Development/Plasticity/Repair

Acute and Chronic Dopamine Receptor Stimulation Modulates AMPA Receptor Trafficking in Nucleus Accumbens Neurons Cocultured with Prefrontal Cortex Neurons

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Postsynaptic interactions between dopamine (DA) and glutamate receptors in the nucleus accumbens (NAc) are critical for addiction. To determine the effect of acute and repeated DA receptor stimulation on AMPA receptor (AMPAR) synaptic targeting in medium spiny NAc neurons, we developed a model system consisting of rat NAc neurons cocultured with prefrontal cortex neurons from enhanced green fluorescent protein-expressing mice. Cortical neurons restore excitatory input onto NAc neurons but are distinguishable based on fluorescence. First, we showed that brief D_1-like agonist exposure increased AMPAR insertion onto extrasynaptic regions of NAc neuronal processes through a mechanism requiring protein kinase A. This facilitated the Ca^{2+}/calmodulin dependent protein kinase II (CaMKII)-dependent synaptic incorporation of AMPARs in response to subsequent NMDA receptor (NMDAR) stimulation. Through this mechanism, DA may promote reward- and drug-related plasticity in the NAc. Then, to model effects of repeated *in vivo* cocaine exposure, we treated cocultures with DA (1 μM, 30 min) on days 7, 9, and 11 in culture. On day 15, NAc neurons exhibited increased synaptic AMPAR levels. This was associated with CaMKII activation and was blocked by the CaMKII inhibitor KN-93 (*N*-[2-[*N*-(4-chlorocinnamyl)-*N*-methylaminomethyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt). Furthermore, D_1-like agonist exposure on day 15 no longer increased AMPAR surface expression. This refractoriness was associated with decreased D_1 receptor surface expression. NMDAR surface expression was not altered by acute or repeated DA receptor stimulation. These results suggest that (1) after repeated DA treatment, NAc neurons are more responsive to glutamate inputs but D_1-like receptor regulation of plasticity is impaired, and (2) NAc/prefrontal cortex cocultures are useful for studying dopamine-induced neuroadaptations.

Key words: D_1 receptor; CaMKII; GluR1; LTP; plasticity; protein kinase A

Introduction

Repeated exposure to psychomotor stimulants can produce persistent changes in drug-seeking behavior in rodents that resemble those associated with human addiction (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004; Bossert et al., 2005). This appears to involve glutamate-dependent plasticity in neuronal circuits that mediate drug seeking and craving (Wolf et al., 2004; Kauer and Malenka, 2007). The goal of this study was to investigate the effects of acute and chronic dopamine (DA) receptor stimulation on synaptic trafficking of AMPA receptors (AMPARs) in neurons of the rat nucleus accumbens (NAc). Activity-dependent regulation of AMPAR levels in excitatory synapses is a critical mechanism for changing synaptic strength during long-term potentiation (LTP), long-term depression, and synaptic scaling (Malinow and Malenka, 2002; Turrigiano and Nelson, 2004).

The NAc plays a central role in neuronal circuits that mediate motivated behaviors related to natural rewards and drugs of abuse (Kelley, 2004). Neurons in the NAc consist of two major classes: GABAergic medium spiny neurons (90%), the projection neurons of the NAc, and at least four populations of interneurons (10%) (Meredith and Totterdell, 1999). The importance of medium spiny neurons in generating motivated behaviors reflects their role as an interface between limbic and motor systems. Medium spiny neurons receive inputs related to motivational state from DA neurons originating in the ventral mesencephalon and from glutamate neurons originating in the prefrontal cortex (PFC) and limbic regions such as the hippocampus and amygdala. They send projections to the ventral pallidum and ventral mesencephalon, regions involved in motor execution of motivated behaviors (Mogenson et al., 1987; Groenewegen et al., 1999; Kelley, 1999).

DA and glutamate inputs converge on common spines of medium spiny neurons (Jay, 2003; Sesack et al., 2003), and postsyn-

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aptic interactions between these transmitter systems are critical for the effects of psychomotor stimulants (Wolf, 1998; Schmidt et al., 2005). We have conducted a series of studies to test the hypothesis that DA receptors, by activating signaling pathways that modulate AMPAR trafficking, influence plasticity at adjacent glutamate synapses on medium spiny neurons. Using postnatal NAc cultures, we found that brief incubation with a D_1-like agonist increased phosphorylation of the AMPAR subunit glutamate receptor subtype 1 (GluR1) at the protein kinase A (PKA) site (Chao et al., 2002a) and also increased GluR1 insertion onto the cell surface through a PKA-dependent mechanism (Chao et al., 2002b; Mangiavacchi and Wolf, 2004). However, we were unable to examine the consequences of D_1-like receptor stimulation for synaptic targeting of AMPARs because NAc cultures do not contain glutamate neurons and thus do not contain glutamate synapses.

Here we describe the establishment of NAc/PFC cocultures, in which excitatory inputs to NAc neurons are restored by PFC neurons. Using NAc/PFC cocultures, we first investigated the facilitating effect of acute D_1-like receptor stimulation and PKA activation on synaptic AMPAR trafficking in NAc neurons. Then, to explore possible consequences of repeated exposure to DA-releasing psychomotor stimulants, we studied the effect of repeated DA treatment on AMPAR synaptic expression, signaling pathways involved in AMPAR trafficking, and the ability of D_1-like receptors to modulate AMPAR trafficking.

Materials and Methods

Animals. All animal use procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the Rosalind Franklin University of Medicine and Science. Pregnant Sprague Dawley rats (Harlan, Indianapolis, IN; Zivic Miller, Pittsburgh, PA), obtained at 18–20 d of gestation, were housed individually in breeding cages. One-day-old offspring were decapitated and used to obtain NAc neurons. PFC cells were obtained from enhanced green fluorescent protein (EGFP)-expressing mice [strain: C57BL/6-TgN(ACTbEGFP)1Osb; The Jackson Laboratory, Bar Harbor, ME]. The EGFP transgenic mouse strain was maintained by mating a male hemizygous carrier with a female C57BL/6J mouse. The EGFP-expressing offspring were identified under a fluorescence microscope on postnatal day 1 and decapitated to obtain cells from the prefrontal cortex. In some experiments, PFC cells were obtained from enhanced cyan fluorescent protein (ECFP)-expressing mice [strain: B6.129(ICR)-Tg(ACTB-ECFP)1Nagy/J; The Jackson Laboratory]. The ECFP transgenic mouse strain was maintained by mating homozygous ECFP male and female mice. All offspring express ECFP.

Postnatal NAc/PFC cocultures. The NAc of postnatal day 1 rats was removed, dissociated with papain (20–25 U/ml; Worthington Biochemical, Lakewood, NJ) at 37°C, and plated at a density of 30 000 cells per well onto coverslips coated with poly-D-lysine (100 μg/ml; Sigma, St. Louis, MO) in 24-well culture plates as described previously (Mangiavacchi and Wolf, 2004). The medial PFC of postnatal day 1 EGFP mice was isolated and dissociated with papain (20–25 U/ml) as described previously for rat PFC (Sun et al., 2005). PFC cells were plated at a density of 20,000 cells per well with the NAc cells described above. NAc/PFC cocultures were grown in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM GlutaMAX, 0.5% Gentamicin, and 2% B27 (Invitrogen). One-half of the medium was replaced with this Neurobasal growth medium every 4 d. Cultures were used for experiments between weeks 2 and 3.

In developing this coculture system, we needed to add PFC neurons in sufficient number to restore glutamate input to NAc neurons while at the same time maintaining a cell density sufficiently low to allow image analysis of single neurons. To achieve this, preliminary studies were conducted in which we plated different ratios of PFC neurons (fluorescent cells) to NAc neurons (nonfluorescent cells), as determined by cell counting before plating, and investigated the cells after 2 weeks *in vitro*. When 100,000, 80,000, 60,000, or 40,000 PFC neurons were plated with 30,000 NAc neurons, cortical innervation was high but it was difficult to conduct quantitative image analysis because many cells were confluent and the processes appeared as a dense network. When 10,000 PFC neurons were plated with NAc neurons, analysis of single medium spiny neurons was possible but cortical innervation was low. We found that 20,000 or 30,000 PFC neurons plated with 30,000 NAc neurons yielded the optimal results. The cell density was optimal for image analysis, and many medium spiny neurons were in close proximity to PFC neurons (Fig. 1*A–C*).

Immunocytochemistry. Cell surface GluR1 was labeled by incubating live cultures with antibody recognizing the extracellular N-terminal domain of GluR1 (amino acids 271–285; RTSDSRDHTRVDWKR; 1:15; Calbiochem, San Diego, CA) in Neurobasal media for 30 min at room temperature. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min, blocked with 5% donkey serum in PBS for 60 min, and incubated with donkey anti-rabbit secondary antibody conjugated to cyanine 3 (Cy3) (1:500; Jackson ImmunoResearch, West Grove, PA) for 60 min under nonpermeant conditions. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 15 min, blocked with 5% goat serum in PBS for 60 min, and incubated with monoclonal antibody to the synaptic marker synaptobrevin (SB)/vesicle-associated membrane protein 2 (1:2000; overnight at 4°C; Synaptic Systems, Goettingen, Germany) followed by goat anti-mouse secondary antibody conjugated to Alexa 350 (1:200; 1 h at room temperature; Invitrogen). A punctate pattern of staining was observed for both GluR1 and synaptobrevin on medium spiny neurons in NAc/PFC cocultures. Methods for quantifying total surface GluR1 expression and synaptic GluR1 expression are described below (Data analysis for immunocytochemistry). For cell surface D_1, D_5, or D_2 receptor staining, live cells were incubated for 30 min with polyclonal antibody recognizing the second extracellular domain of the D_1 receptor, the third extracellular domain of the D_5 receptor, or the N terminal of the D_2 receptor (D_1 antibody, 1:100; D_5 antibody, 1:250; D_2 antibody, 1:200; gifts from Dr. Marjorie Ariano, Rosalind Franklin University of Medicine and Science). The subtype specificity of the D_1, D_5, and D_2 receptor antibodies has been demonstrated (McVittie et al., 1991; Ariano and Sibley, 1994; Ariano et al., 1997a). Then the cells were fixed and incubated with donkey anti-rabbit secondary antibody conjugated to Cy3 (1:500; Jackson ImmunoResearch) at room temperature for 1 h. To determine the location of D_1 receptors, the cells were further immunostained for synaptobrevin as described above.

In some studies, we measured glycine-induced synaptic incorporation of GluR1 as described by Lu et al. (2001). Cells were treated with glycine (1 μM) for 3 min at room temperature in bathing solution (140 mM NaCl, 1.3 mM $CaCl_2$, 5.0 mM KCl, 25 mM HEPES, 10 mM glucose, and 0.5 μM TTX, pH 7.4) and then transferred to bathing solution without glycine for 15 min. Cells were then immunostained for newly externalized GluR1 and synaptobrevin as described below.

In experiments shown in Figures 3, 4, and 5*A* (and supplemental Figs. 2*A*, 3, available at www.jneurosci.org as supplemental material), we selectively detected insertion of new GluR1-containing receptors onto the cell surface using a preblocking method modified from Lu et al. (2001). To block existing cell surface GluR1, live cells were first incubated with N-GluR1 antibody (1:15 in Neurobasal growth media) for 30 min and then incubated with nonconjugated goat anti-rabbit secondary antibody (5 μg/ml; Sigma) for 30 min at 15°C in a 3% CO_2 refrigerated incubator (Tritech Research, Los Angeles, CA). Then, cells were brought to room temperature, in either control media or media containing test drugs, to allow insertion of new AMPARs into the cell membrane. After this incubation, cultures were rinsed and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. To detect newly inserted GluR1, cultures were subsequently incubated with N-GluR1 antibody (1:15) at room temperature for 1 h, blocked with 5% donkey serum, and incubated with Cy3-conjugated donkey anti-rabbit secondary antibody (1:500) at room temperature for 1 h. To determine the location of newly inserted GluR1, synapses were immunostained with mouse monoclonal antibody to synaptobrevin (1:2000) and Alexa 350 donkey anti-mouse IgG (1:200) after permeabilization with 0.1% Triton X-100 in PBS for 15

min and incubation with 5% goat serum in PBS for 1 h. For coculture experiments in which the PFC was obtained from ECFP-expressing mice, Alexa 488 donkey anti-mouse IgG (1:1000; Invitrogen) was used as the secondary antibody.

Data analysis for immunocytochemistry. Images were acquired and analyzed with an imaging system consisting of a Nikon (Melville, NY) inverted microscope, ORCA-ER digital camera, and MetaMorph software (Universal Imaging, Downingtown, PA). Images for all experimental groups were taken using identical acquisition parameters. All groups to be compared were processed simultaneously using cells from the same culture preparation. For each experimental group, cells from at least four different wells were used, and approximately six cells from each well were analyzed. Neuronal subtypes in the NAc were distinguished based on previously defined morphological criteria (Shi and Rayport, 1994; Chao et al., 2002b). Medium spiny neurons, the predominant cell type in the NAc, were identified by a soma diameter of ~10–15 μm, with two to four relatively closely projecting processes. Interneurons were identified by a soma diameter >15 μm with extended processes over 10 times the length of the soma. The soma was excluded from analysis because, in the intact brain, glutamate synapses from PFC onto medium spiny neurons occur exclusively on processes (Meredith and Totterdell, 1999), and nearly all GluR1 immunostaining is observed on processes, not on the soma (Chen et al., 1998). Neuronal processes were selected for analysis under phase-contrast imaging to avoid the possibility of experimenter bias based on the intensity of fluorescence staining. For each image, the total area of fluorescently labeled surface GluR1 in a fixed length of process (15 μm) was measured using a threshold set at least two times higher than average background fluorescence in processes of untreated cells. The same approach was used to define the area of synaptobrevin staining (SB area). Nonsynaptic GluR1 area was defined as the area of GluR1 staining (in arbitrary units) that did not overlap with synaptobrevin staining. Synaptic GluR1 area was defined as the area of GluR1 staining (in arbitrary units) that overlapped with synaptobrevin staining. Another parameter, the percentage of synaptic area containing new GluR1 (or synaptic GluR1 incorporation), was quantified as the percentage of total synaptobrevin staining that overlapped with new GluR1 staining [(SB + GluR1 area)/total SB area × 100]. Because of limitations of light microscopy, a concern is that colocalization with a synaptic marker may not be sufficient to distinguish between synaptic and perisynaptic GluR1. However, analysis of striatal medium spiny neurons using the postembedding immunogold method revealed low levels of perisynaptic GluR1, whereas ~85% of the GluR1 immunoparticles on the postsynaptic surface of the spine were located within the postsynaptic density (Bernard et al., 1997). In Figure 2*F*, we were unable to normalize to synaptobrevin area because it was altered by the experimental manipulation, so synaptic GluR1 incorporation was expressed as the percentage of cell surface GluR1 area found in synapses [(SB + GluR1 area)/surface GluR1 area × 100]. All values refer to mean ± SEM. Independent group *t* tests were used for comparing two groups. For multiple groups, we used ANOVA followed by an appropriate *post hoc* test (n = number of cells analyzed).

Surface receptor crosslinking with bis(sulfosuccinimidal)suberate. After two washes with HBSS (Invitrogen), cultures were incubated with 2 mM bis(sulfosuccinimidal)suberate (BS^3) (Pierce, Rockford, IL) in HBSS for 10 min with agitation at 37°C (Hall and Soderling, 1997a,b; Hall et al., 1997). Crosslinking was terminated by quenching the reaction with 100 mM glycine for 10 min at 4°C.

Western blotting. Cultures were scraped into ice-cold buffer containing protease and phosphatase inhibitors [25 mM HEPES, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, 20 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 μM microcystin-LF, 1 μM okadaic acid, 1× protease inhibitor cocktail set I (Calbiochem), and 0.1% Nonidet P-40 (v/v)]. Homogenates were obtained by sonicating culture suspensions three times for 5 s each time on ice and centrifuging at 12,000 × *g* for 5 min. The supernatant was then aliquotted and stored at − 80°C. Protein concentration was determined by the Bio-Rad assay (Bio-Rad, Hercules, CA). Samples (20 μg total protein per lane) were run on a 3–8% gradient Tris-acetate gel (Invitrogen) or 10% bis-Tris gel (Invitrogen) under reducing conditions, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for immunoblotting. Membranes were washed in double-distilled H_2O (ddH_2O) and blocked with 1% goat serum or 5% nonfat dry milk in TBS–Tween 20 (TBS-T), pH 7.4, or with 3% BSA (Sigma) in TBS-T for 1 h at room temperature. Membranes were then incubated with one of the following phospho-antibodies overnight at 4°C: P-Ser845 GluR1 (1:500; Phosphosolutions, Aurora, CO), P-Ser831 GluR1 (1:500; Millipore Bioscience Research Reagents, Temecula, CA), P-Thr286 Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) (1:10,000; Phosphosolutions), phosphorylated extracellular signal-regulated kinase 44/42 (P-ERK44/42) (1:10 000; Millipore Bioscience Research Reagents), or an antibody recognizing phosphorylated PKA substrates (1:2500; Cell Signaling, Danvers, MA). The PKA substrate antibody was produced by immunizing rabbits with a synthetic phospho-PKA substrate peptide and purified over an affinity column using the same peptide. Overlapping substrate specificity with other Arg-directed protein kinases with similar Arg requirements [e.g., AKT and to a lesser extent protein kinase C (PKC)] relative to the phosphorylated Ser/Thr (Kennelly and Krebs, 1991; Pearson and Kemp, 1991) is a potential concern for experiments with the PKA substrate antibody. To assess this, we compared the PKA substrate antibody with the AKT substrate antibody (Cell Signaling Technology) in Western blotting experiments using rat NAc tissue and found that the antibodies produced completely different banding patterns. Of the nine major bands detected under our conditions with the PKA substrate antibody, none were detected with the AKT substrate antibody. Furthermore, only the PKA substrate antibody showed increased immunoreactivity by Western blotting in tissue that was stimulated with the PKA activator Sp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (SpcAMPS) or the D_1-like agonist SKF 81297 [(±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide] (SKF). Similarly, the PKC substrate antibody (Cell Signaling Technology) produced a significantly different banding pattern, and the peptide used to raise the PKC substrate antibody did not diminish immunoreactivity detected with the PKA substrate antibody (data not shown). After incubation with primary antibodies, membranes were washed extensively with TBS-T solution, incubated for 60 min with HRP-conjugated anti-rabbit IgG (1:10,000; Upstate Cell Signaling, Lake Placid, NY), and washed extensively again in TBS-T. Membranes were then rinsed with ddH_2O and immersed in enhanced chemiluminescence (ECL) detecting reagent that included luminol as a substrate and phenols as enhancers (GE Healthcare, Little Chalfont, UK) for 1 min. Images were collected on a Versa Doc Model 5000 (Bio-Rad) for 1 s to 5 min. Phospho-antibodies were stripped from the membrane by incubating in 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol at 65°C for 60 min or in Re-blot Plus Solution (Millipore Bioscience Research Reagents) at room temperature for 30 min. Blots were then probed with a phosphorylation-independent antibody to GluR1 (1:500; Millipore Bioscience Research Reagents), αCaMKII (1:20,000; Millipore Bioscience Research Reagents), βCaMKII (1:500; Abcam, Cambridge, MA), or ERK44/42 (1:10,000; Millipore Bioscience Research Reagents). We conducted positive control experiments to confirm specificity of the phospho-specific antibodies based on the ability of D_1-like receptor stimulation to activate PKA and ERK (Neve et al., 2004) and the ability of phorbol esters to activate PKC. Immunoreactivity detected with P-845 GluR1 and P-ERK44/42 antibodies (as well as the PKA substrate antibody; see above) was significantly greater in tissue incubated with SKF 81297 (10 μM, 15 min), and P-831 GluR1 immunoreactivity was significantly greater in tissue incubated with phorbol-12-myristate-13-acetate (1 μM, 3 h; Calbiochem) (data not shown). For protein crosslinking experiments, samples were loaded onto 4–15% Bio-Rad Tris-HCl gels and transferred to PVDF membranes as described above. Blots were probed with antibodies to NR2A (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and NR2B (1:500, Calbiochem). For supplemental Figure 2*B* (available at www.jneurosci.org as supplemental material), blots were probed with antibody to Arc (1:1000; Santa Cruz Biotechnology). To measure total D_1 receptor protein after repeated DA treatment, we quantified a doublet at ~74 kDa (1:200; Santa Cruz Biotechnology); previous studies have shown that, when cells are prepared in lysis buffer containing DTT to dissociate dimers (Lee et al., 2003b), this D_1 receptor antibody reacts predominantly with two bands of ~74 kDa,

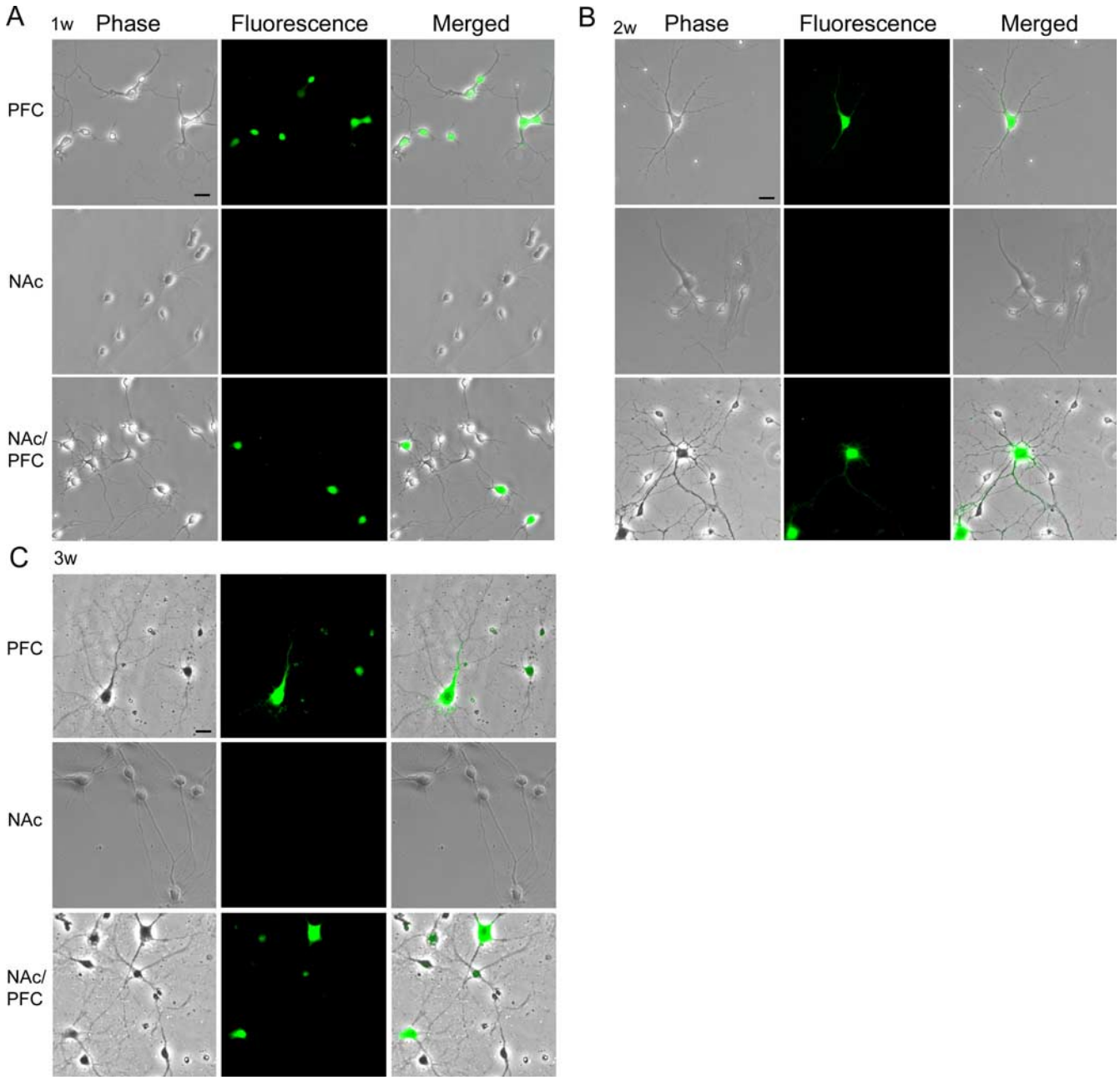

Figure 1. Comparison of PFC cultures, NAc cultures, and NAc/PFC cocultures after 1 week (***A***), 2 weeks (***B***), or 3 weeks (***C***) in culture. PFC cells were obtained from EGFP mice and NAc cells from rats. In cocultures, cells with the morphology of medium spiny neurons (the predominant cell type in NAc) were not fluorescent, whereas cells with the morphology of pyramidal neurons (from PFC) expressed EGFP. Many medium spiny neurons cluster near pyramidal neurons and their processes were sometimes apposed. Scale bar, 20 μm.

which corresponds to the size of glycosylated D_1 receptors (Bergson et al., 1995; Karpa et al., 1999). For all experiments, total protein in each lane was determined by staining membranes with Ponceau S (Sigma). The optical density of each band was determined using TotalLab (Nonlinear Dynamics, Newcastle, UK). For the PKA substrate antibody, the entire lane was scanned so that our assessment would be based on multiple substrates. The control and repeated DA treatment groups were compared by a *t* test. Significance was set at $p < 0.05$.

Results

Characterization of NAc/PFC cocultures

To enable the study of excitatory synapses onto NAc neurons while preserving the ability to identify NAc neurons, we established a coculture system consisting of rat NAc neurons and PFC neurons from EGFP transgenic mice. In pure PFC cultures prepared from EGFP transgenic mice, all neurons express EGFP, which can be readily detected by fluorescence microscopy after 1, 2, or 3 weeks in cultures (Fig. 1*A–C*, respectively). In pure NAc cultures prepared from rats, no neurons express EGFP (Fig. 1*A–C*). In NAc/PFC cocultures, cells with the morphology of pyramidal neurons from PFC express EGFP, whereas cells with the morphology of medium spiny neurons from NAc do not (Fig. 1*A–C*). Many medium spiny neurons are located near pyramidal cells, and their processes are sometimes apposed (Fig. 1*A–C*). Thus, this coculture system enables us to easily distinguish NAc cells from PFC cells under fluorescence microscopy. Our approach can be widely applied to other studies that require distinguishing between two cell sources.

To determine how addition of PFC cells might influence the

properties of NAc neurons, we conducted several experiments comparing pure NAc cultures and NAc/PFC cocultures. First, we determined the relative number of medium spiny neurons and interneurons, based on analysis of ~300 cells for each culture system (cells were from randomly selected fields in four wells). In NAc/PFC cocultures, ~78% of the NAc cells were medium spiny neurons and ~22% were interneurons. This is similar to results in pure NAc cultures (~80% medium spiny neurons and ~20% interneurons) and with our previous report (Chao et al., 2002b). Next, we determined whether restoring excitatory inputs by adding PFC neurons influenced DA receptor surface expression on medium spiny NAc neurons. Cell surface D_1 and D_5 DA receptors were labeled by incubating live cultures (3 weeks old) with antibodies to extracellular epitopes of these receptors. Medium spiny neurons in NAc/PFC cocultures exhibited punctate cell surface expression of D_1 and D_5 DA receptors (Fig. 2*A*). Similar staining was observed for pure NAc cultures (data not shown). To determine the percentage of medium spiny neurons that exhibited D_1 or D_5 receptor surface expression, we analyzed ~500 neurons from each culture system (cells were from randomly selected fields in six wells). In NAc/PFC cocultures, ~70% of medium spiny neurons were D_1 receptor positive, ~85% were D_5 receptor positive, and ~87% were labeled when D_1 and D_5 antibodies were used together. Very similar results were obtained for pure NAc cultures, in which ~75% of medium spiny neurons were D_1 receptor positive, ~85% were D_5 receptor positive, and ~89% were labeled when D_1 and D_5 antibodies were used together. The expression of cell surface D_2 receptors in the two culture systems was also compared, and no differences were found (~80% of medium spiny neurons were D_2 receptor positive in both pure NAc cultures and NAc/PFC cocultures). These results indicate a high degree of D_1 and D_2 receptor colocalization in cultured NAc medium spiny neurons. However, the degree of colocalization of D_1 and D_2 receptors *in vivo* remains controversial. Some studies reported nearly complete segregation or that only a small portion (<20%) of projection neurons in dorsal striatum or NAc expressed both D_1 and D_2 receptors (Gerfen et al., 1990; Le Moine et al., 1991; Le Moine and Bloch, 1995; Deng et al., 2006). However, other studies found that D_1 and D_2 receptors were coexpressed by a greater percentage of dorsal striatal projection neurons, albeit at different levels in different striatal populations, with estimates ranging from 30 to 40% (Meador-Woodruff et al., 1991; Ariano et al., 1992; Ariano and Sibley, 1994) to nearly all striatal neurons (Aizman et al., 2000).

Double immunostaining of the presynaptic marker synaptobrevin and cell surface D_1 receptors in NAc/PFC cocultures demonstrated that most surface D_1 receptors were located at extrasynaptic sites on medium spiny NAc neurons (Fig. 2*B*), consistent with previous studies of striatal neurons (see Discussion). From this point forward, we will use the term D_1-like to refer to the D_1 family of receptors (which includes the D_5 receptor) because agonists and antagonists used in our studies act on both D_1 and D_5 receptors.

Finally, we determined whether addition of PFC cells altered GluR1 surface expression or synapse formation onto medium spiny neurons. Surface GluR1 and the synaptic marker synaptobrevin were stained in pure NAc cultures and NAc/PFC cocultures after 3 weeks in culture. Most medium spiny neurons in both culture systems were GluR1 positive, consistent with *in vivo* findings in the adult rat striatum (Bernard et al., 1997; Chen et al., 1998). GluR1 surface expression on process segments, classified according to their distance from the soma (0–15, 15–30, or 30–45 μm), did not differ significantly between medium spiny neurons in the two culture systems, although there was a trend toward an increase in the NAc/PFC cocultures (Fig. 2*D*). Medium spiny neurons in NAc/PFC cocultures showed significant increases in both synaptobrevin expression and the percentage of cell surface GluR1 found in synapses (Fig. 2*C*,*E*,*F*). In addition, we found that the presence of PFC neurons significantly increased spine formation by the medium spiny NAc neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In NAc/PFC cocultures, 40 ± 7% of GluR1-positive puncta was located within spines. Our results are similar to those of Segal et al. (2003) who demonstrated that addition of mouse cortical neurons to cultured rat striatal neurons resulted in increased spine density and the appearance of spontaneous and evoked excitatory synaptic currents in the striatal neurons. The existence of synaptic GluR1 on medium spiny neurons in NAc/PFC cocultures establishes the coculture system as useful for studying AMPAR synaptic trafficking.

The D_1-like receptor agonist SKF 81297 increases cell surface GluR1 but not GluR1 synaptic incorporation in medium spiny neurons

We showed previously that the D_1-like receptor agonist SKF 81297 increased GluR1 insertion onto the surface of medium spiny neurons in pure NAc cultures (Mangiavacchi and Wolf, 2004). In the present study, we extended these results by determining the effect of SKF 81297 on GluR1 synaptic targeting using NAc/PFC cocultures. A preblocking method modified from Lu et al. (2001) was used to selectively detect newly inserted GluR1 on medium spiny neurons. Briefly, preexisting cell surface GluR1 was preblocked by incubating cultures with primary antibody and nonconjugated secondary antibody at 15°C. Then, cells were brought to room temperature for 15 min to allow insertion of new GluR1-containing receptors (timing based on Mangiavacchi and Wolf, 2004). Newly inserted GluR1 was detected with a second round of immunostaining under nonpermeant conditions with Cy3-conjugated secondary antibody. Finally, cells were stained for synaptobrevin under permeant conditions to determine whether the insertion of GluR1 occurred at synapses.

In NAc/PFC cocultures, medium spiny neurons treated with SKF 81297 (1 μM) for 15 min exhibited higher levels of new surface GluR1 area than control cultures (Fig. 3*A*,*B*). This effect was blocked when the D_1-like receptor antagonist SCH 23390 [*R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride] (10 μM) was added 5 min before SKF 81297. When given alone, SCH 23390 had no effect on GluR1 insertion (Fig. 3*B*). SKF did not increase the percentage of synaptic area containing new GluR1 (Fig. 3*C*) or new synaptic GluR1 area (Fig. 3*D*), whereas nonsynaptic GluR1 area was significantly increased by SKF 81297 treatment (Fig. 3*E*). These results indicate that SKF 81297 increased GluR1 insertion onto the cell surface but not its synaptic incorporation in medium spiny neurons. Based on the fact that D_1-like receptor agonists also increased GluR1 insertion in pure NAc cultures (above), we conclude that D_1-like receptor agonists are increasing GluR1 insertion in NAc/PFC cocultures by interacting directly with D_1-like receptors on NAc neurons rather than indirectly via D_1-like receptors on PFC neurons.

A caveat should be noted regarding methods used to analyze synaptic GluR1 in Figure 3 and subsequent experiments. Although results in Figures 4 and 5 provide positive controls for our ability to detect an increase in synaptic GluR1, it is possible that the sensitivity of our methods is adequate to detect GluR1 addition to silent synapses but not GluR1 addition to synapses already

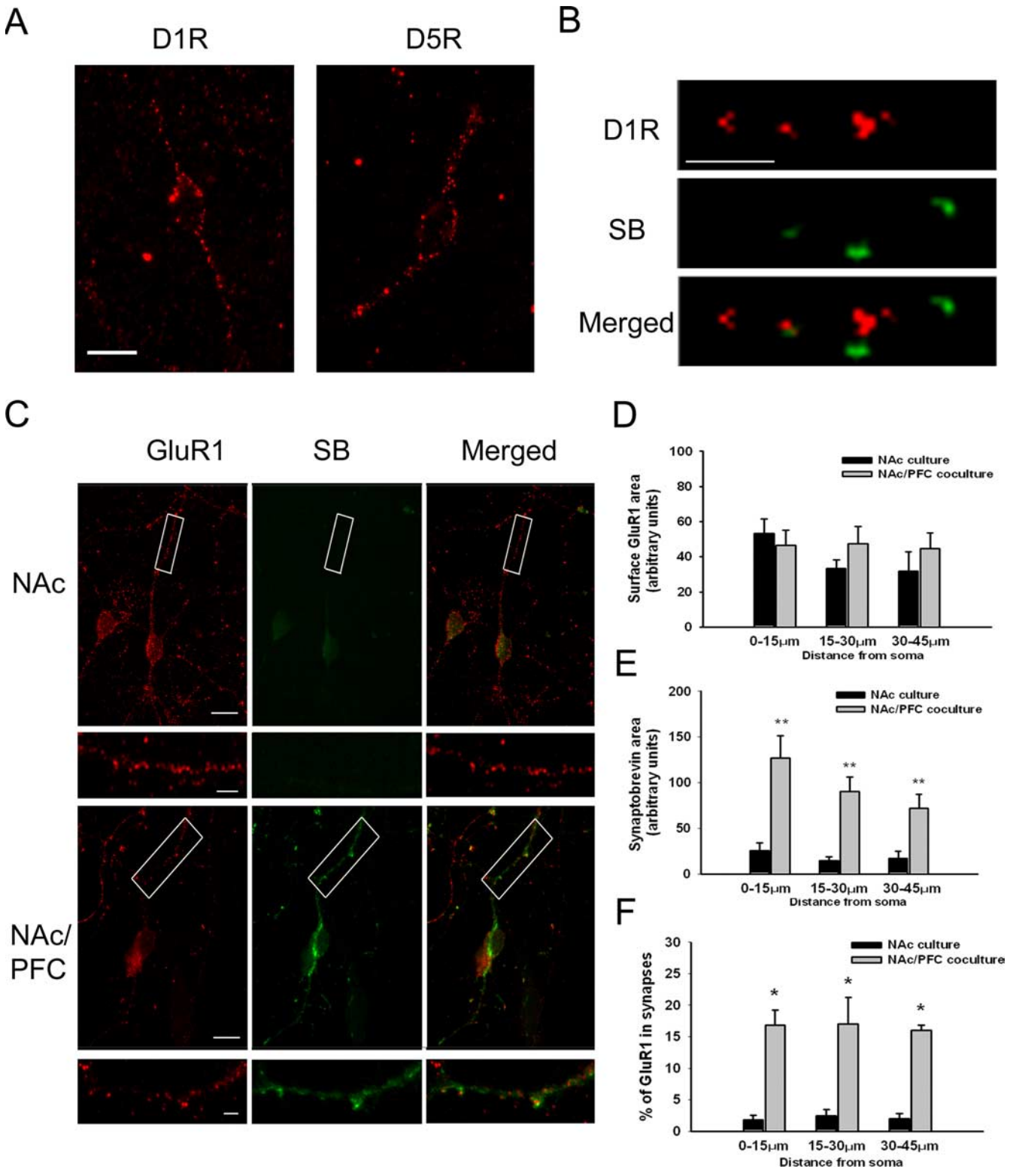

Figure 2. Cell surface expression of D_1 DA receptors, D_5 DA receptors, and GluR1-containing AMPARs on medium spiny NAc neurons in NAc/PFC cocultures. After 3 weeks *in vitro*, cultures were immunostained for cell surface D_1 or D_5 receptors, cell surface GluR1, and the synaptic marker SB as described in Materials and Methods. ***A***, Medium spiny neurons in NAc/PFC cocultures exhibited punctate cell surface expression of D_1 receptors and D_5 receptors. Scale bar, 20 μm. ***B***, Double staining of SB and D_1 receptors showed that most D_1 receptors are located at extrasynaptic sites on the processes of medium spiny neurons. D_1 receptors were detected with Cy3 secondary antibody (red), whereas synaptobrevin was detected with Alexa 350 (original color was blue; green pseudocolor is used for clarity). Scale bar, 2 μm. ***C***, Representative images of surface GluR1 and SB double staining in pure NAc cultures and NAc/PFC cocultures. GluR1 was detected with Cy3 secondary antibody (red), whereas SB was detected with Alexa 350 (green pseudocolor). Scale bars, 20 μm for large panels. Higher-power views of selected areas (white squares) are also shown (scale bars, 5 μm). ***D***, Comparison of GluR1 surface expression on process segments (0–15, 15–30, or 30–45 μm from the soma) of medium spiny neurons in pure NAc cultures ($n = 19$) and NAc/PFC cocultures ($n = 22$). Coculture of NAc neurons with PFC neurons did not significantly change GluR1 surface expression. ***E***, Coculture of NAc neurons with PFC neurons increased SB expression on medium spiny neuron processes (**$p < 0.01$, *t* test, compared with pure NAc cultures). ***F***, Coculture increased the percentage of cell surface GluR1 found in synapses [(GluR1 + SB area)/surface GluR1 area × 100] (*$p < 0.05$, *t* test, compared with pure NAc cultures).

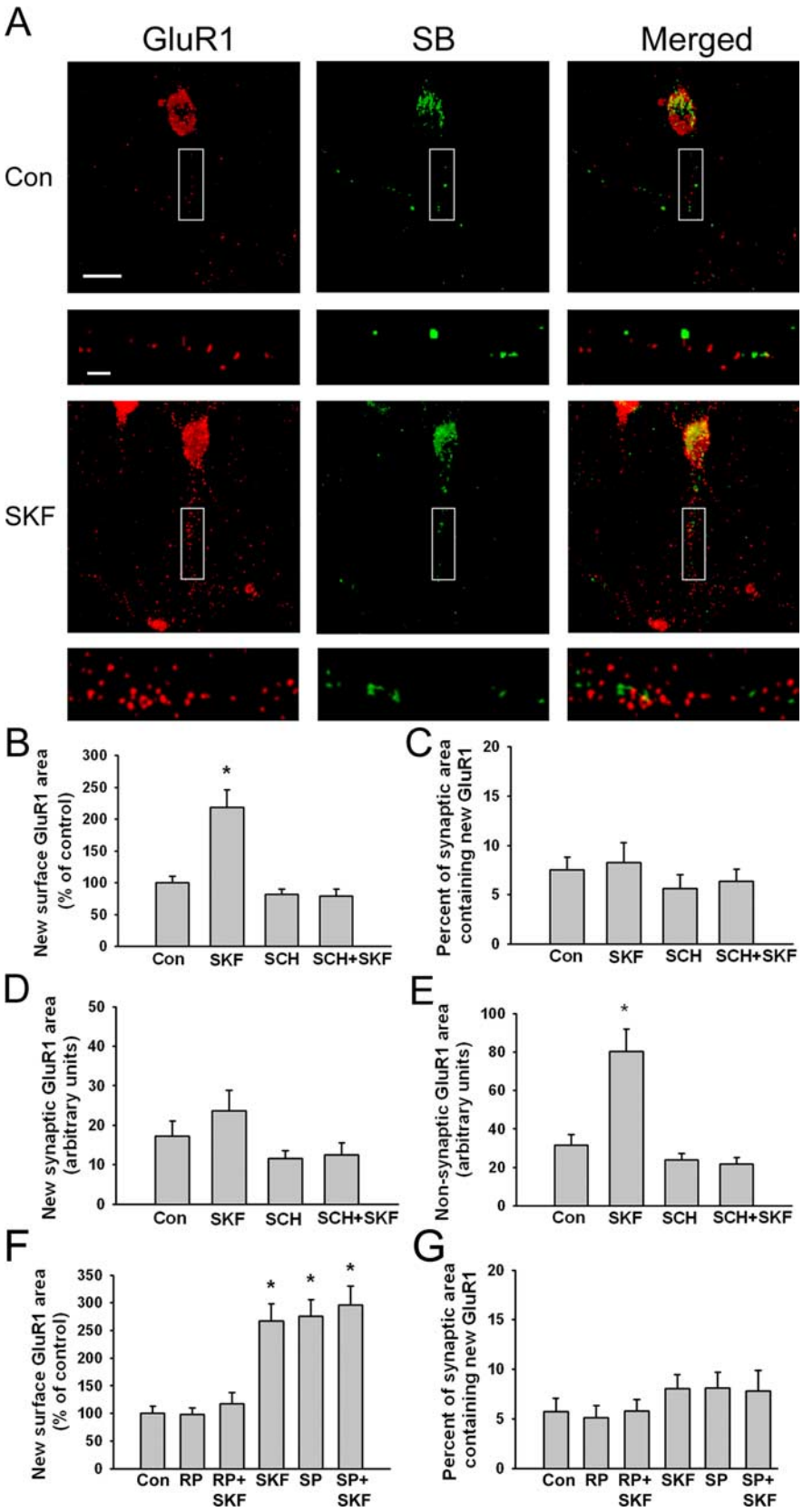

Figure 3. The D_1-like receptor agonist SKF 81297 increased GluR1 insertion onto the extrasynaptic cell surface in medium spiny NAc neurons through a PKA-dependent pathway. A preblocking method was used to selectively detect newly inserted GluR1. ***A***, Examples of newly inserted GluR1 on processes of control (Con) and SKF-treated neurons (1 μM, 15 min), shown with SB staining after permeabilization. GluR1 was detected with Cy3 secondary antibody (red), whereas SB was detected with Alexa 350 (original color was blue; green pseudocolor is used for clarity). Scale bars, 10 μm for large panels. Higher-power views of selected areas (white squares) are also shown (scale bar, 5 μm). ***B***, Effect of SKF on GluR1 surface insertion ($n = 17$–24, Dunn's test, $^*p < 0.05$ compared with control group, SCH group, and SCH + SKF group). Results are presented as the mean area of GluR1 puncta, normalized to controls. Total incubation time was 20 min. Vehicle or the D_1-like antagonist SCH 23390 (SCH; 10 μM) were present throughout, and SKF (1 μM) was added for the final 15 min. ***C***, The percentage of synaptic area containing new GluR1 was determined as the percentage of total SB staining that overlaps with new GluR1 staining [(SB + GluR1 area)/total SB area × 100]. This parameter was not altered by SKF treatment ($n = 17$–24; ANOVA, $p > 0.05$). ***D***, Quantification of synaptic GluR1 area (area of surface GluR1 staining that overlapped with SB staining) ($n = 17$–24; ANOVA, $p > 0.05$). ***E***, Quantification of nonsynaptic GluR1 area (area of surface GluR1 staining that did not overlap with SB) ($n = 17$–24; Dunn's test, $^*p < 0.05$ compared with control group, SCH group, and SCH + SKF group). ***F***, The PKA activator SpcAMPS occluded the effect of the D_1-like agonist SKF 81297 on GluR1 cell surface insertion, and the PKA inhibitor RpcAMPS blocked the increase in GluR1 insertion produced by SKF 81297. Total incubation time was 20 min. SpcAMPS and RpcAMPS were present throughout (10 μM), and SKF (1 μM) was added for the final 15 min. Results are presented as the mean area of GluR1 puncta, normalized to controls. The SKF, SpcAMPS, and SpcAMPS + SKF groups differed significantly from the control group ($n = 19$–31; Dunn's test, $^*p < 0.05$ compared with control group, RpcAMPS group, and RpcAMPS + SKF group). ***G***, The percentage of synaptic area containing new GluR1 [(SB + GluR1 area)/total SB area × 100] was not altered by SKF, SpcAMPS, RpcAMPS, or combined treatment with SKF and SpcAMPS or RpcAMPS ($n = 19$–31; ANOVA, $p > 0.05$).

expressing GluR1. It is therefore possible that we failed to detect a D_1-like agonist effect on GluR1-containing synapses in Figure 3, although it seems unlikely that D_1-like receptors would selectively influence GluR1-containing synapses but not silent synapses.

Increased GluR1 cell surface insertion induced by D_1-like receptor stimulation requires PKA activation

D_1 family receptors are positively coupled to adenylyl cyclase (Neve et al., 2004). In pure NAc cultures, we showed that PKA activity was required for D_1-like agonist-induced increases in GluR1 surface expression (Chao et al., 2002b; Mangiavacchi and Wolf, 2004). Using cocultures, we extended these studies by examining the role of PKA in AMPAR synaptic targeting. After preblocking, NAc/PFC cocultures were incubated for 15 min with SpcAMPS (10 μM), a potent membrane-permeable PKA activator. SpcAMPS significantly increased new surface GluR1 area on medium spiny neurons but did not significantly alter GluR1 synaptic incorporation (Fig. 3*F*,*G*). Thus, as was found for D_1-like receptor stimulation, PKA activation is sufficient for increasing surface expression, but not synaptic delivery, of GluR1-containing AMPARs. To determine whether PKA activation mediates the ef-

fect of D_1-like receptor stimulation, we examined the effect of PKA inhibitors and activators in combination with SKF 81297. After preblocking, cultures were incubated for 20 min with Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (RpcAMPS) (10 μM), a membrane-permeable PKA inhibitor, or a maximally effective concentration of SpcAMPS (10 μM). Then the D_1-like agonist SKF 81297 (1 μM) was added for the final 15 min of the incubation. RpcAMPS had no effect on its own but blocked the D_1-like agonist-induced increase in new GluR1 cell surface insertion (Fig. 3*F*). After PKA activation by SpcAMPS, SKF 81297 failed to further increase GluR1 insertion (Fig. 3*F*). Thus, PKA inhibition prevented the effect of D_1-like receptor stimulation, whereas PKA activation occluded it. The effect of these experimental manipulations on GluR1 synaptic incorporation was also determined. GluR1 synaptic incorporation was not altered by SKF 81297, SpcAMPS, RpcAMPS, or the combined administration of RpcAMPS or SpcAMPS with SKF 81297 (Fig. 3*G*). This was confirmed by analysis of synaptic GluR1 area and nonsynaptic GluR1 area (data not shown).

Regulation of AMPAR trafficking in medium spiny NAc neurons by D_1 family receptors is independent of protein synthesis

Smith et al. (2005) reported that incubation of hippocampal cultures with a D_1-like agonist [100 μM SKF 38393 [(±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride] or 10 μM dihydrexidine; 15 min] increased GluR1 surface expression through a mechanism that required PKA activity and protein synthesis. To test the role of protein synthesis in D_1-like agonist effects on NAc neurons, NAc/PFC cocultures were pretreated for 20 min with media or the protein synthesis inhibitor anisomycin (40 μM), followed by incubation with the D_1-like agonist SKF 81297 (1 μM) for 15 min. Pretreatment with anisomycin did not affect basal levels of GluR1 cell surface insertion nor the increased GluR1 insertion induced by SKF 81297 (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material). As a positive control, we demonstrated that this anisomycin concentration was sufficient to inhibit BDNF-induced translation of the immediate early gene Arc (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material). This result and others (Karachot et al., 2001) indicate that our anisomycin protocol is sufficient to inhibit protein synthesis. Thus, the new GluR1 surface expression induced by D_1-like receptor activation is attributable to the insertion of preexisting AMPARs rather than the synthesis of new receptors. We similarly reported that brief D_1-like agonist exposure increased GluR1 cell surface insertion in hippocampal neurons through a mechanism that was independent of protein synthesis (Gao et al., 2006). Possible explanations for different outcomes in our experiments, compared with results of Smith et al. (2005), include differences in D_1-like agonist treatment (drug, concentration, and timing of some experiments) and higher cell density in their cultures, perhaps leading to differences in the ongoing level of synaptic transmission. Together, these results suggest that activation of the D_1-like receptor–PKA pathway may increase AMPAR levels through both protein synthesis-dependent and -independent mechanisms. Further supporting a protein synthesis-independent mechanism, brief PKA activation increased surface GluR1 expression but not total GluR1 protein in cortical cultures (Man et al., 2007).

The D_1-like receptor agonist SKF 81297 facilitates glycine-induced synaptic incorporation of GluR1 in medium spiny neurons

Considerable evidence suggests that PKA-mediated increases in GluR1 surface expression prime GluR1 for synaptic delivery (Esteban et al., 2003; Sun et al., 2005; Gao et al., 2006; Oh et al., 2006; Man et al., 2007). We thus hypothesized that D_1-like receptors, by increasing extrasynaptic levels of GluR1, would facilitate GluR1 synaptic delivery in response to subsequent NMDA receptor (NMDAR) activation. To test this hypothesis, we adapted an assay developed by Lu et al. (2001) in which glycine, an obligatory coagonist at the NMDAR, is added briefly to cultured neurons in a bathing solution containing TTX. TTX reduces glutamate release to allow the release of only a few quanta of transmitter from each terminal, confining release to the synapse. Under these conditions, glycine selectively potentiates synaptic NMDAR transmission, resulting in synaptic incorporation of GluR1-containing AMPARs and LTP (Lu et al., 2001). In our study, we used a subthreshold concentration of glycine (1 μM) that does not produce significant AMPAR incorporation on its own (Sun et al., 2005; Gao et al., 2006). After preblocking, NAc/PFC cocultures were pretreated with the D_1-like agonist SKF 81297 (1 μM, 15 min), rinsed, incubated with the subthreshold concentration of glycine (1 μM, 3 min), rinsed, transferred to glycine-free bathing solution for 15 min, and then stained for GluR1 and synaptobrevin (SKF → Gly group). Other experimental groups were treated with SKF only, glycine only, or glycine and SKF in the reverse order (Gly → SKF). Effects on GluR1 surface expression and synaptic incorporation are shown in Figure 4, *A* and *B*, respectively, with representative images shown in Figure 4*C*. As expected, SKF increased insertion of new GluR1 onto the cell surface, whether alone or in combination with glycine (Fig. 4*A*), whereas neither glycine nor SKF alone was sufficient to significantly increase GluR1 synaptic incorporation (Fig. 4*B*). However, cells treated with SKF followed by glycine showed a significant increase in GluR1 synaptic incorporation (Fig. 4*B*). A trend toward an increase was observed when drugs were applied in the reverse order (Gly → SKF group) or after SKF alone. Analysis of synaptic and nonsynaptic GluR1 area confirmed the above findings (data not shown). To confirm that glycine was working via NMDARs, we demonstrated that inclusion of the NMDAR antagonist APV in the bath significantly decreased GluR1 synaptic delivery in cells treated with SKF and glycine but did not prevent SKF from increasing GluR1 cell surface insertion (supplemental Fig. 3*A*,*B*, available at www.jneurosci.org as supplemental material). This observation is consistent with the fact that LTP induction in NAc neurons requires NMDAR stimulation (Pennartz et al., 1993; Kombian and Malenka, 1994). Overall, our results indicate that D_1-like and NMDA receptors work cooperatively to induce GluR1 synaptic incorporation.

The above findings support our hypothesis that PKA-mediated increases in the extrasynaptic GluR1 pool facilitate NMDAR-dependent synaptic GluR1 delivery. However, other mechanisms could also contribute to this facilitating effect. One possibility is that PKA activation induced by SKF treatment enhances the response of NMDARs to glycine, and this enables a subthreshold concentration of glycine to produce GluR1 synaptic incorporation. Supporting this possibility, D_1-like receptors enhance NMDAR transmission through several mechanisms that include direct physical interactions and second-messenger-mediated effects on NMDAR currents and trafficking (for review, see Cepeda and Levine, 2006). We focused on a series of studies in dorsal striatal tissue showing that D_1-like receptor stimulation

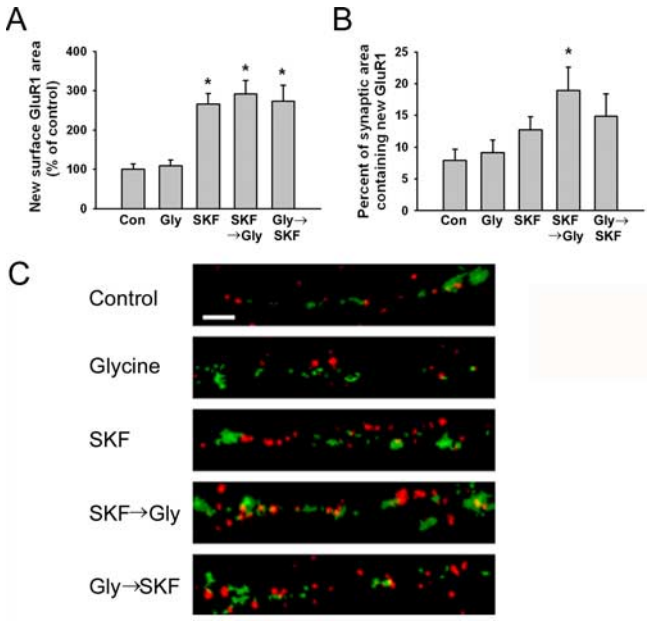

Figure 4. The D_1-like receptor agonist SKF 81297 facilitated NMDAR-dependent synaptic incorporation of GluR1 in medium spiny NAc neurons. We used a subthreshold concentration of the NMDAR coagonist glycine (1 μM) that on its own does not induce GluR1 synaptic delivery. ***A***, SKF significantly increased GluR1 insertion onto the cell surface whether added before or after glycine (Gly). Cultures in the SKF 81297 → glycine group were treated 15 min with 1 μM SKF, rinsed, and treated 3 min with 1 μM glycine. Cultures in the glycine → SKF group were treated in the reverse order. Results are presented as the mean area of GluR1 puncta, normalized to controls (Con; $n = 19–25$; Dunn's test, $^*p < 0.05$ compared with control group and 1 μM glycine group). ***B***, SKF significantly increased GluR1 synaptic incorporation when added before glycine. The SKF, glycine, and glycine → SKF groups did not differ significantly from controls. Results are presented as the percentage of synaptic area containing new GluR1 [(SB + GluR1 area)/total SB area × 100] ($n = 19–25$; Dunn's test, $^*p < 0.05$ compared with control group). ***C***, Examples of colocalization of GluR1 with SB on processes of control neurons and neurons treated with glycine (1 μM, 3 min), SKF (1 μM, 15 min), SKF → glycine, and glycine → SKF. GluR1 was detected with Cy3 secondary antibody (red), whereas SB was detected with Alexa 350 (original color was blue; green pseudocolor is used for clarity). Scale bar, 2.5 μm.

increased NMDAR surface expression (Dunah and Standaert, 2001; Dunah et al., 2004; Hallett et al., 2006). To determine whether a similar effect occurred in NAc/PFC cocultures, we used a BS^3 protein crosslinking assay that has been used to study glutamate receptor trafficking in dissociated cultures (Hall and Soderling, 1997a,b; Hall et al., 1997; Archibald et al., 1998). No NR1 antibody exists that is suitable for this assay (or for live-cell labeling), so we focused on NR2A and NR2B. We found no significant changes in surface, intracellular, or total levels of either subunit after treatment with SKF 81297 (1 μM, 15 min) (NR2A: surface, 115 ± 19.1; intracellular, 85.4 ± 14.8; total, 99.9 ± 13.8; NR2B: surface, 102.8 ± 20.1; intracellular, 91.8 ± 5.4; total, 97.3 ± 9.9; all values expressed as percentage of vehicle treated cultures, $n = 7–11$ per group). As a positive control, we have used the same assay to demonstrate increased NR2B surface expression after D_1-like agonist treatment of PFC cultures (C. Gao and M. E. Wolf, unpublished findings). Differences between our results in NAc/PFC cocultures and those obtained in striatal tissue (Dunah and Standaert, 2001; Dunah et al., 2004; Hallett et al., 2006) could reflect use of a different D_1-like agonist and concentration in striatal tissue (SKF 82958 [(±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide]; 50 μM), the use of brain tissue rather than cultured neurons, and differences between ventral (accumbens) and dorsal striatum. Although the quantity of surface NMDARs is not altered by D_1-like receptor stimulation in NAc/PFC cocultures, it remains possible that NMDAR function is enhanced by the D_1-like receptor–PKA pathway and that this contributes to facilitation of glycine action (see Discussion).

Repeated DA treatment decreases D_1 receptor surface expression in NAc/PFC cocultures

One goal of this study was to use primary cultures to obtain a better understanding of how AMPAR trafficking in NAc neurons may be altered by repeated exposure to cocaine. We could not use cocaine in our studies, because it acts by blocking the DA transporter on DA nerve terminals, and our cultures do not contain DA neurons. Therefore, we investigated the effect of repeated treatment with DA itself on AMPAR trafficking in NAc/PFC cocultures. Cocultures were treated with either vehicle (control) or DA (1 μM, 30 min) on days 7, 9, and 11 in culture. Four days after discontinuing repeated treatment (day 15), cells were "challenged" with vehicle or SKF 81297 (1 μM, 15 min). SKF significantly increased GluR1 insertion onto the cell surface in vehicle-treated cultures, although it failed to do so after repeated DA treatment (Fig. 5*A*). Thus, repeated DA treatment eliminated the ability of D_1-like receptors to regulate AMPAR trafficking.

We ruled out toxic effects of DA as an explanation for refractoriness to the D_1-like agonist by showing that repeated DA treatment had no effect on cell viability (control, 91.5 ± 2.8%; DA, 88.9 ± 2.0%; *t* test, $p > 0.05$; assessed with Live/Dead/Viability/Cytotoxicity Assay; Invitrogen). Then we determined whether the effect of repeated DA treatment was DA receptor-mediated by applying DA together with the D_1-like receptor antagonist SCH 23390 and the D_2-like receptor antagonist raclopride on days 7, 9, and 11. Under these conditions, SKF retained its ability to increase GluR1 surface expression on day 15, demonstrating that repeated DA treatment eliminated the D_1-like agonist response through a mechanism requiring DA receptor stimulation (supplemental Fig. 4, available at www.jneurosci.org as supplemental material).

We hypothesized that cells become refractory to SKF 81297 because repeated D_1 receptor stimulation leads to D_1 receptor internalization. To test this, NAc/PFC cocultures were treated repeatedly with vehicle or DA on days 7, 9, and 11, and D_1 receptor surface expression was measured on day 15 using antibody to an extracellular epitope of the D_1 receptor. Repeated DA treatment significantly decreased D_1 receptor surface expression compared with the control group (Fig. 5*B*,*C*), suggesting that D_1 receptors internalize during repeated DA treatment and that D_1 receptor surface expression remains low even 4 d after the last DA exposure. Interestingly, total D_1 receptor protein levels, determined by Western blotting, were significantly increased on day 15 after repeated DA treatment (control, 100 ± 13; DA, 263 ± 75; data expressed as percentage of vehicle-treated cultures; *t* test, $p < 0.05$; $n = 6$), despite the fact that cell surface D_1 receptor expression was reduced (see Discussion). Refractoriness to the D_1-like agonist could also reflect impaired PKA regulation of GluR1 trafficking. However, this possibility was eliminated by demonstrating that repeated DA treatment did not influence the ability of SpcAMPS, a direct PKA activator, to increase GluR1 surface expression (Fig. 5*A*). This experiment also showed that refractoriness to the D_1-like agonist was not attributable to a ceiling level of GluR1 surface expression.

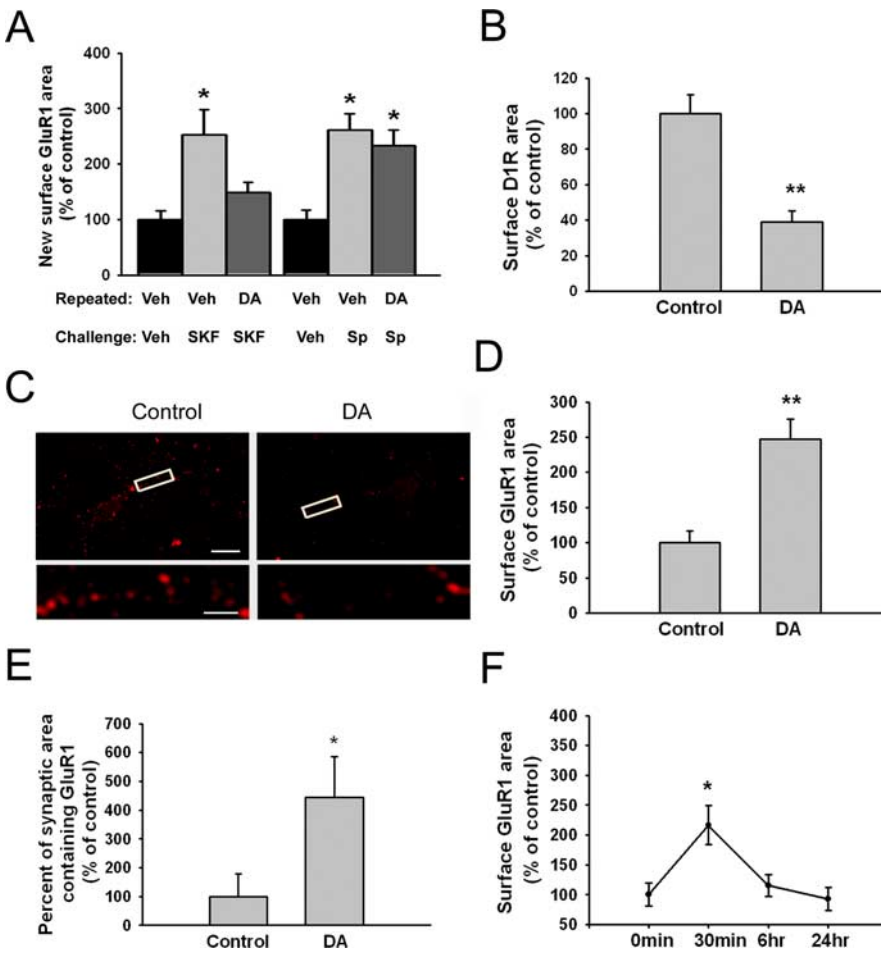

Figure 5. Repeated DA treatment altered the regulation of DA and AMPAR surface expression on medium spiny NAc neurons. ***A–E*** compare two pretreatment conditions, termed Control and DA. Control, NAc/PFC cocultures were treated with vehicle on days 7, 9, and 11 in culture. DA, NAc/PFC cocultures were treated with DA (1 μM, 30 min) on days 7, 9, and 11. ***A***, On day 15, control and DA-treated cultures were challenged with vehicle (Veh), SKF 81297 (1 μM, 15 min), or SpcAMPS (Sp; 10 μM, 15 min). In control cultures, SKF and SpcAMPS significantly increased the area of newly inserted GluR1 on the cell surface. After repeated DA treatment, D_1-like receptor stimulation was no longer able to increase GluR1 cell surface insertion, whereas SpcAMPS maintained its ability to increase GluR1 insertion. All data are normalized to control group ($n = 17–31$; t test, $^*p < 0.05$ compared with vehicle + vehicle group). ***B***, For this and ***C–E***, cells were treated as described in ***A*** except that no vehicle or SKF 81297 challenge was administered on day 15. ***B*** shows that D_1 receptor surface expression was significantly decreased in the repeated DA group on day 15 ($n = 19–23$; t test, $^{**}p < 0.01$ compared with vehicle-treated group). ***C***, Representative images of D_1 receptor surface expression on day 15 after repeated vehicle or DA treatment. Scale bars, 10 μm for large panels. Higher-power views of selected areas (white squares) are also shown (scale bar, 2 μm). ***D***, Quantification of GluR1 surface expression on day 15 after repeated vehicle or DA treatment ($n = 22–27$; t test, $^{**}p < 0.01$ compared with vehicle treated group). ***E***, Quantification of the percentage of synaptic area containing GluR1 on day 15 after repeated vehicle or DA treatment ($n = 22–27$; t test, $^*p < 0.05$ compared with vehicle treated group). ***F***, Time course of GluR1 surface expression after a single DA treatment (1 μM, 30 min) on day 11 *in vitro* ($n = 17–25$ for each time-point; Dunn's test, $^*p < 0.05$ compared with 0 min group).

Repeated DA treatment increases GluR1 surface and synaptic expression in NAc/PFC cocultures

We also measured GluR1 surface expression after repeated DA treatment and found that both surface and synaptic GluR1 levels were increased on day 15 (Fig. 5*D*,*E*). In light of the ability of D_1-like receptors to acutely increase GluR1 surface expression (Fig. 3*A*,*B*), we considered the possibility that GluR1 upregulation after repeated DA treatment was a direct and persistent effect of the final DA treatment on day 11 (although if this was the case, an increase in synaptic GluR1 would not have been expected). To evaluate this possibility, we examined the time course of GluR1 surface expression after a single DA exposure on day 11. GluR1 surface expression was significantly increased immediately after DA treatment (1 μM, 30 min) but returned to basal levels after 6 h and remained at basal levels 24 h after DA treatment (Fig. 5*F*). This indicates that the enhanced GluR1 surface expression induced by a single exposure to DA is transient, and thus the persistently enhanced GluR1 surface expression observed in Figure 5*D* is the consequence of repeated DA treatment. A final conclusion that can be drawn from Figure 5*F* is that DA has the same effect on GluR1 surface expression as the D_1-like agonist SKF 81297 (both produce an increase), establishing that effects we observed with a D_1-like agonist are relevant to endogenous DA transmission.

The observed increase in GluR1 surface and synaptic expression after repeated DA treatment is of interest because AMPAR surface expression is also increased in the NAc after *in vivo* cocaine treatment (see Discussion). To investigate mechanisms that might underlie the effects of repeated DA treatment, NAc/PFC cocultures were treated repeatedly with vehicle or DA as described above and harvested on day 15 (without challenge) for Western blot analysis of three signaling pathways important for both addiction and regulation of AMPAR trafficking: PKA, ERK, and CaMKII. Measuring signaling pathway activation 4 d after the last DA exposure is justified based on evidence that repeated *in vivo* administration of psychomotor stimulants can produce changes in protein kinase activity that persist days to weeks after discontinuing drug exposure (Gnegy, 2000; Hope et al., 2005; Boudreau et al., 2007).

PKA phosphorylation of GluR1 is associated with AMPAR surface expression (Ehlers, 2000; Chao et al., 2002a,b; Esteban et al., 2003; Lee et al., 2003a; Lu et al., 2003; Oh et al., 2006; Man et al., 2007). We assessed the overall level of PKA phosphorylation using an antibody that recognizes phosphorylated PKA substrates (see Materials and Methods). We also examined GluR1 phosphorylation at the PKA site (Ser845) using a phospho-specific antibody. Repeated DA treatment failed to alter the overall level of PKA phosphorylation or the ratio of P-845/total GluR1 (Fig. 6*A*,*B*). Total GluR1 protein was unchanged (Fig. 6*B*). We also measured GluR1 phosphorylation at Ser831, a site phosphorylated by CaMKII and PKC (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997). Phosphorylation at Ser831 potentiates AMPAR transmission by increasing single-channel conductance (Derkach et al., 1999) but is not required for AMPAR synaptic incorporation (Hayashi et al., 2000). As with Ser845, we found no significant effect of repeated DA treatment (Fig. 6*B*). However, these negative results should be interpreted with caution, because we may have missed effects confined to a particular cellular compartment, such as the postsynaptic density. We did not examine

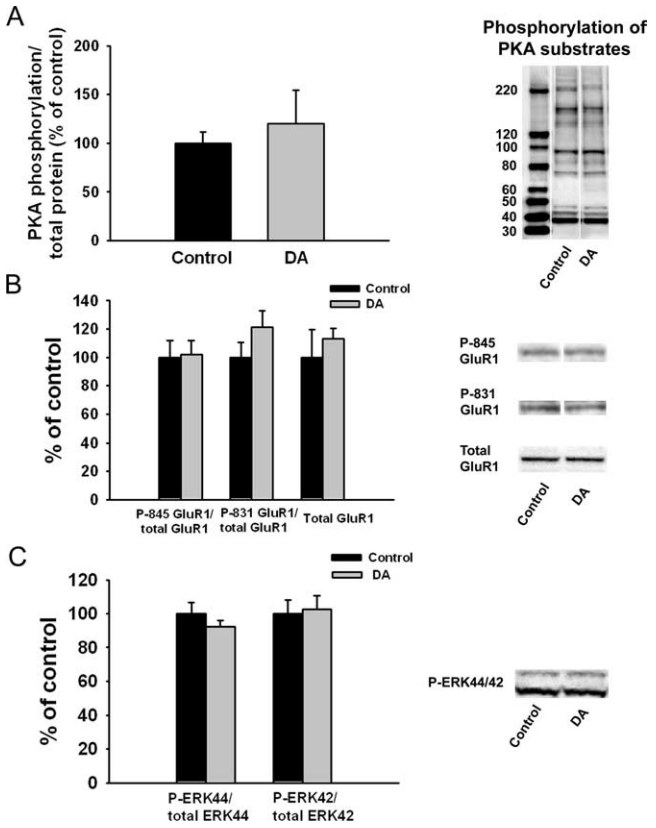

Figure 6. Repeated DA treatment did not alter phosphorylation of PKA substrates, phosphorylation of ERK, or phosphorylation of GluR1 at Ser845 or Ser831. NAc/PFC cocultures were harvested for Western blotting on day 15 after repeated vehicle or DA treatment. Representative blots are shown for all experiments. ***A***, PKA phosphorylation was measured using an antibody that recognizes the phosphorylated PKA consensus sequence (see Materials and Methods). Data are normalized to total protein in the lane and presented as percentage of the control group ($n = 6$; t test, $p > 0.05$). ***B***, GluR1 phosphorylation and total GluR1 expression. Phospho-specific antibodies were used to determine the ratio of P-845 GluR1/total GluR1 and P-831 GluR1/total GluR1. Data are presented as percentage of the control group ($n = 6$; t test, $p > 0.05$). ***C***, ERK44/42 activation was measured as the ratios of P-ERK44/total ERK44 and P-ERK42/total ERK42. Data are presented as percentage of the control group ($n = 6$; t test, $p > 0.05$).

Ser818, a PKC phosphorylation site on GluR1 implicated in AMPAR synaptic incorporation during hippocampal LTP (Boehm et al., 2006).

CaMKII and ERK are required for AMPAR synaptic delivery during hippocampal LTP (Hayashi et al., 2000; Zhu et al., 2002), so we measured their activation state after repeated DA treatment by using Western blotting to assess phosphorylation of CaMKII (P-CaMKII/total CaMKII) and ERK44/42 (P-ERK44/total ERK44 and P-ERK42/total ERK42). Although repeated DA treatment had no effect on ERK phosphorylation (Fig. 6*C*), it significantly increased phosphorylation of CaMKII (Fig. 7*A*). Total expression of CaMKII and ERK44/42 remained unchanged after repeated DA treatment (data not shown). In contrast, acute DA treatment failed to produce activation of CaMKII (Fig. 7*A*). Activation of CaMKII after repeated but not acute DA treatment may explain why only repeated DA treatment is sufficient to increase synaptic GluR1 levels. The observation that GluR1 Ser831 phosphorylation is not increased after repeated DA treatment (Fig. 6*B*) despite activation of CaMKII (Fig. 7*A*) is consistent with similar results in hippocampal neurons (no increase in GluR1 Ser831 phosphorylation despite activation of CaMKII) that were attributed to different compartmentalization of CaMKII and GluR1 in the postsynaptic density (Tsui and Malenka, 2006).

To determine whether CaMKII activation was required for increased GluR1 surface expression after repeated DA treatment, the CaMKII inhibitor KN-93 (*N*-[2-[*N*-(4-chlorocinnamyl)-*N*-methylaminomethyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt) (Sumi et al., 1991) was added to the media immediately after the last DA treatment on day 11 and washed out just before GluR1 immunostaining on day 15. KN-93 blocked the increase in GluR1 surface and synaptic expression produced by repeated DA treatment (Fig. 7*B*,*C*). KN-93 treatment had no effect on cell viability (control, 88.3 ± 2.0%; KN-93, 87.3 ± 1.4%; t test, $p > 0.05$; assessed with Live/Dead/Viability/Cytotoxicity Assay; Invitrogen). These results suggest that activation of CaMKII after repeated DA treatment is required for the observed increase in GluR1 surface expression.

The question of how repeated DA treatment activates CaMKII is intriguing. As noted above, D_1-like receptors can increase surface expression of NMDARs in striatal tissue (Dunah and Standaert, 2001; Dunah et al., 2004; Hallett et al., 2006), which might lead to increased Ca^{2+} signaling and activation of CaMKII. Although we did not observe an increase in NMDAR surface expression after acute DA treatment (above), we tested the effect of repeated DA treatment on NR2A and NR2B surface expression using a BS^3 protein crosslinking assay. We found no significant differences in surface, intracellular, or total protein expression of either subunit after repeated DA treatment (NR2A: surface, 96.7 ± 15.0; intracellular, 111.2 ± 12.6; total, 102.9 ± 7.3; NR2B: surface, 98.7 ± 8.9; intracellular, 105.7 ± 7.4; total, 101.9 ± 7.3; all values expressed as percentage of vehicle-treated cultures; $n =$ 6–7 per group). It remains possible that D_1-like receptor activation enhances NMDAR transmission or Ca^{2+} signaling through other mechanisms, contributing to CaMKII activation (see Discussion).

Discussion

Acute D_1-like receptor modulation of AMPAR trafficking in NAc neurons

We used NAc/PFC cocultures to study dopaminergic regulation of excitatory synapses on medium spiny NAc neurons. We found that brief incubation with the D_1-like agonist SKF 81297 (1 μM, 15 min) increased AMPAR insertion on the extrasynaptic cell surface. This required PKA activity but not protein synthesis. The same D_1-like agonist treatment facilitated AMPAR synaptic delivery in response to subsequent activation of synaptic NMDARs with glycine. We hypothesize that this facilitation occurred because D_1-like agonist treatment increased the size of the extrasynaptic GluR1 pool, thereby facilitating GluR1 translocation into the synapse. This hypothesis is consistent with a two-step model for AMPAR synaptic incorporation (surface insertion followed by synaptic incorporation) (Passafaro et al., 2001) and with evidence that PKA phosphorylation of GluR1 increases its surface insertion and thereby primes its synaptic delivery (Esteban et al., 2003; Sun et al., 2005; Gao et al., 2006; Oh et al., 2006; Man et al., 2007).

An alternative explanation for facilitation of GluR1 synaptic delivery is that D_1-like receptor stimulation potentiates NMDAR transmission. This can occur through many mechanisms (Cepeda and Levine, 2006), including increased NMDAR surface expression (Dunah and Standaert, 2001). We tested this possibility but failed to detect increased NMDAR surface expression after D_1-like receptor stimulation. Future studies could determine whether other mechanisms linking the D_1 receptor–PKA path-

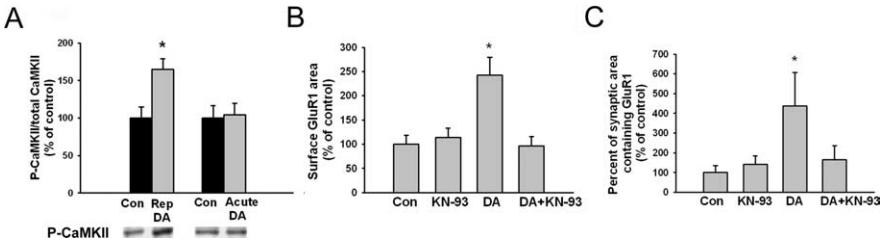

Figure 7. CaMKII activation was increased after repeated DA treatment, and CaMKII activity was required for increased GluR1 surface expression after repeated DA treatment. ***A***, Repeated (Rep) DA treatment increased CaMKII activation but acute DA treatment did not. NAc/PFC cocultures were harvested for Western blotting on day 15 after repeated treatment (days 7, 9, and 11; 30 min/d) with vehicle or DA (1 μM) or after acute treatment (30 min) with vehicle or DA (1 μM). CaMKII activation was measured as the ratio of P-CaMKII/total CaMKII. Data are presented as percentage of the control group (Con; $n = 6$, *t* test; $^*p < 0.05$ compared with control group). Representative blots are shown. Data shown are for αCaMKII but we also examined βCaMKII and found no effect of either acute or repeated DA treatment (data not shown). ***B***, The CaMKII inhibitor KN-93 blocked the increased GluR1 surface expression produced by repeated DA treatment. NAc/PFC cocultures were treated repeatedly with vehicle or DA as described above. After the last DA application on day 11, KN-93 (5 μM) was added to the media until day 15, when it was washed out before immunostaining for surface GluR1. Results are presented as the mean area of GluR1 puncta, normalized to controls ($n = 24–29$; Dunn's test, $^*p < 0.05$ compared with control group, KN-93 group, and repeated DA + KN-93 group). ***C***, The CaMKII inhibitor KN-93 blocked the increased synaptic GluR1 expression produced by repeated DA treatment. Results are presented as the percentage of synaptic area containing GluR1, normalized to the control group ($n = 20–27$; Dunn's test, $^*p < 0.05$ compared with control group).

way to NMDAR transmission contribute to D_1-like receptor facilitation of GluR1 synaptic incorporation. For example, the D_1-like receptor-PKA pathway enhances NMDAR currents in striatal neurons by modulating L-type Ca^{2+} channels (Cepeda and Levine, 2006) and PKA increases Ca^{2+} conductance of NMDARs in hippocampal neurons (Skeberdis et al., 2006). Another possible explanation is suggested by reports that PKA activity increases presynaptic release probability at some glutamate synapses (Evans et al., 2001). Arguing against this is electrophysiological evidence that D_1-like receptor activation decreases glutamate release from terminals in the NAc (Pennartz et al., 1992; Nicola et al., 1996; Nicola and Malenka, 1997).

Regardless of the mechanism, our results suggest that D_1-like and NMDA receptors work cooperatively to promote AMPAR synaptic delivery. This may underlie the requirement for coordinated D_1-like and NMDA receptor stimulation in the NAc for reward-related learning (Kelley et al., 1997; Smith-Roe and Kelley, 2000; Beninger and Gerdjikov, 2004; Dalley et al., 2005; Hernandez et al., 2005; Heusner and Palmiter, 2005).

D_1-like receptor regulation of AMPAR trafficking is lost after repeated DA treatment

Repeated cocaine exposure alters D_1-like receptor sensitivity and PKA signaling (White and Kalivas, 1998; Hope et al., 2005), suggesting that D_1-like receptor regulation of AMPAR trafficking in NAc neurons may also be altered. To test this, NAc/PFC cultures were treated repeatedly with DA (days 7, 9, and 11 *in vitro*). Four days later (day 15), cultures were incubated with the D_1-like agonist SKF 81297 (1 μM, 15 min). Although this D_1-like agonist treatment increased GluR1 surface expression in control cultures (above), it failed to do so after repeated DA treatment. If refractoriness similarly develops after repeated *in vivo* cocaine exposure, one consequence could be loss of the ability of DA to facilitate plasticity in the NAc during cocaine withdrawal.

To determine whether refractoriness reflects D_1 receptor internalization, we measured D_1 receptor surface expression on day 15 after repeated DA or vehicle treatment. Control cultures showed robust D_1 receptor surface expression at extrasynaptic sites on medium spiny neurons, but this was significantly decreased after repeated DA treatment. D_1 receptor internalization also occurs after acute D_1 receptor stimulation (Ariano et al., 1997b; Dumartin et al., 1998; Martin-Negrier et al., 2000; Bloch et al., 2003; Hara and Pickel, 2005). However, after acute agonist exposure, D_1 receptor distribution normalizes within 20 min of agonist removal (Martin-Negrier et al., 2006). This suggests that decreased D_1 receptor surface expression on day 15 is not attributable to agonist-induced internalization resulting from the final DA exposure on day 11 but is more likely an adaptation resulting from repeated DA exposure. Interestingly, total D_1 receptor protein levels were increased on day 15. D_1 receptors are resistant to degradation after internalization (Vargas and von Zastrow, 2004), so they may have accumulated intracellularly. Another recent study showed that 6 d of *in utero* cocaine exposure in rabbits persistently decreased striatal D_1 receptor surface expression and signaling, although total D_1 receptor levels were unchanged (Stanwood and Levitt, 2007).

GluR1 surface expression increases after repeated DA treatment: an *in vitro* model for cocaine-induced increases in AMPAR surface expression?

GluR1 surface expression was significantly increased after repeated DA treatment. This was attributable to repeated DA treatment, not to lingering effects of the final DA exposure. This is reminiscent of results obtained in rats treated repeatedly with cocaine to produce behavioral sensitization, a persistent enhancement of behavioral responses mediated by plasticity in addiction-related neuronal circuits (Boudreau and Wolf, 2005, 2006; Boudreau et al., 2007). Using a protein crosslinking assay, we showed that cocaine-sensitized rats exhibited increased cell surface levels of GluR1/2-containing AMPARs in the NAc after 1–3 weeks of withdrawal from cocaine. On withdrawal day 1, rats expressed locomotor sensitization but surface AMPAR levels were normal, indicating that AMPAR upregulation does not underlie locomotor sensitization. Rather, we proposed that increased AMPAR surface expression increases the responsiveness of NAc neurons to cortical and limbic glutamate inputs that trigger drug seeking and thereby contributes to sensitization of the incentive-motivational effects of cocaine (Boudreau and Wolf, 2005). A sensitization-related increase in AMPAR synaptic transmission in the NAc has also been detected electrophysiologically (Kourrich et al., 2007).

We investigated possible mechanisms of increased GluR1 surface expression after repeated DA treatment by measuring activation of CaMKII, ERK, and PKA in NAc/PFC cocultures treated repeatedly with DA or vehicle and harvested 4 d later (day 15). We focused on CaMKII, ERK, and PKA based on their roles in AMPAR synaptic targeting (Zhu et al., 2002; Esteban et al., 2003; Sun et al., 2005; Oh et al., 2006) and addiction (Gnegy, 2000; Hope et al., 2005; Lu et al., 2006). ERK phosphorylation and phosphorylation of PKA substrates were not altered by repeated DA treatment. It should be noted that our negative findings for PKA were based on averaging signals from many PKA-phosphorylated substrates. Although this approach has the advantage of not relying on a single protein as an index of PKA activity, it could overlook specific effects on relevant substrates.

In contrast, repeated DA treatment produced a significant increase in CaMKII phosphorylation at Thr286. CaMKII activation is necessary to drive AMPARs into synapses during hippocampal LTP (Hayashi et al., 2000), so we tested the hypothesis that CaMKII activation is necessary for increased GluR1 surface expression after repeated DA treatment. We found that GluR1 upregulation was prevented by inhibiting CaMKII during the 4 d period after discontinuing DA treatment. These results suggest that CaMKII is activated after repeated DA treatment and that this is necessary for GluR1 upregulation. Furthermore, they strengthen the idea that repeated DA treatment of NAc/PFC cocultures provides a model for repeated cocaine treatment *in vivo*. In addition to the fact that both treatments produce AMPAR upregulation (above), we observed CaMKII activation in the NAc at the earliest withdrawal time from cocaine (day 7) that AMPAR surface expression is increased (A. C. Boudreau and M. E. Wolf, unpublished findings). Other *in vivo* studies provide additional support for the idea that CaMKII is activated in the NAc during cocaine withdrawal and contributes to increased AMPAR transmission and other adaptations (Pierce and Kalivas, 1997; Pierce et al., 1998; Singer et al., 2006).

It is unclear how repeated DA treatment activates CaMKII, although DA receptors are linked to several signaling pathways that regulate Ca^{2+} channels or mobilize intracellular Ca^{2+} (Nicola et al., 2000; Neve et al., 2004). Once activated, CaMKII may promote AMPAR synaptic incorporation through mechanisms related to those involved in hippocampal LTP, although the CaMKII substrate important in AMPAR insertion during LTP is unknown. One possibility is that CaMKII decreases the activity of a synaptic RasGAP, leading to activation of ERK and AMPAR synaptic insertion (Rumbaugh et al., 2006 and references therein), but we did not observe ERK activation after repeated DA treatment. Interestingly, ERK is activated in the NAc of cocaine-sensitized rats (Boudreau et al., 2007), demonstrating that repeated DA treatment does not reproduce all aspects of cocaine withdrawal, as expected. Alternatively, the important CaMKII substrate could be a transmembrane AMPAR regulatory protein (TARP). TARPs are phosphorylated by CaMKII (Tsui and Malenka, 2006), and this may contribute to AMPAR synaptic incorporation during LTP (Tomita et al., 2005).

Conclusions

In medium spiny NAc neurons cocultured with PFC neurons, acute D_1-like receptor stimulation increased extrasynaptic AMPAR levels, facilitating their NMDAR-dependent synaptic incorporation. Repeated DA treatment abolished this response by decreasing D_1 receptor surface expression. Repeated DA treatment also increased AMPAR surface expression; this was associated with CaMKII activation and prevented by CaMKII inhibition. These findings, combined with *in vivo* results, suggest that psychomotor stimulants, by increasing DA levels, may initially facilitate plasticity in the NAc, perhaps contributing to learning of drug-seeking behaviors. After drug withdrawal, this regulatory mechanism may be impaired, but accumbens neurons may be more responsive to glutamate inputs that trigger drug seeking.

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