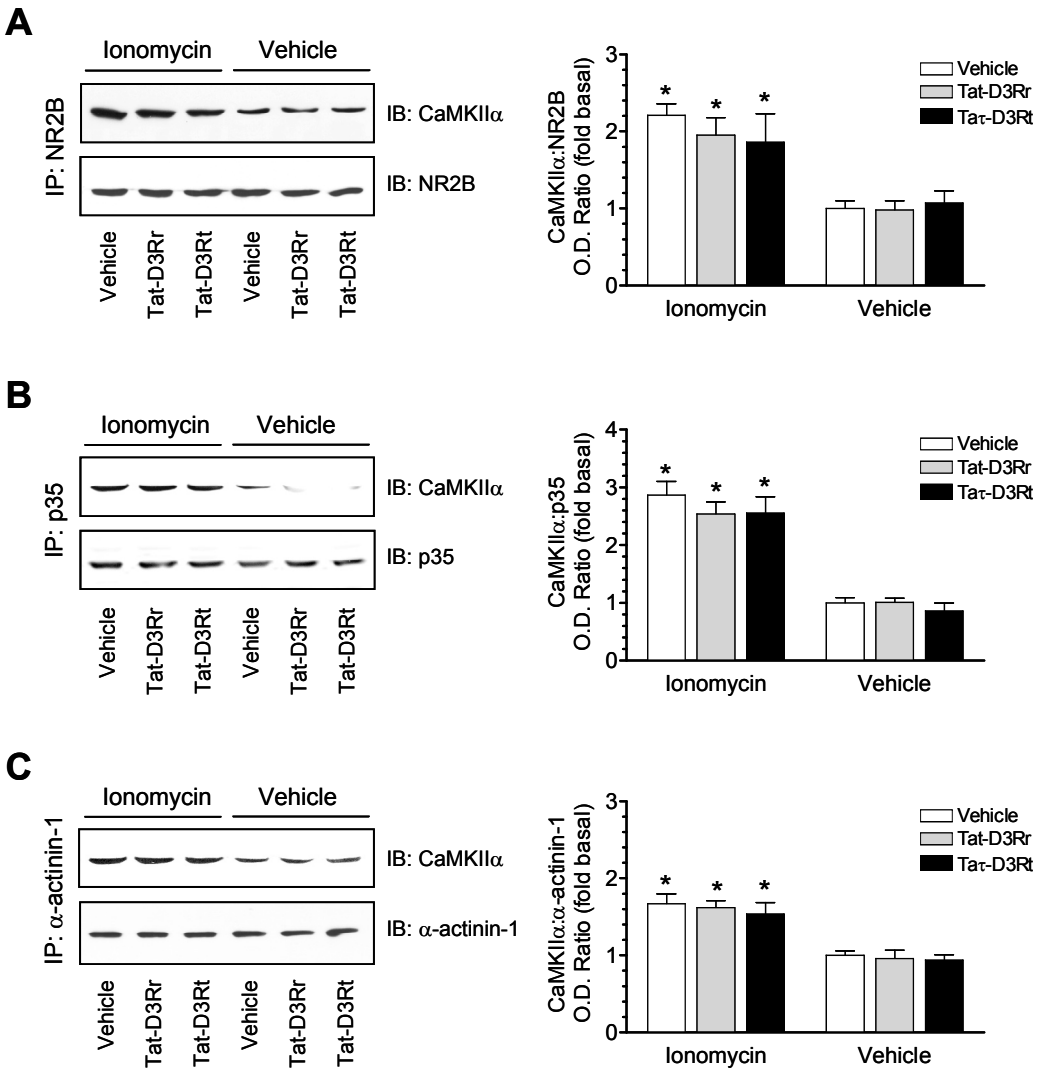


**Figure S7. The effect of Tat peptides on the $Ca^{2+}$-regulated association of CaMKIIα with NR2B (A), p35 (B), and α-actinin-1 (C).** Rat accumbal slices were treated with Tat-D3Rr or Tat-D3Rt (10 μM) 1 h prior to a 15-min treatment with ionomycin (10 μM). After immunoprecipitation (IP) with an antibody against NR2B, p35, or α-actinin-1, proteins of interest in these precipitates were detected by immunoblots (IB) using a specific antibody. Data are presented as means ± SEM for 4-6 experiments per group. *$p < 0.05$ versus vehicle + vehicle.

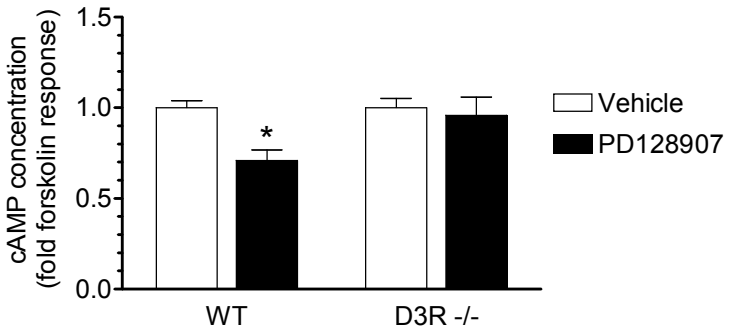


**Figure S8.** Effects of PD128907 on forskolin-stimulated cAMP accumulation in accumbal slices prepared from WT and D3R mutant mice. PD128907 (1-3 nM) was co-treated with forskolin (1 μM, 20 min). Data are presented as means ± SEM for 4-5 experiments per group. *$p < 0.05$ versus forskolin alone.

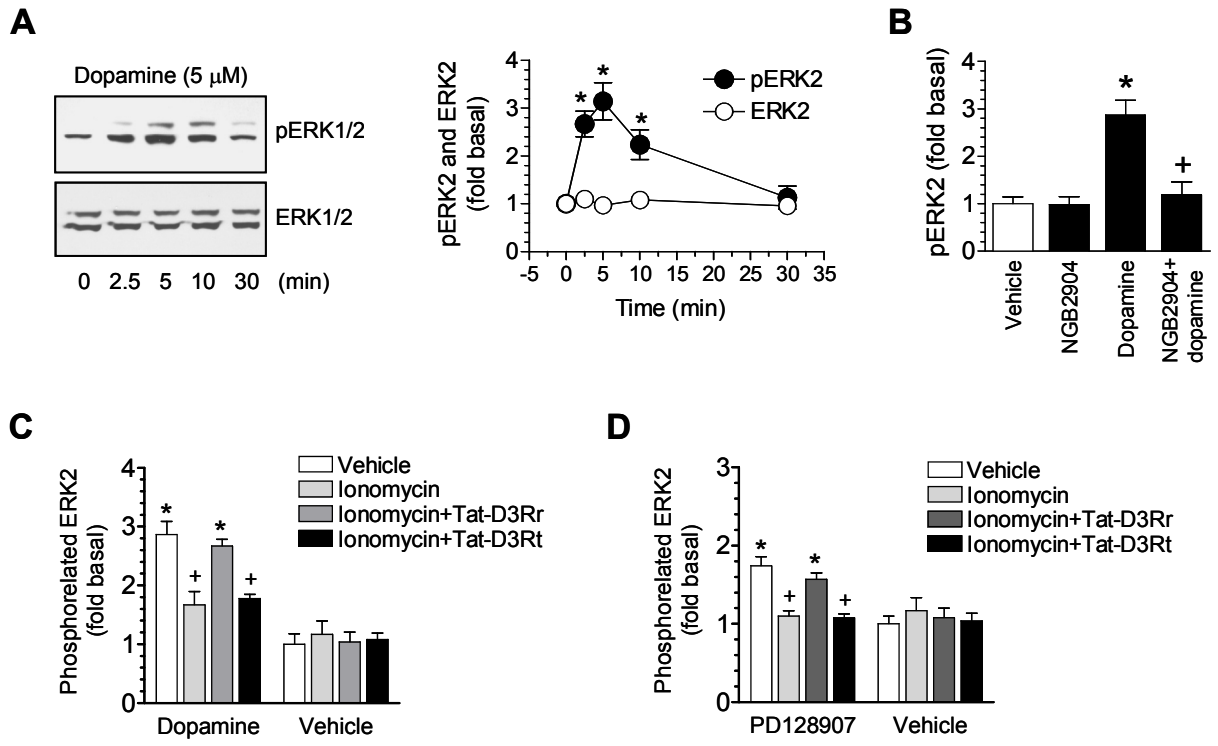


**Figure S9.** Dopamine increases phosphorylation of ERK1/2 in HEK293 cells transiently transfected with D3Rs and accumbal slices. **(A)** Dopamine (5 μM) induced a rapid and transient increase in cellular levels of phosphorylated ERK1/2 (pERK1/2) in HEK293 cells. **(B)** NGB2904 blocked the dopamine-stimulated ERK2 phosphorylation in HEK293 cells. NGB2904 (50 nM) was applied 10 min prior to dopamine (5 μM, 5 min). **(C)** Tat-D3Rr reversed the effect of ionomycin on the dopamine-stimulated ERK2 phosphorylation in HEK293 cells transiently co-transfected with D3Rs and CaMKIIα. **(D)** Tat-D3Rr reversed the effect of ionomycin on the PD128907-stimulated ERK2 phosphorylation in accumbal slices. Ionomycin (2 μM with 1 mM $CaCl_2$, 3 min) was applied 30 min prior to dopamine (5 μM, 5 min) or PD128907 (3 nM, 5 min). The selection of a 30-min interval allows a complete recovery of ERK1/2 phosphorylation induced by a brief incubation of ionomycin. In the experiments in which the Tat-fusion peptide was employed, the peptide at 8 μM was applied 1 h prior to ionomycin. Data are presented as means ± SEM for 4-5 experiments per group. *$p < 0.05$ versus ERK2 at the corresponding time point (**A**), vehicle (**B**), or vehicle + vehicle (**C** and **D**). +$p < 0.05$ versus dopamine or PD128907.

## Supplemental Experimental Procedures

*Surgeries for intravenous cannulation and intra-accumbal injection.* Chronic intravenous cannulation was made after rats were deeply anesthetized with 2.5-3% isoflurane. A PE-50 catheter was implanted into the right jugular vein and the free end was tunneled subcutaneously to the rats' scapulae and connected to a plastic connector. After the surgery, the cannulae were regularly flushed with heparinized (100 U/ml) saline to ensure patency. For intra-accumbal injection, rats were anesthetized and placed in a stereotaxic holder. Two 26-gauge guide cannulae were bilaterally implanted to the NAc (1-1.6 mm anterior to bregma, 1.6 mm lateral to midline, and 4.2 mm below surface of skull) based on the atlas of Paxinos and Watson (1997). Rats were allowed at least 7 days for recovery. On the day of the experiment, a 33-gauge injector replaced the inner sealing wire and protruded 2.5 mm beyond the guide cannula. Drugs were infused into the NAc in freely moving rats in 0.4 μl. After completing injection, the cannula was left in place for 3 min. The standard histological examination was conducted after experiments to verify the injection sites.

*Subcellular PSD fraction preparation*. Isolation of PSDs was performed essentially as described by Carlin et al. (1980) with a modification of adding protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstain, 5 μg/ml aprotinin, and 0.2 mM PMSF) to all extraction buffers (Liu et al., 2006). Accumbal tissue of 25 rat brains were pooled and homogenized and then subjected to several steps of differential centrifugation to obtain crude synaptosomal fractions (P2). The P2 pellet was separated by 0.85/1.0/1.2 M sucrose density gradient centrifugation. The synaptosomes were obtained from the 1.0/1.2 M sucrose interface. After being washed with 0.5% Triton X-100, synaptosomal pellets were collected by centrifugation and then subjected to a second 1.0/1.5/2.0 M sucrose density gradient centrifugation. The PSDs were obtained from the 1.5/2.0 M interface of the sucrose gradients. The PSD fraction was diluted with an equal volume of 1% Triton X-100/150 mM KCl solution, mixed for 5 min, and centrifuged at 201,800 *g* for 1 h. The resultant PSD pellet was resuspended in a 0.5% Triton X-100/75 mM KCl solution containing sodium orthovanadate and protease inhibitors. All steps were performed at 4°C. The yield of the PSD preparation was ~ 0.3 mg/2 g initial wet wt tissue. The purity of the PSD fraction was confirmed by our routine detection (Liu et al., 2006) of the lack of immunoreactivity for PKCε, a known PKC isozyme present exclusively in the presynaptic compartment.

*Nondenaturing solubilization of membrane proteins.* Nondenaturing solubilization was performed as described previously (Luo et al., 1997; Mao et al., 2005). The P2 pellets and isolated PSDs were resuspended in sample buffer and solubilized in 0.5% sodium deoxycholate. After incubation at 4$^{o}$C for 20 min, Triton X-100 was added to a final concentration of 0.5%. Insoluble proteins were sedimented at 50,000 *g* at 4$^{o}$C for 30 min. The supernatants were used

for coimmunoprecipitation.

*Coimmunoprecipitation.* Solubilized NAc samples (P2 or PSD) were incubated with a rabbit antibody against CaMKIIα (Santa Cruz), D3Rs (Chemicon, Temecula, CA), NR2B (Chemicon), or p35 (Santa Cruz Biotechnology), or a monoclonal antibody against α-actinin-1 (Santa Cruz Biotechnology). The complex was precipitated with 50% protein A or G agarose/sepharose bead slurry (Amersham). Proteins were separated on Novex 4-12% gels and probed with a mouse anti-CaMKIIα (Santa Cruz), anti-phosphoserine (Sigma), or anti-α-actinin-1 (Santa Cruz Biotechnology) antibody, a goat anti-CaMKIV antibody (Santa Cruz), or a rabbit anti-D3R (Chemicon), anti-D1R (Santa Cruz), anti-NR2B (Chemicon), or anti-p35 antibody. HRP-conjugated secondary antibodies and enhanced chemiluminescence were used to visualize proteins.

*Western blot analysis.* The equal amount of protein was separated on SDS NuPAGE Bis-Tris 4-12% gels (Invitrogen). Proteins were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) and blocked in blocking buffer (5% nonfat dry milk in PBS and 0.1% Tween 20) for 1 h. The blots were incubated with primary antibodies usually at 1:500-2000 overnight at 4$^{o}$C. This was followed by 1 h incubation in goat or bovine horseradish peroxidase-linked secondary antibodies (Jackson Immunoresearch Laboratory, West Grove, PA or Santa Cruz Biotechnology, Santa Cruz, CA) at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ), and captured into Kodak Image Station 2000R. The MagicMark XP Western protein standard (Invitrogen) or the kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) was used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software, and bands were normalized to actin protein values where appropriate.

*Identification of the 50 kDa $D3R_{IL3}$-binding protein.* The 50 kDa band was excised and digested by trypsin. The resulting peptide fragments were analyzed by a nano-LC/MS/MS method, which identified the 50 kDa protein as rat CaMKIIα (accession #: P11275).

*Phosphorylation site mapping using LC/MS/MS.* Phosphorylated $D3R_{IL3}$ was resolved by SDS-PAGE, excised, and digested by trypsin. Peptides were separated by HPLC with a capillary column (Monitor C18, 100 μm x 11 cm, 5 micron, 100 Å, Column Engineering). MS/MS scans of peptides were acquired by a ThermoFinnigan LTQ linear ion trap mass spectrometry with a ThermoFinnigan Surveyor LC pump and NanoSpray source (Thermo Electron) in an isolation width of 3 m/z, an activation time of 30 ms, activation Q of 0.250, and 30% normalized collision energy using 1 microscan and ion time of 100 for each MS/MS scan. The mass spectrometry was tuned prior to analysis to spray voltage of 1.8KV, a capillary temperature of 150$^{o}$C, a

capillary voltage of 50V, and tube lens 100V. Tandem MS analysis was conducted by data-dependent scanning in which one full MS spectra (mass range of 200-2000 amu) was followed by three MS/MS spectra. Peptides and modification were identified with the SEQUEST algorithm with SEQUEST Browser software (Thermo Electron, San Jose, CA). Candidate modifications found by software were verified by visual inspection of corresponding spectra.

*GST-fusion protein and synthetic peptide phosphorylation in vitro.* GST-fusion proteins, GST alone, or synthetic peptides at 0.1 μM or indicated concentrations were incubated with purified CaMKIIα for 10 min or indicated times at 30°C, in a final volume of at least 30 μl of the reaction buffer containing 10 mM HEPES pH7.4, 10 mM $MgCl_2$, 1 mM $Na_3VO_4$, 1 mM DTT, 0.1 mg/ml BSA, 50 μM ATP, and 2.5 μCi/tube [$\gamma$-$^{32}$P]ATP (~3000 Ci/mmol, PerkinElmer) with or without 0.5 mM $CaCl_2$ and 1 μM CaM. The phosphorylation reactions were stopped by adding LDS sample buffer and boiling for 5 min. Phosphorylated proteins were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and visualized by autoradiography. Alternatively, target bands on Coomassie Brilliant Blue-stained gels were excised, and radioactivity was determined by liquid scintillation counting. Phosphorylation stoichiometry was calculated by the division of the radioactivity per mole of substrate by the radioactivity per mole of phosphate (moles phosphate per moles substrate). Kinetic parameters were calculated using the Michaelis-Menten model, whereby $V = V_{max}[S]/([S]+K_m)$ and $V_{max} = k_2[E_{\text{Total}}]$. At least three experiments were performed for each analysis.

*Cell cultures and transfection.* HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum and 10 μg/ml gentamicin (Sigma) at 37°C with 5% $CO_2$. Transfections were performed on a ~70-80% confluent monolayer using Lipofectamine Plus™ (Invitrogen) according to the manufacturer's instructions. Cells were used in pharmacological experiments 24-30 h after the transfection. Mammalian expression vectors include wild-type human D3R in pcDNA3.1+ bearing the CMV promoter (Missouri S&T cDNA Resource Center, Rolla, MO) and wild-type rat CaMKIIα in pCS2+ bearing the CMV promoter (Addgene).

*Expression of CaMKII mutants.* HEK293 cells were transfected with Lipofectamine Plus™ (Invitrogen) and a mammalian SRα-CaM kinase expression vector carrying the cDNA encoding CaMKIIα T305/306D, T286A, or T286D mutant (gifts from Dr. H. Schulman and Dr. K.U. Bayer) as described previously (Yang and Schulman, 1999) or a pCS2+ vector carrying WT rat CaMKIIα. Cells were harvested 36-48 h after transfection. Cell pellets were obtained by centrifugation (200 *g*), and were lysed by sonication. The homogenates were centrifuged at 100,000 *g* for 2 h at 4°C, and the supernatant was frozen at -80°C. The quantity of mutant kinases present in HEK293 cell lysates was determined by immunoblots using a mouse anti-

CaMKIIα antibody, which demonstrated stable expression of all the mutant kinases with full-length at high levels.

*Autophosphorylation of CaMKIIα:* Autophosphorylation reaction was performed in 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 0.3 mM $CaCl_2$, 50 μM ATP, and 1 μM CaM for 1 min at 30°C. The reaction was stopped by adding EGTA (3 mM final concentration) on ice. Aliquots (5 μl) of the autophosphorylated kinases were immediately used in *in vitro* binding assays (50 μl total).

*Biotinylated peptide pull-down assay:* Biotinylated peptides (5 μg) were immobilized on 10 μl of streptavidin Sepharose beads (Amersham) in binding buffer (200 mM NaCl, 0.2% Triton X-100, 0.1 mg/ml BSA, 50 mM Tris, pH 7.5, 1 mM $CaCl_2$, and 1 μM CaM) overnight at 4°C. The beads were washed three times with binding buffer and then incubated with CaMKIIα or rat NAc lysates for 2-3 h with shaking at 4°C. Beads were washed four times with binding buffer. Bound CaMKIIα proteins were eluted with 2X LDS loading buffer, resolved by SDS-PAGE, and immunoblotted with a specific antibody against CaMKIIα.

*Peptide synthesis.* All peptides, including Tat-fusion peptides, were synthesized and purified by Abgent (San Diego, CA) or Sigma (Sigma-Genosys). Tat-fusion peptides gain cell permeability by containing an arginine-enriched cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) Tat protein (YGRKKRRORRR) (Schwarze et al., 1999). The CaMKII inhibitory peptide, a 21-mer CaMKIINtide (KRPPKLGQIGRSKRVVIEDDR), is derived from CaMKIIN (Chang et al., 1998). The 32-mer Tat-fusion CaMKIINtide (Tat-CaMKIINtide) can be easily synthesized by a routine peptide synthesis facility (Vest et al., 2007).

*Measurement of Tat-peptide concentrations in striatal tissue.* An $NH_2$-terminal fluorescein isothiocyanate (FITC)-glycine group was added to the Tat peptides to serve as a fluorescent reporter. After an intravenous injection of FITC-Tat-D3Rr (3 nmol/g), rats were anesthetized and perfused with saline to wash out residual peptides in the blood and sacrificed at the indicated times. Brains were removed and striati were dissected and homogenized in the RIPA buffer. The homogenates were centrifuged at 15,000 *g* for 30 min at 4°C. Soluble proteins in the supernatant were measured and adjusted to a concentration of 10 μg/μl. The fluorescent intensity of fluorescein-Tat-D3Rr in protein supernatants was measured in a 96-well microplate (100 μl of protein supernatant/well) using a fluorescence microplate reader at 490 nm excitation and 520 nm emission wavelengths. Peptide abundance (pg/mg total protein) was calculated from a standard curve constructed with known concentrations of FITC-Tat-D3Rr peptide.

*Histological examination of intracellular uptake of FITC-conjugated Tat-peptides.* Rats received a single intravenous injection of FITC-Tat-D3Rr or FITC-Tat38-48 peptides at a dose of 3 nmol/g, and were anesthetized 1.5 h after injection. After decapitation, brains were removed and cut into coronal sections (50 μm) using a vibratome (Vibratome, St. Louis, MO). Intracellular uptake of fluorescent peptides was examined by analyzing fluorescent images with confocal microscopy (Nikon C1 laser scanning confocal microscope, Nikon, Tokyo, Japan).

*Accumbal slice preparation*. Accumbal slices were prepared as previously described (Snyder et al., 2000). Briefly, rats were anesthetized and decapitated. The brains were removed rapidly and placed in ice-cold artificial CSF (ACSF) containing (in mM): 10 glucose, 124 NaCl, 3 KCl, 1.25 $KH_2PO_4$, 26 $NaHCO_3$, 2 $MgSO_4$, and 2 $CaCl_2$, bubbled with 95% $O_2$-5% $CO_2$, pH 7.4. Coronal slices (350 μm) were prepared using a vibratome. The NAc was dissected from the slices in ice-cold ACSF. Each slice was transferred to a polypropylene incubation tube with 2 ml of fresh ACSF for a preincubation at room temperature or 30$^{o}$C under constant oxygenation with 95% $O_2$-5% $CO_2$ for 60 min. The solution was replaced with fresh ACSF for an additional preincubation (10-20 min). Drugs as specified in each experiment were added and incubated at room temperature or 30$^{o}$C. After drug treatment, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at -80$^{o}$C until assayed.

*Yeast two-hybrid assays.* The cDNAs encoding $D3R_{IL3}$(R210-Q375), $D3R_{IL3}$(R210-P239), or $D3R_{IL3}$(Q240-Q375) were amplified from full-length cDNA clones by PCR and subcloned into pDBLeu vectors (Invitrogen) for expression as GAL4 DNA binding domain fusion proteins (bait proteins). A cDNA encoding the N-terminal (residues 1-316) domain of CaMKIIα was generated by PCR and subcloned into pPC86 vectors (Invitrogen) for expression as GAL4 activation domain fusion proteins. MaV203 yeast cells (Invitrogen) containing *HIS3*, *LacZ*, and *URA* reporter genes under the control of the GAL4-activating sequences were used. They were cotransformed with pDBLeu vectors containing D3R fragments and pPC86 vectors containing CaMKIIα fragments by the lithium acetate method. Interactions were detected by growth on Leu-, Trp-, and His- media containing 3-amino-1,2,4-triazol (3-AT, 20 mM). β-Galactosidase (LacZ) assay was used to compare the strength of interaction among the proteins encoded by cotransformed cDNAs. Colonies that turned blue within 1 h contain strongly interacting proteins (+++). The ++ (moderate) and + (weak) symbols were used to designate colonies that turned

blue within 1-8 h and 8-24 h, respectively. The - symbol was used to indicate colonies that did not turn blue within 24 h.

*Antibodies and pharmacological agents.* Antibodies used in this study include a rabbit antibody against D3Rs (Millipore, Billerica, MA), D1Rs (Santa Cruz Biotechnology, Santa Cruz, CA), CaMKIIα(T286) (Santa Cruz), NR2B (Millipore), CaM (Zymed/Invitrogen), p35 (Santa Cruz Biotechnology), pCREB (Santa Cruz Biotechnology), CREB (Santa Cruz Biotechnology), pERK1/2 (Cell Signaling, Beverly, MA), ERK1/2 (Cell Signaling), pGluR1(S845) (Millipore), GluR1 (Millipore), or c-Fos (Oncogene Research Products, San Diego, CA), a mouse antibody against CaMKIIα (Santa Cruz Biotechnology), phosphoserine (Sigma), GST (Sigma, St. Louis, MO), PSD-95 (UC Davis/NINDS/NIMH, NeuroMab Facility), or α-actinin-1 (Santa Cruz Biotechnology), or a goat antibody against CaMKIV (Santa Cruz Biotechnology), PSD-93 (Santa Cruz Biotechnology), SAP97 (Santa Cruz Biotechnology), or SAP102 (Santa Cruz Biotechnology). Pharmacological agents include ionomycin, PD128907, SCH23390, NMDA, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d]cyclohepten-5,10-imine maleate (MK801), 4-(8-methyl-9*H*-1,3-dioxolo[4,5-hr][2,3]benzodazepin-5-yl)-benzenamine dihydrochloride (GYKI52466), nifedipine, SKF81297, and SKF83959 that were purchased from Tocris Cookson Inc. (Ballwin, MO). Cocaine hydrochloride, KN93, KN92, dopamine, L-glutamate, and 2,5-dimethyl-4-[2-(phenylmethyl)ben-zoyl]-1*H*-pyrrole-3-carboxylic acid methylester (FPL64176) were purchased from Sigma. U73122 and U73343 were purchased from Calbiochem (San Diego, CA). NGB2904 was a gift from Dr. Amy Hauck Newman (Medicinal Chemistry Section, NIDA-IRP) and Dr. Zheng-Xiong Xi (NIDA-IRP). All drugs were freshly prepared at the day of experiments. Cocaine was dissolved in physiological saline and its dose was calculated as a salt.

## Supplemental References


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