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Dopamine D1 receptors mediate CREB phosphorylation via phosphorylation of the NMDA receptor at Ser897–NR1

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Abstract

Addictive drugs such as amphetamine and cocaine stimulate the dopaminergic system, activate dopamine receptors and induce gene expression throughout the striatum. The signal transduction pathway leading from dopamine receptor stimulation at the synapse to gene expression in the nucleus has not been fully elucidated. Here, we present evidence that D1 receptor stimulation leads to phosphorylation of the transcription factor Ca^{2+} and cyclic AMP response element binding protein (CREB) in the nucleus by means of NMDA receptor-mediated Ca^{2+} signaling. Stimulation of D1 receptors induces the phosphorylation of Ser897 on the NR1 subunit by protein kinase A (PKA). This phosphorylation event is crucial for D1 receptor-mediated CREB phosphorylation. Dopamine cannot induce CRE-mediated gene expression in neurons transfected with a phosphorylation-deficient NR1 construct. Moreover, stimulation of D1 receptors or increase in cyclic AMP levels leads to an increase in cytosolic Ca^{2+} in the presence of glutamate, but not in the absence of glutamate, indicating the ability of dopamine and cyclic AMP to facilitate NMDA channel activity. The recruitment of the NMDA receptor signal transduction pathway by D1 receptors may provide a general mechanism for gene regulation that is fundamental for mechanisms of drug addiction and long-term memory.

Keywords: calcium/calmodulin kinase, CREB, cyclic AMP, dopamine D1 receptor, NMDA, protein kinase A.

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Stimulation of D1 receptors initiates a sequence of molecular events that modify gene expression. Transcription factors such as c-Fos, FosB and the cyclic AMP response element (CRE) binding protein (CREB), are activated by dopamine and mediate the expression of genes that shape the structure and function of neurons (Robinson and Kolb 1999; Hyman and Malenka 2001; Nestler 2001). Long-lasting adaptations to increased dopamine receptor stimulation are also translated into behavioral responses. For example, cocaine and amphetamine, two drugs that facilitate dopamine release (Wise 1984), induce sensitization and drug tolerance, which contribute to drug-seeking behavior and the addictive properties of these drugs (Robinson and Berridge 2000). The connection between the molecular changes and behavioral responses to stimulation of the midbrain dopaminergic system is not fully understood.

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Abbreviations used: AM, acetoxymethyl; APV, (–)2-amino-5-phosphonovalerate; CRE, cyclic AMP response element; CREB, cyclic AMP response element biding protein; DMEM, Dulbecco's modified Eagle medium; DNQX, 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione; DTT, dithiothreitol; ER, endoplasmic reticulum; FBS, fetal bovine serum; GYKI 52466, 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride; H89, (*N*-[2-((p-Bromocinnamyl)amino) ethyl]-5-isoquinolinesulfonamide HCl; IP, immunoprecipitation; KN62, 1-[N,O-bis(5-Isoquinolinesulfpnyl-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PD 98059, 2′-Amino-3′-methoxyflavone; PEI, polyehtylenimine; PFC, pre-frontal cortex; PKA, protein kinase A; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; U0126, 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; VTA, ventral tegmental area.

One candidate molecule that has been connected to the behavioral consequences of addiction to amphetamine and cocaine is the transcription factor CREB (Konradi *et al*. 1994; Cole *et al*. 1995; Carlezon *et al*. 1998; Self *et al*. 1998; Andersson *et al*. 2001; Pliakas *et al*. 2001). CREB is constitutively bound to DNA and is activated by the phosphorylation of Ser133 (Montminy *et al*. 1990). Phosphorylation of Ser133 can be mediated by protein kinase A (PKA), by Ca^{2+}/calmodulin-dependent kinase (CamK, Montminy *et al*. 1990; Dash *et al*. 1991; Sheng *et al*. 1991) or by the mitogen-activated protein kinase (MAPK) pathway (Xing *et al*. 1996, 1998; Choe and McGinty 2000, 2001). The analysis of the intracellular signal transduction pathway that leads from stimulation of dopamine receptors to CREB phosphorylation is an important step toward elucidating the relationship between molecular adaptations and behavioral consequences.

While it has long been accepted that the dopaminergic system plays a central role in the molecular and behavioral aspects of drug addiction (Hyman 1996; Wise 1996; Koob *et al*. 1998), it has also become apparent that other neurotransmitter systems may be necessary for the behavioral expression of addiction. The glutamate system, for example, seems to be critically involved in drug addiction (Wolf 1998; Vanderschuren and Kalivas 2000). Inhibition of the NMDA-type glutamate receptor blocks the development of behaviors such as sensitization (Wolf and Jeziorski 1993; Haracz *et al*. 1995; Kim *et al*. 1996; Li *et al*. 1999; Battisti *et al*. 2000) and locomotor hyperactivity (Karler *et al*. 1989; Pulvirenti *et al*. 1991; Bristow *et al*. 1994; Bespalov and Zvartau 1996; Druhan and Wilent 1999; Uzbay *et al*. 2000), although the relationship is complex and depends on the experimental conditions (Tzschentke and Schmidt 1998). NMDA antagonists also block drug tolerance (De Montis *et al*. 1992) and cocaine- and amphetamine-mediated gene expression (Torres and Rivier 1993; Konradi *et al*. 1996).

Anatomical evidence demonstrates an extensive interaction between dopaminergic and glutamatergic neurons in a network that involves cortical, midbrain and striatal pathways. For instance, excitatory projections from the prefrontal cortex (PFC) to the ventral tegmental area (VTA) regulate the activity of dopaminergic VTA neurons (Carr and Sesack 2000). Dopaminergic neurons in the VTA synapse onto glutamatergic neurons in the PFC, which, in turn, project to the nucleus accumbens (Carr *et al*. 1999). In the striatum (in which we include the nucleus accumbens), descending cortical inputs release glutamate and ascending midbrain inputs release dopamine at synapses of medium spiny neurons (Smith and Bolam 1990; Carr *et al*. 1999). Associative activation of these two pathways leads to heterosynaptic plasticity of the descending corticostriatal pathway, which is disrupted after dopamine denervation (Centonze *et al*. 2001). These findings suggest an important physiological interplay between glutamate and dopamine within the individual neurons of the striatum, in addition to their interplay within the neuronal circuit. Indeed, a physical interaction between D1 and NMDA receptor has recently been reported (Fiorentini *et al*. 2003). Moreover, there is evidence of a cross-talk in striatal neurons between the second messenger pathways of these two systems (Konradi *et al*. 1996; Konradi 1998; Leveque *et al*. 2000). Here we demonstrate that a molecular locus of the interaction between dopamine receptors and glutamate receptors in striatal neurons is phosphorylation of the NR1 subunit of the NMDA receptor at Ser897, a PKA phosphorylation site (Tingley *et al*. 1997). This phosphorylation event has been shown to enhance glutamate-evoked currents through NMDA receptors in heterologous systems (Westphal *et al*. 1999). The role of NMDA receptor phosphorylation for D1 receptor-mediated signal transduction and gene expression is tested both *in vitro* and *in vivo*.

Materials and methods

Animals

Animals were housed on a 12/12-h light/dark cycle with free acces to food and water. All animal experiments were carried out in accordance with the rules and regulations of the McLean Animal Care and Use Committee, which follows NIH guidelines.

Drugs and antibodies

Dopamine, SKF-38393, NMDA, dizocilpine maleate [(+) MK801 hydrogen maleate], (–)2-amino-5-phosphonovalerate (APV), 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466), 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) and 6-OHDA hydrobromide were purchased from Sigma-Aldrich (St Louis, MO, USA). KN62 1-[N,O-bis(5-Isoquinolinesulfpnyl-*N*-methyl-L-tyrosyl]-4-phenylpiperazine), H89 (*N*-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (HCl), 2′-Amino-3′-methoxyflavone (PD 98059), and 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) were purchased from Calbiochem (San Diego, CA, USA). Polyclonal antisera against Ser133 CREB (Ginty *et al*. 1993), CREB, and the NR1 subtype of the NMDA receptor were purchased from Upstate (Charlottesville, VA, USA). A monoclonal Ser133 CREB antibody from Cell Signaling Technology (Beverly, MA, USA) was used for comparison purposes in immunoblots, and gave identical results to the polyclonal antiserum. The antibody to NR1 phosphorylated on Ser897 (Tingley *et al*. 1997) was a gift from Richard L. Huganir (Johns Hopkins University) and is commercially available from UBI. Isoflurane was purchased from Abbott Laboratories (North Chicago, IL, USA).

Primary striatal cultures

Striata were dissected under a stereomicroscope from 18-day-old Sprague–Dawley rat fetuses (Taconic Farms, Germantown, NY, USA). Tissue was resuspended in 2 mL of defined medium [50% Dulbecco's modified Eagle medium (DMEM)/F12 and 50% DMEM; Cellgro-Mediatech, Herndon, VA], with the following supplements per litre of medium: 4.5 g dextrose, 1 × B27 (Invitro-

gen, Carlsbad, CA, USA), 10 mL penicillin/streptomycin liquid (Sigma-Aldrich), 25 mM HEPES. The tissue was mechanically dissociated with a pasteur pipette, the cells were resuspended in defined medium to 1.2×10^6 cells/mL, and plated in 12-well plates (Becton-Dickinson, Franklin Lakes, NJ) at 1.2×10^6 cells/well. Plates were pre-treated with 1 mL of 1 : 500 diluted sterile solution of polyethylenimine (PEI) in water for 24 h, washed twice with sterile water, coated with 2.5% serum-containing phosphate-buffered saline (PBS) solution for at least 4 h and aspirated just prior to plating. All experiments were performed in duplicates with cells 6–8 days in culture (except for Ca^{2+} imaging) and repeated at least once in an independent dissection. As determined by high-performance liquid chromatography (HPLC) analysis, glutamate levels in the medium on the day of the experiments ranged from 1 to 5 μM. Neuron to astroglia ratio was below 25 : 1, as established by immunocytochemical staining with the glial fibrillary acid protein (Dako, Carpinteria, CA, USA), and counterstaining with 1% cresyl violet.

For experiments in calcium-free medium, cells were transferred to salt solutions with or without 1.8 mM $CaCl_2$ 18 h before the experiment to avoid false results due to media change. Neurons were carefully monitored for neuronal death. Salt solutions contained 110 mM NaCl, 5.3 mM KCl, 2 mM $MgSO_4$, 400 μM glycine, 0.5% phenol red, 3 g/L dextrose and 20 mM HEPES–KOH. The Ca^{2+}-free medium contained 1 mM EGTA.

For Ca^{2+} imaging, cells were grown for 12–13 days on PEI-coated two-well borosilicate chambered glass coverslips (Nalge Nunc, Rochester, NY, USA) in defined medium at 2×10^5 cells/chamber. Because neurons can be easily distinguished from glia under the microscope, 0.5% fetal bovine serum (Sigma), which can promote glial growth, was added to the medium to support neuronal viability in these low-density cultures. The cultures were re-fed with defined medium containing additives plus 0.25% fetal bovine serum (FBS) on the ninth day *in vitro*.

Immunoblots

Primary rat striatal neurons were harvested in hot sample buffer [62.5 mM Tris–HCl, pH 6.8, 3% sodium dodecyl sulfate (SDS), 200 mM dithiothreitol (DTT), 10% glycerol, 0.025% bromophenol blue] 15 min after treatment, sonicated for 10 s, centrifuged for 10 min at 16 000 *g*, and supernatants were loaded onto polyacrylamide gels. For preparation of rat striatal samples, rats were injected intraperitoneally (i.p.) with amphetamine (4 mg/kg free base) and killed 20 min later. Striata were rapidly dissected and quickly frozen in liquid nitrogen. Frozen striata were weighed, taken up in hot sample buffer (0.1 mg/10 μL) and treated like the primary striatal neurons. Equal volumes of the lysates were size-separated on 12% polyacrylamide gels for Ser133 phospho-CREB and CREB immunoblots, or 8% gels for Ser897 phospho-NR1 immunoblots. Protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-p, 0.45 mM; Millipore, Bedford, MA, USA) and blocked in blocking buffer (5% non-fat dry milk in PBS, 0.1% Tween-20) for 1–2 h. The blots were incubated in primary antibody (1 : 2000 dilution anti-Ser133 phospho-CREB, 1 : 20 000 anti-CREB, 1 : 5000 anti NR1 or 1 : 9000 anti Ser897 phospho-NR1) for 2 h followed by six washes for 5–10 min in blocking buffer. Blots were incubated for 1 h in goat anti-rabbit horseradish peroxidase-linked IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1 : 3000 for Ser133 phospho-CREB and CREB, 1 : 5000 for NR1 and 1 : 10 000 for Ser897 phospho-NR1. Blots were washed five times for 5–10 min in 1 × PBST, developed with Western Lightning Chemiluminescence Reagent PLUS (Perkin Elmer, Boston, MA, USA), and exposed to autoradiographic film (Kodak, Rochester, NY, USA). Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA, USA) were used for protein size determination. Ser133 phospho-CREB and CREB bands were detected at 43 kDa and NR1 and phospho-NR1 bands at 120 kDa.

For immunoprecipitation (IP), animals were killed 20 min after injection (i.p) of drugs. Brains were quickly frozen, and striatal tissue was dissected in a cryostat from frozen sections. Tissue was weighed and disrupted by brief sonication in TEVP buffer (10 mM Tris pH 7.5, 50 mM NaF, 2 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 1 μM Microcystine) which contained 1% SDS. It was determined that protein represented 10% of tissue mass; this estimate was used to get a final concentration of 10 μg protein/μL. SDS was diluted to 0.1% with IP buffer (150 mM NaCl, 50 mM Na_3PO_4 pH 7.2, 1% Na deoxycholate, 2 mM EDTA, 1% Triton X-100, 50 mM NaF, 2 mM Na_3VO_4) and centrifuged for 3 min at 16 000 *g*. The supernantant was incubated with antiphosphoserine antibody (Sigma) according to company protocol, and rotated for 3 h at 4°C, followed by an incubation with protein A sepharose beads for 2–4 h at 4°C. Beads were washed with ice-cold IP buffer four times, suspended in an equal volume of 2× sample buffer and boiled for 5 min. Samples were centrifuged at 16 000 × *g* for 10 min and the supernatant used for immunoblotting.

Calcium phosphate transfections

Transfection of primary striatal neurons was performed as described previously (Xia *et al*. 1996) with minor modifications (Rajadhyaksha *et al*. 1999). The *3xCRE-luciferase* construct contains three CRE sites (TGACGTCA), fused to a minimal Rous sarcoma virus promoter (enhancerless) in the pA3Pluc vector (Maxwell *et al*. 1989), 5′ of a luciferase reporter gene. The *3xCRE-luciferase* construct was provided by Susan E. Lewis (Massachusetts General Hospital). The NR1 wild-type and NR1 S896/897A constructs were a gift from Richard L. Huganir (Johns Hopkins University, School of Medicine). The NR1 constructs are cloned into the pRK5 mammalian expression vector. NR1 S896/897A construct contains a serine to alanine mutation at residues 896 and 897 (Ehlers *et al*. 1995). The expression vector for the heat-stable inhibitor of the cAMP-dependent protein kinase A (PKI) was a gift from Dr Richard A. Maurer (Oregon Health Sciences University). The PKI gene is driven by the Rous sarcoma virus promoter in the pBR322 vector (Day *et al*. 1989). For all transfections, a total of 3 μg DNA was used per well of a 12-well plate. In cotransfections with the NR1 and kinase mutants, a construct or a control vector (β-galactosidase) was added in a fourfold molar excess over the *3xCRE-luciferase* for a total of 3 μg.

Luciferase assay

Luciferase activity was analyzed with the Promega Luciferase Assay kit (Promega, Madison, WI). Cells were lysed in lysis reagent (25 mM Tris–phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexame-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100) and luciferase activity was measured in a luminometer (Dynex Technologies, Chantilly, VA, USA).

Ca^{2+} imaging

All drug treatments and imaging were performed on days *in vitro* (DIV) 12 or DIV 13 with the cell-permeable Ca^{2+} indicator, fluo-3 acetoxymethyl (AM) ester (Molecular Probes, Inc., Eugene, OR, USA). Cultures were rinsed of serum with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM D-Glucose, 5 mM HEPES; pH 7.3) and loaded for 30 min with 2.5 μM fluo-3 AM stock, in Locke's buffer. The cells were washed twice with Sodium Locke's Buffer followed by a 15-min incubation to allow for ester hydrolysis.

All fluorescence recordings were made at 40× magnification using an inverted Leica TCS NT confocal microscope (Leica Microsystems, Bannockburn, IL, USA). Pharmacological compounds were added directly to the cells under the microscope in Locke's Buffer after several baseline frames were taken. A 488-nm argon laser, filtered at 530 ± 30 nm was used. The total imaging session consisted of 250 frames, collected with approximately 3.4 s between frames. Numerical data were acquired from the series using the Leica TCS NT software physiology package (Leica Microsystems). Data are presented as average fluorescence intensity in the soma of all identified neurons in the frame. Glia were occasionally found in the cultures but were easily identified and excluded from analysis.

In a series of preliminary experiments, dual imaging of Fluo-3 and Fura Red was compared to imaging of Fluo-3 alone (data not shown). Fura Red was used to control for photobleaching. Because no significant photobleaching was observed in our paradigm, neurons for the experiments shown were loaded with and imaged for Fluo-3 only.

6-OHDA lesions

Sprague–Dawley rats (Taconic Farms) were anaesthetized with isoflurane vapor, and 6-OHDA hydrobromide (5 mg/mL free base) was delivered to the medial forebrain bundle via two injections, 3.0 μL to AP −0.44; ML −0.13; DV −0.78; toothbar −0.24, and 2.5 μL to AP −0.40; ML −0.09; DV −0.80; toothbar + 0.34 (coordinates from bregma).

The animals were allowed to recover for 4–5 weeks before the administration of amphetamine (4 mg/kg, i.p.; Sigma) or apomorphine (0.05 mg/kg in 0.1% ascorbic acid, s.c., Sigma). A second, identical injection was administered 1–2 weeks after the first injection. At this time, rotations (ipsilateral for apomorphine, contralateral for amphetamine) were recorded for 15 min, rats were anesthetized, and perfused with 4% paraformaldehyde in 1 × PBS. At least 50 net rotations per 15 min was considered an adequate lesion.

Immunocytochemistry

Brain tissue was sectioned at 45 μm on a freezing microtome and immunocytochemistry was performed as previously described for phosphorylation-specific antibodies (Konradi *et al*. 1994). The dilution for primary antisera was 1 : 9000 for Ser897 NR1, 1 : 2500 for Ser133 CREB.

Statistical analyses

Blots were developed on a Kodak Imaging station 440, or on film. Autoradiographic films were scanned with the Microtek Scanmaker 4. Data were analyzed with one-way ANOVAS. The Tukey–Kramer HSD (honestly significant difference) was used to analyze differences between the groups. The JMP computer program (SAS Institute, Cary, NC) was used for data analysis.

Results

NMDA receptors and Ca^{2+} are involved in intraneuronal signal transduction by D1 receptors

Activation of primary striatal cultures with either dopamine (50 μM), or the D1 receptor agonists SKF 38393 (50 μM) and SKF 82958 (50 μM; not shown) induced phosphorylation of the transcription factor CREB (p-CREB; Figs 1a–c). This phosphorylation was blocked by the D1 antagonist, SCH23390 (not shown). The non-competitive NMDA antagonist MK801 (1 μM; Huettner and Bean 1988) brought CREB phosphorylation down to control levels. Because MK801 by itself reduced p-CREB levels to almost half of the control levels, a significant difference between MK801 treatment and dopamine or SKF 38393 with MK801 was also observed ($p <= 0.05$). However, a similar situation was observed with NMDA-mediated (50 μM) CREB phosphory-

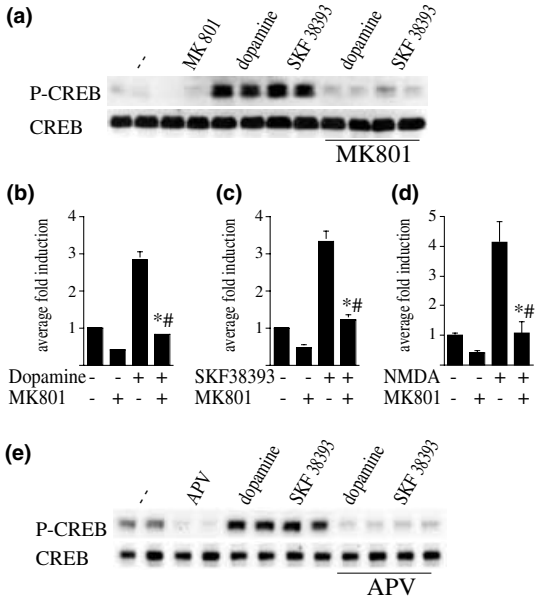

Fig. 1 D1 receptors mediate CREB phosphorylation in an NMDA receptor-dependent manner. (a) In primary striatal cultures, treatment with dopamine (50 μM) or SKF 38393 (50 μM) led to an increase of phosphorylation of the transcription factor CREB at Ser133 (upper panel). Pre-treatment with the NMDA antagonist MK801 (1 μM) blocked CREB phosphorylation. Total levels of CREB protein were not affected by the treatments (lower panel). (b–d) Average fold induction of Ser133-CREB levels ± SEM, and inhibition by MK801 after (b) dopamine, $n = 18$ (c) SKF 38393, $n = 10$, or (d) NMDA, $n = 9$. *$p = 0.001$ compared to agonist, #$p = 0.05$ compared to antagonist. (e) The non-competitive NMDA antagonist, APV (50 μM), blocked dopamine and SKF 38393-mediated CREB phosphorylation.

lation, i.e. MK801 brought NMDA receptor-mediated p-CREB levels down to control levels, while, by itself, it reduced p-CREB levels to 50% of control levels (Fig. 1d). Thus, MK801 blocked CREB phosphorylation mediated by the D1 receptor with comparable strength to CREB phosphorylation mediated by the NMDA receptor. Moreover, CREB phosphorylation by the D1 receptor was also blocked by the non-competitive NMDA antagonist, APV (50 μM) (Fig. 1e). The observation that MK801 does not fully prevent NMDA-mediated CREB phosphorylation may be explained by the fact that MK801 binds in the channel pore and requires the NMDA receptor to open before it can block (Huettner and Bean 1988). Thus, some ions may pass and activate the signal transduction pathway and CREB phosphorylation, before MK801 enters and blocks the pore. APV, on the other hand, competes with ambient glutamate for the NMDA receptor and has decreasing potency with increasing glutamate concentrations (not shown but see Konradi *et al*. 1996).

We also tested whether the residual CREB phosphorylation is due to the activation of alternative receptors and pathways such as AMPA/kainate receptors or the MAP kinase pathway. Because the glutamate receptor subunit *Glu*R1 is phosphorylated after stimulation of D1 receptors (Chao *et al*. 2002), and because kainate-evoked current amplitude was reportedly enhanced by D1 agonists (Price *et al*. 1999), we examined whether AMPA/kainate receptors contribute significantly to the D1 receptor-mediated signal transduction pathway in our experimental system. However, neither the AMPA/kainate receptor antagonist, GYKI 52466 (10–200 μM; Fig. 2a) nor DNQX (10–200 μM; not shown), significantly impaired D1 receptor-mediated CREB phosphorylation, although both blocked AMPA-mediated CREB phosphorylation (not shown, but see Rajadhyaksha *et al*. 1999). Similarly, while the MAP kinase pathway may be involved in dopamine- and glutamate receptor-mediated CREB phosphorylation in some experimental paradigms (Zhen *et al*. 1998; Choe and McGinty 2000, 2001), in our paradigm we did not find inhibition by the MAP kinase kinase (MEK) inhibitor PD 98059 (5–200 μM; Fig. 2b) or the MEK1/MEK2 inhibitor U0126 (1 μM-10 μM), in either dopamine or NMDA-mediated CREB phosphorylation (Fig. 2b). Both inhibitors blocked CREB phosphorylation in striatal culture by human recombinant brain-derived neurotropic factor (BDNF; not shown). These findings are in line with findings in the dopamine-intact striatum, where full D1 dopamine receptor agonists do not activate ERK1/2 MAP kinase although immediate-early genes are induced (Gerfen *et al*. 2002).

To further expand on the role of the NMDA receptor in dopamine-mediated CREB phosphorylation, we looked at the requirement for extracellular Ca^{2+}, as Ca^{2+} entering through the NMDA receptor may be an important part of the signal transduction cascade (MacDermott *et al*. 1986). Indeed, in Ca^{2+}-free medium, neither dopamine nor SKF 38393 were able to induce CREB phosphorylation at Ser133 (Figs 3a and b). These data support a role for Ca^{2+} and the NMDA receptor in dopamine-mediated CREB phosphorylation, and are in line with previously published reports (Konradi *et al*. 1996).

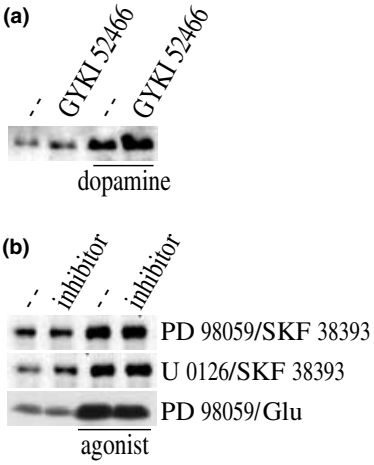

Fig. 2 Inhibition of AMPA/kainate receptors, and inhibition of the MAP kinase pathway does not affect D1 receptor-mediated CREB phosphorylation. (a) CREB phosphorylation by dopamine (50 μM) was not affected by the AMPA antagonist GYKI 52466 (50 μM). (b) The MEK inhibitor PD 98059 (20 μM) and the MEK1/2 inhibitor U0126 (1 μM) did not affect SKF 38393-mediated CREB phosphorylation. PD 98059 did also not affect glutamate-mediated CREB phosphorylation in primary culture. Specific inhibitors and agonists are shown to the right of each panel.

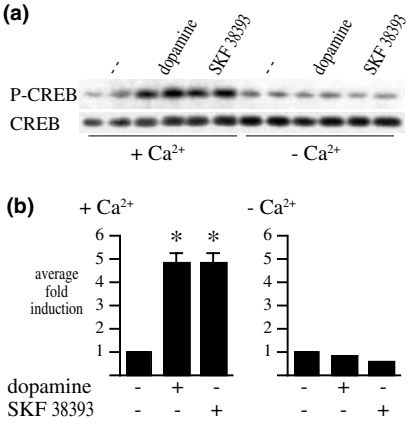

Fig. 3 Ca^{2+} is essential for D1 receptor-mediated signal transduction. (a) In Ca^{2+}-free defined salt solution neither dopamine (50 μM) nor SKF 38393 (50 μM) were able to induce CREB phosphorylation, whereas both agonists induced in defined salt solution with 1.8 mM Ca^{2+} chloride (upper panel). Total levels of CREB protein were not affected by the salt solutions (lower panel). (b) Average fold induction of CREB phosphorylation, ± SEM in the presence or absence of Ca^{2+}, of $n = 6$ samples.

Activation of D1 receptors leads to phosphorylation of the NR1 subunit of the NMDA receptor, at Ser897

A possible link between the dopamine D1 signal transduction pathway and the NMDA signal transduction pathway is phosphorylation of the NMDA receptor. It is known that stimulation of D1 receptors activates PKA (Monsma *et al*. 1990), and that PKA phosphorylation can enhance currents through NMDA receptors (Westphal *et al*. 1999). Of the various NMDA receptor subunits, NR1 is the only one known to have a PKA phosphorylation site, Ser897 (Tingley *et al*. 1997; Fig. 4a). Activation of D1 receptors in primary striatal cultures caused phosphorylation of Ser897, while total levels of NR1 protein were unaffected (Fig. 4b). Activation of the PKA pathway with the adenylate cyclase activator forskolin (Seamon *et al*. 1981) led to Ser897-NR1 phosphorylation in the same cultures (Fig. 4c). PKA-dependence was further demonstrated with a PKA inhibitor, H89. Inhibition of PKA activity with H89 (20 μM) attenuated phosphorylation of Ser897 by the D1 receptor pathway (Fig. 4d) to the same extent it attenuated forskolin-mediated (2.5 μM) Ser897-NR1 phosphorylation (Fig. 4e).

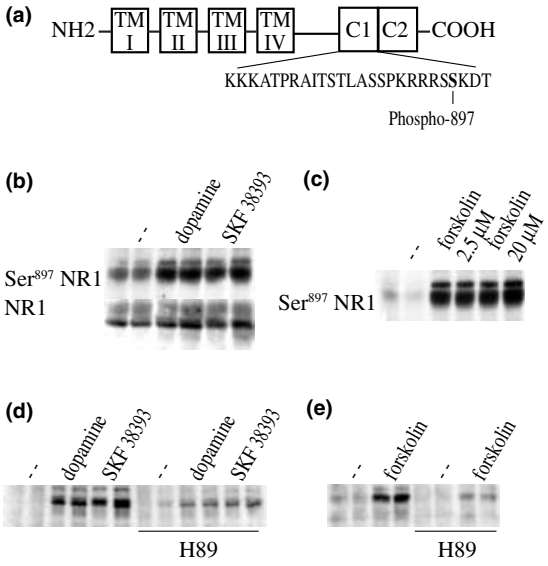

Fig. 4 Activation of the dopamine D1 receptor second messenger pathway, or of PKA, leads to the phosphorylation of Ser897 of the NMDA receptor subunit, NR1. (a) Schematic representation of the NR1 subunit. Downstream of the fourth transmembrane domain (TMIV), the NR1 subunit has two amino acid stretches that can be alternatively spliced, C1 and C2 (Sugihara *et al*. 1992; Hollmann *et al*. 1993). The C1 cassette has a variety of serine residues that are phosphorylated under stimulation (Tingley *et al*. 1997). Only Ser897 is phosphorylated by PKA. (b) With a phosphorylation-specific antiserum, an increase in phospho-Ser897 is shown after treatment of primary striatal cultures with dopamine (50 μM) or SKF 38393 (50 μM). Levels of NR1 protein are unchanged (lower panel). (c) Forskolin at 2.5 and 20 μM also increases phospho-Ser897 in primary striatal cultures. Representative experiments from $n = 6$ (for dopamine or SKF 38393), $n = 20$ (forskolin 2.5 μM and forskolin 20 μM) are shown. (d) Dopamine-induced (50 μM) and SKF 38393-induced (50 μM) phosphorylation of Ser897-NR1 is attenuated by the PKA inhibitor H89 (20 μM) to the same extent as forskolin-mediated NR1-phosphorylation is mediated (e).

Both PKA and Ca^{2+}/CamK are required for D1 receptor-mediated signal transduction to the CRE

Whereas activation of D1 receptors stimulates PKA activity (Monsma *et al*. 1990), activation of NMDA receptors causes Na^{+} and Ca^{2+} influx (MacDermott *et al*. 1986) and stimulation of CamK activity (Fukunaga *et al*. 1992). Because the Ser133 phosphorylation site of CREB is a PKA and CamK consensus site (Lonze and Ginty 2002), we wanted to know if PKA or CamK are required for D1 receptor-mediated CREB phosphorylation. The PKA inhibitor H89 (20 μM) blocked dopamine- and SKF 38393-mediated CREB phosphorylation (Figs 5a–d). H89 was also used at 10 μM (not shown), but was inconsistent in blocking D1 receptor-mediated – or forskolin (2.5 μM)-mediated CREB phosphorylation (Fig. 5e). The involvement of PKA in D1 receptor-mediated gene expression was further verified in a co-transfection assay of a luciferase construct under the control of three CRE sites (*CRE-luciferase* construct) with the dominant-negative PKA construct, PKI (Day *et al*. 1989). Consistent with the CREB phosphorylation results, a two to threefold increase in luciferase activity induced by dopamine or SKF 38393 was blocked by PKI (Fig. 5f). However, if PKA, stimulated by D1 receptors, directly phosphorylates CREB, CamK, which play a role in NMDA receptor-mediated plasticity (Soderling 2000) should be superfluous. We investigated the role of CamK in D1 receptor-mediated CREB phosphorylation by incubating with the CamKe II and IV inhibitor KN62. KN62 (30 μM) significantly reduced dopamine-mediated (Figs 6a and c) and SKF 38393-mediated (Figs 6b and d) CREB phosphorylation. KN62, at 10 μM, had an inconsistent effect on NMDA-mediated (50 μM) CREB phosphorylation (Fig. 6e), but reliably blocked at 30 μM NMDA- and D1 receptor-mediated CREB phosphorylation in a comparable fashion (Figs 6a, b and f). This finding is in line with *in vivo* studies in rat striatum, where amphetamine-mediated CREB phosphorylation was blocked by KN62 (Choe and Wang 2002). We therefore hypothesized that PKA activity is important to activate NMDA receptors, whereas CamK is actually involved in CREB phosphorylation.

Activation of D1 receptors, or activation of PKA, facilitates glutamate-mediated Ca^{2+} influx into striatal neurons

The putative mechanism by which PKA-mediated phosphorylation of the NMDA receptor leads to CREB phosphorylation and gene transcription is a facilitation of NMDA receptor activity. In order to test this hypothesis, we

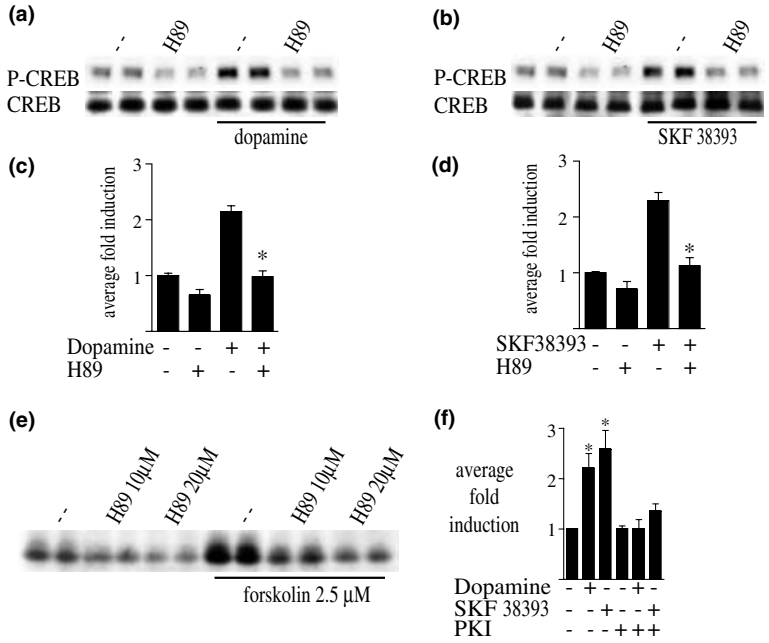

Fig. 5 PKA is necessary for D1 receptor-mediated CREB phosphorylation. (a) Dopamine-mediated (50 μM) CREB phosphorylation is blocked by the PKA inhibitor H89 (20 μM). (b) CREB phosphorylation by the D1 agonist SKF 38393 is also blocked by H89 (20 μM). (c and d) Average fold induction ± SEM of $n = 28$ samples for dopamine (c), $n = 14$ samples for SKF 38393 (d), $^*p = 0.01$ compared to agonist. (e) Choice of concentration for H89: CREB phosphorylation by forskolin (2.5 μM) was partially blocked by 10 μM H89, but consistently blocked by 20 μM H89. (f) Primary striatal cultures were transfected with a luciferase construct under the control of three CRE promoter sites. Dopamine-induced (50 μM) and SKF 38393-induced (50 μM) CRE activity two to threefold, as shown by the induction of luciferase activity. Co-transfection with the dominant negative PKA inhibitor, PKI, blocked the activation of the luciferase construct. Average ± SEM of $n = 12$ for dopamine, $n = 18$ for SKF 38393. PKI transfected neurons treated with dopamine or SKF 38393 had the same levels of luciferase activity than untreated neurons transfected with PKI. $^*p = 0.01$ compared to control, dopamine + PKI, or SKF 38393 + PKI (f).

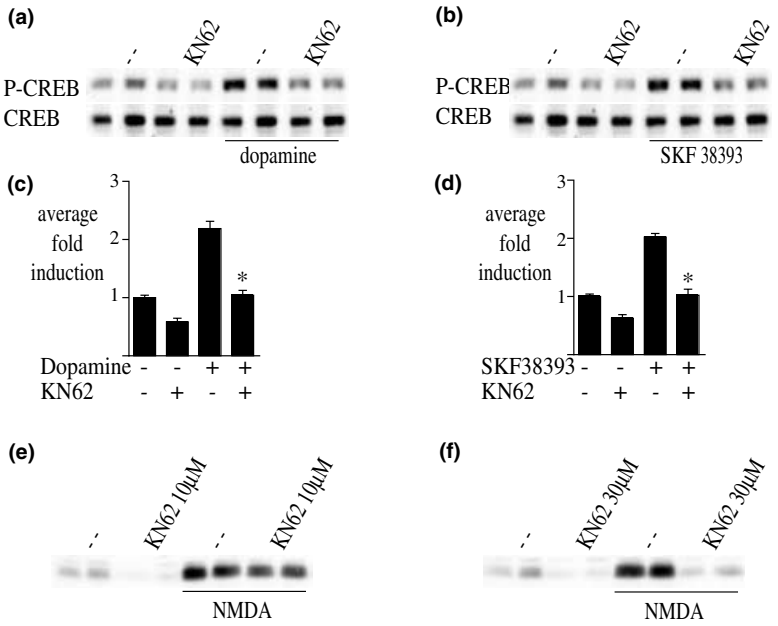

Fig. 6 CamK is necessary for D1 receptor-mediated CREB phosphorylation. (a) Dopamine-mediated (50 μM) CREB phosphorylation is blocked by the CamK inhibitor, KN62 (30 μM). (b) CREB phosphorylation by the D1 agonist SKF 38393 is also blocked by KN62 (30 μM). (c and d) Average fold induction ± SEM of $n = 14$ samples for dopamine (c), $n = 12$ samples for SKF 38393 (d), $^*p = 0.01$ compared to agonist. (e and f) Choice of concentration for KN62: CREB phosphorylation by NMDA (50 μM) was partially blocked by 10 μM KN62, but consistently blocked by 30 μM KN62.

used Ca^{2+} imaging and confocal microscopy of primary striatal neurons. SKF 38393 alone did not induce Ca^{2+} influx into striatal neurons in glutamate-free medium (Fig. 7a, open diamonds; SKF 38393 was added at 'drug 1' arrow). Glutamate, in contrast, had a profound effect on Ca^{2+} influs (Fig. 7a, filled squares; glutamate was added at 'drug 1' arrow). In line with our hypothesis, SKF 38393 *was* able to induce Ca^{2+} influs when glutamate was present in the medium (Fig. 7a, filled squares; glutamate was added at 'drug 1' arrow, SKF 38393 was added at 'drug 2' arrow 3 min after glutamate). All Ca^{2+} imaging esperiments were carried out in the presence of 2 μM TTX, to avoid spreading depolarization and effects due to neurotransmitter release.

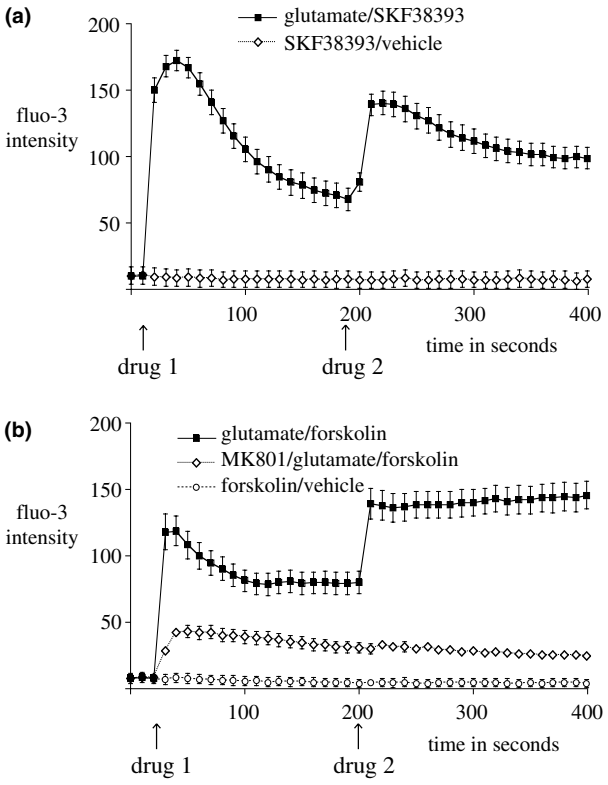

Fig. 7 Activation of D1 receptors or of PKA potentiates Ca^{2+} influx via NMDA receptors. Ca^{2+} influx into neurons was measured with Fluo-3 with a confocal microscope. (a) ■: glutamate (10 μM) was added at the 'drug 1' arrow, SKF 38393 (50 μM) was added at the 'drug 2' arrow. ◇: SKF 38393 (50 μM) was added at the 'drug 1' arrow, medium was added at the 'drug 2' arrow. (b) ■: glutamate (10 μM) was added at the 'drug 1' arrow, forskolin (10 μM) was added at the 'drug 2' arrow. ○: Forskolin (10 μM) was added at the 'drug 1' arrow, medium was added at the 'drug 2' arrow. ◇: Cells were pre-incubated with MK801 (2 μM) for 30 min before start of the imaging and during imaging. Glutamate (10 μM) was added at the 'drug 1' arrow, forskolin (10 μM) was added at the 'drug 2' arrow.Fluo-3 intensity of all neurons within the field is shown. Average ± SEM of $n = 11$–17 neurons. Approximately 20–22 measurements were taken per minute, for a total of 15 min. For better clarity of the graph, every 10-second data point is shown. No significant changes were observed after 400 s. All experiments were performed in the presence of 2 μM TTX. Experiments were repeated two to six times in independent dissections.

The PKA activator, forskolin, induced a pattern of Ca^{2+} influx similar to the D1 agonist. By itself, forskolin had no effect on Ca^{2+} influx into neurons (Fig. 7b, open circles; forskolin was added at 'drug 1' arrow). However, if given after glutamate, forskolin was able to potentiate Ca^{2+} influx (Fig. 7b, filled squares; glutamate was added at 'drug 1' arrow, forskolin was added at 'drug 2' arrow 3 min after glutamate). Pre-treatment of the neurons with the NMDA antagonist, MK801, attenuated the glutamate-mediated Ca^{2+} influx, and blocked the effect of forskolin (Fig. 7b, open diamonds, glutamate was added at 'drug 1' arrow, forskolin was added at 'drug 2' arrow).

These results are consistent with the hypothesis that stimulation of the dopamine signaling pathway enhances the response of NMDA receptors to tonic levels of glutamate stimulation. However, changes in fluorescence intensity were measured at the soma rather than the synapse. While we do not have direct measurements of Ca^{2+} at the synapse, the sensitivity of these effects to MK-801 suggest that the Ca^{2+} signal is initiated at the sites where NMDA receptors are located, i.e. the synapse. The detection of somatic changes in intracellular Ca^{2+} confirms the propagation of the signal to the soma and identifies Ca^{2+} as a putative second-messenger. This type of analysis is similar to that of other investigators who examined Ca^{2+} signaling and gene expression (e.g. Dolmetsch *et al*. 2001).

Mutation of Ser897 to alanine on the NR1 subunit disrupts the D1 signal transduction pathway

We have shown that the phosphorylation of Ser897 is correlated with SKF-, DA- and forskolin-mediated gene expression and with increased Ca^{2+} influx, and we have hypothesized that PKA, stimulated by D1 receptors, recruits the NMDA receptor signal transduction pathway rather than directly phosphorylating CREB in striatal neurons. If Ser897 phosphorylation of NR1 is necessary for gene expression, a point mutation of Ser897 should be sufficient to disrupt signaling. The functional importance of the Ser897 NR1 phosphorylation site for the D1 signal transduction pathway was assessed in a co-transfection assay in primary striatal culture. In this assay, the *CRE-luciferase* construct was co-transfected with either the wild-type NR1 construct (*NR1wt*), or an NR1 construct in which the two neighboring serines, Ser896 and Ser897 (of which only Ser897 is a consensus PKA phosphorylation site, Tingley *et al*. 1997), were mutated to alanine (Fig. 8a; NR1S896/897A). Activity of the *CRE-luciferase* construct was induced twofold after treatment with SKF 38393 (Fig. 8b). Co-transfection with the wildtype NR1 construct significantly increased the ability of SKF 38393 to transactivate the CRE promoter (Fig. 8b, fourfold induction of luciferase activity). Co-transfection

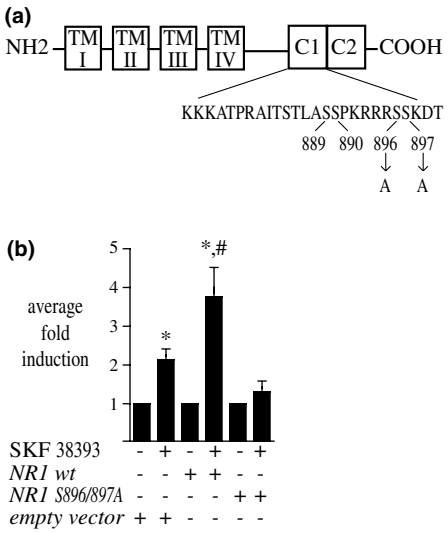

Fig. 8 Phosphorylation of Ser897 is necessary for D1 receptor-mediated gene expression. (a) In the *NR1 S896/897A* mutant construct, two adjacent serines in the C1 exon, Ser896 and Ser897, were changed to alanine, whereas Ser889 and Ser890 were left unchanged. (b) Striatal cultures were cotransfected with the *3*×CRE-luciferase construct and control vector, NR1 wild-type (*NR1wt*) or *NR1 S896/897A*. Treatment with SKF 38393 induced luciferase activity twofold, which was enhanced to fourfold in the presence of excess *NR1wt*. The S896/897A mutant did not permit luciferase gene expression after D1 receptor stimulation. The average fold induction of luciferase activity (± SEM) over control levels is shown. Asterisks indicate statistically significant differences from untreated cells transfected with a control vector or *NR1wt*, respectively. Hatchmark (#) indicates significant difference to SKF 38393/control vector cultures ($n = 9$).

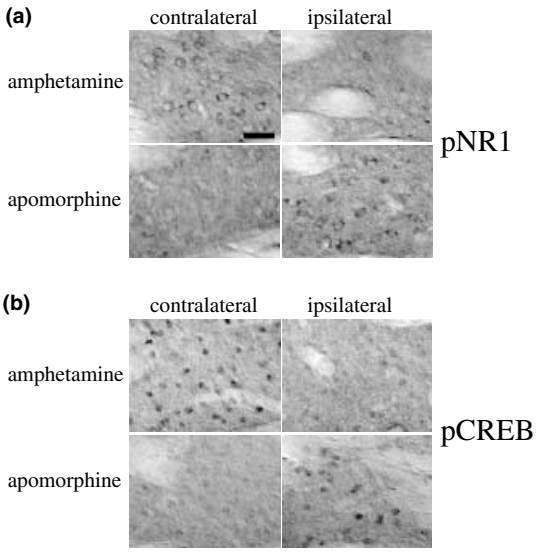

Fig. 9 *In vivo* induction of Ser897-NR1 phosphorylation and Ser133-CREB phosphorylation in 6-OHDA-lesioned rats. 6-OHDA-lesioned rats were injected with amphetamine or apomorphine. Immunohistochemistry was performed with the Ser897-NR1 antiserum (a), or the Ser133-CREB antiserum (b). (a) Amphetamine (4 mg/kg, i.p), increases Ser897-NR1 on the contralateral side, whereas apomorphine (0.05 mg/kg, s.c) increases Ser897-NR1 on the ipsilateral side. (b) Immunohistochemistry was performed with the Ser133-CREB antiserum in consecutive slices. A similar pattern as in (a) was observed. A representative experiment for $n = 3$ for each treatment is shown. Scale bar = 100 μM.

with the mutated NR1 blocked the ability of SKF 38393 to induce the *CRE-luciferase* construct (Fig. 8b). These findings demonstrate that (a) PKA stimulated by the D1 receptor cannot activate CREB independently of the Ser897 NR1 site, that (b) Ser897 is necessary for recruitment of NMDA-mediated signaling and (c) suggest that NR1S896/897 A has a dominant negative effect. *In vivo* stimulation of dopamine receptors leads to phosphorylation of NR1 at Ser897.

In order to investigate if dopamine receptor-activation *in vivo* leads to Ser897-NR1 phosphorylation, we used a phosphorylation-specific antiserum. We have found that phosphorylation states are difficult to maintain and are extremely sensitive to minute experimental variations. Therefore, we initially used a paradigm in which, within the same experimental animal, changes in striatal neurons in one brain hemisphere could be compared to a baseline provided by striatal neurons in the other hemisphere. Unilaterally 6-OHDA lesioned rats were treated either with amphetamine or with apomorphine (Fig. 9). Phosphorylation of Ser897-NR1 on the side contralateral to the lesion was increased after amphetamine treatment, due to the release of dopamine from the intact terminals (Fig. 9a). Apomorphine treatment increased phosphorylation on the ipsilateral side, due to the development of supersensitivity of dopamine receptors after the loss of pre-synaptic dopamine release (Thornburg and Moore 1975; Fig. 9a). Phosphorylation of CREB on Ser133 followed a similar pattern (Fig. 9b), confirming the correlation between Ser897-NR1 induction and Ser133-CREB phosphorylation established with the *in vitro* experiments.

In a second set of experiments, rats were acutely injected with amphetamine and compared to rats injected with saline (Fig. 10). Treatment with amphetamine increased staining of neurons with the Ser897-NR1 antiserum (Fig. 10a). In immunoblots of striatal protein extracts a significant increase of three bands at and below the 128 kDa marker was observed in amphetamine-treated rats (Fig. 10b). When immunoprecipitation (IP) was performed with a phosphoserine antibody, and the blot was developed with an NR1 antiserum, the IP precipitate showed a predominant band at the expected site, close to the 128 kDa marker (Fig. 10c, P), confirming the specificity of the antibody. Again, the intensity of this band was increased in amphetamine-treated rats (Fig. 10d).

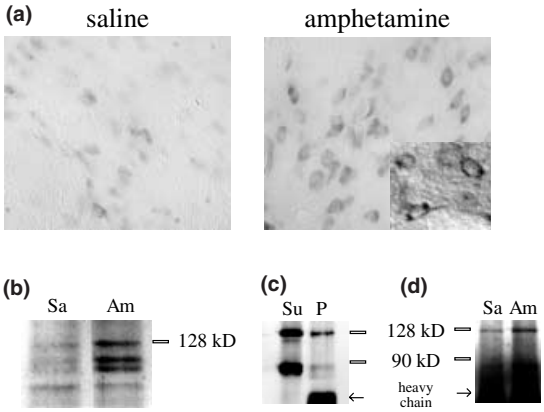

Fig. 10 *In vivo* induction of Ser897-NR1 phosphorylation in amphetamine-treated rats. Intact rats were injected i.p. with either amphetamine (4 mg/kg) or saline. (a) Immunohistochemistry shows an induction of Ser897-NR1 after treatment with amphetamine (right panel). Insert: Staining of the pericarya, but not of the nucleus is observed, $n = 4$. (b) In immunoblots, amphetamine-treated rats showed an increase in three bands around and below the 128 kDa marker, $n = 3$. (c) Immunoprecipitation (IP) was performed with a phospo-serine specific antibody, and blot was developed with an NR1 antiserum. A specific band was observed slightly below the 128 kDa marker. Non–specific interaction was observed with the heavy chain of the antiserum used for IP. Su, supernatant of the IP reaction; p, pellet. (d) IP reaction was performed with saline-treated (Sa) and amphetamine-treated (Am) rats. The band below the 128 kDa marker was induced in the amphetamine-treated rats ($n = 3$).

Discussion

The dopamine D1 signal transduction pathway, activation of the transcription factor CREB, and dopamine-mediated gene expression are critically involved in drug addiction (Nestler 2001). Interruption of this pathway can interfere with behaviors associated with drug addiction (Carlezon *et al*. 1998; Pliakas *et al*. 2001) and can potentially be used for intervention. It is therefore important to identify the elements that link the activation of dopamine receptors at the synapse to the phosphorylation of CREB in the nucleus. We have shown previously that inhibition of NMDA receptors blocks the second messenger pathway of D1 dopamine receptors (Konradi *et al*. 1996). We show here that dopamine recruits the NMDA receptor second messenger pathway to its own second messenger pathway by causing phosphorylation of Ser897 of the NR1 subunit. This phosphorylation correlates with nuclear CREB phosphorylation *ex vivo* in primary striatal neurons after treatment with dopamine, and *in vivo* in the rat striatum after i.p. administration of amphetamine or apomorphine. In many of the experiments described, transcription assays were performed that showed a correlation between CREB phosphorylation and CRE-mediated gene expression (some data not shown). Whereas it has been demonstrated in heterologous systems that CREB phosphorylation is necessary, but not sufficient, for gene transcription through the CRE enhancer element (Hu *et al*. 1999), we have not found evidence for a dissociation between CREB phosphorylation and CRE-mediated transcription in striatal neurons.

We also present evidence that phosphorylation of NR1 at Ser897 is necessary for D1 receptor-mediated gene transcription. Mutation of the Ser897-NR1 phosphorylation site blocks dopamine-mediated induction of gene transcription from the CRE enhancer in primary cultures of striatal neurons. Recent findings in striatal slices suggest that the D1 receptor regulates NMDA receptor sensitivity via DARPP-32, a potent inhibitor of protein phosphatase-1 (Snyder *et al*. 1998; Maldve *et al*. 2002). Our findings are consistent with these results, although we would postulate that the primary phosphorylation event of NR1 is accomplished by PKA, and DARPP-32 is recruited to stabilize this phosphorylation.

How does phosphorylation of Ser897–NR1 enhance NMDA receptor activity and facilitate gene transcription? The findings in neuronal culture suggest that phosphorylation increases the sensitivity of the NMDA receptor to glutamate. This hypothesis is in agreement with reports that dopamine receptor agonists or stimulation of PKA enhance NMDA currents (Cepeda *et al*. 1998; Westphal *et al*. 1999; Centonze *et al*. 2001), and we provide here the first molecular evidence for the mechanism of this potentiation. The Ca^{2+} imaging studies presented here show increased Ca^{2+} influx through the NMDA receptor when D1 receptors or PKA are activated, even though glutamate levels remain unchanged. The changes in intracellular Ca^{2+} concentration, though induced by glutamate, are consistent with NMDA currents because (1) the NMDA antagonist MK801 blocked Ca^{2+} influx, (2) NMDA receptors are the only glutamate receptors in the striatum with significant permeability to Ca^{2+} (Götz *et al*. 1997), and (3) AMPA receptors are rapidly desensitizing in the presence of glutamate and are unlikely to be active on the timescales examined (Mayer and Westbrook 1987).

A variety of mechanisms have been described by which another protein kinase, protein kinase C (PKC), can increase the sensitivity of the NMDA receptor. Among these are the incorporation of more NR1 subunits into the synapse (Scott *et al*. 2001), and the interference with the Mg^{2+} block (Chen and Huang 1992). PKA could have similar effects to PKC (Tingley *et al*. 1993; Tingley *et al*. 1997). For example, PKA could induce the incorporation of newly synthesized NMDA receptors from the endoplasmic reticulum (ER) into the membrane, although the timecourse observed with PKC, 2–3 h after activation (Scott *et al*. 2001), would be at odds with the rapid timecourse observed with PKA in our paradigm. A more plausible explanation for the potentiation of the NMDA receptor by PKA is a reduction of the voltage-dependent Mg^{2+} block and an increased probability of

channel openings, such as has been observed with PKC (Chen and Huang 1992).

In many experimental systems PKA can directly phosphorylate Ser133 CREB (Gonzalez and Montminy 1989; Lonze and Ginty 2002). However, neurons seem to be an exception, which may be a reflection of their unique elongated shape and their highly evolved signal transduction machinery. Although PKA is activated at the synapse, it may not be capable of traveling to the nucleus in order to phosphorylate CREB, but instead recruit better suited signal transduction molecules. Dopamine receptors are located on distal dendrites (Smith and Bolam 1990), at a seemingly prohibitive distance for diffusion or transport of activated PKA to the nucleus, without PKA being inactivated along the way. Moreover, the regulatory subunit of PKA may be largely kept in place by anchoring proteins, which assemble in large, macromolecular complexes at the synapse (Dell'Acqua and Scott 1997; Fraser and Scott 1999). Some of these complexes anchor PKA to the NMDA receptor (Westphal *et al*. 1999; Colledge *et al*. 2000), and D1 receptors are in physical proximity to NMDA receptors (Fiorentini *et al*. 2003). Thus, synaptic PKA and NMDA receptors might be involved in CREB phosphorylation by D1 receptors. A difference in the effect of synaptic PKA and PKA in the cell soma is supported by data in neurons which show that CREB phosphorylation is independent of NMDA receptors only when cells are stimulated with agents that can powerfully activate PKA, such as forskolin at high concentrations (20 μM; Rajadhyaksha *et al*. 1998).

We provide here evidence for an involvement of Ca^{2+} and CamK in the signal transduction pathway from D1 receptor activation to CREB phosphorylation. Although CREB phosphorylation by Ca^{2+} has been shown to involve either CamK or the Ras/ERK pathway (Lonze and Ginty 2002), interruption of the Ras/ERK pathway by MEK inhibitors was of no consequence to D1 receptor-mediated CREB phosphorylation. Therefore, we postulate a D1 signal transduction pathway that involves the activation of PKA, phosphorylation of NR1, recruitment of the NMDA receptor, influx of Ca^{2+}, activation of CamK and phosphorylation of CREB.

There is evidence for interactions between dopaminergic and glutamatergic second messenger pathways in behaviors ranging from drug addiction to long-term plasticity. Currently, the molecular mechanisms that mediate this interaction are unknown. The data presented here demonstrate a specific mechanism of interaction between D1 receptors and NMDA receptors in gene regulation in the striatum. We postulate that the recruitment of NMDA receptors and their associated signaling pathways via phosphorylation provides a general intracellular mechanism by which neuromodulatory inputs interact with glutamatergic signaling in neurons. This form of heterosynaptic plasticity is relevant not only to drug addiction (Nestler 2001) but also to the molecular mechanisms of long-term memory (Bailey *et al*. 2000).

Acknowledgements

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