Dopamine Receptor Stimulation Modulates AMPA Receptor Synaptic Insertion in Prefrontal Cortex Neurons

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Addiction is believed to involve glutamate-dependent forms of synaptic plasticity that promote the formation of new habits focused on drug seeking. We used primary cultures of rat prefrontal cortex (PFC) neurons to explore mechanisms by which dopamine-releasing psychomotor stimulants such as cocaine and amphetamine influence synaptic plasticity, focusing on AMPA receptor trafficking because of its key role in long-term potentiation (LTP). Brief stimulation of D1 dopamine receptors increased surface expression of glutamate receptor 1 (GluR1)-containing AMPA receptors through a protein kinase A-dependent mechanism, by increasing their rate of externalization at extrasynaptic sites. Newly externalized GluR1 remained extrasynaptic under basal conditions but could be translocated into synapses by subsequent NMDA receptor activation. These results suggest that D1 receptors may facilitate LTP by increasing the AMPA receptor pool available for synaptic insertion. However, stimulation of D2 receptors decreased surface and synaptic GluR1 expression. These findings are discussed in the context of evidence that D1 and D2 receptors act independently rather than antagonistically in the intact PFC. D1 receptor facilitation of AMPA receptor synaptic insertion helps explain D1 receptor-dependent facilitation of LTP and learning in the normal brain. Abnormal engagement of this mechanism during unregulated dopamine release may account for maladaptive plasticity after repeated exposure to cocaine or amphetamine.

Key words: addiction; AMPA receptor; dopamine; LTP; plasticity; protein kinase A

Introduction
Addiction is increasingly associated with neuronal plasticity. Therefore, effects of addictive drugs on long-term potentiation (LTP) and long-term depression (LTD) have become a focus of addiction research (Kauer, 2004; Wolf et al., 2004). Dopamine (DA) receptors modulate LTP and LTD in many brain regions important for addiction (Centonze et al., 2003; Jay, 2003; Lovinger et al., 2003), as do DA-releasing psychomotor stimulants such as cocaine (Jones et al., 2000; Thomas et al., 2000, 2001; Ungless et al., 2001; Saal et al., 2003; Dong et al., 2004). However, the mechanisms involved are unclear. This study investigated the possibility that DA receptors in prefrontal cortex (PFC) modulate the synaptic trafficking of AMPA receptors, a critical mechanism for expression of LTP and LTD (Malinow and Malenka, 2002; Song and Huganir, 2002; Breit and Nicoll, 2003).

In hippocampal pyramidal cells, AMPA receptors containing glutamate receptor 1 (GluR1) or GluR4 subunits are delivered to synapses in an activity-dependent manner during LTP, whereas GluR2/GluR3-containing receptors are constitutively delivered, independent of neuronal activity (Passafaro et al., 2001; Shi et al., 2001). Synaptic GluR1 delivery during LTP requires activation of Ca2+-calmodulin-dependent protein kinase II (CaMKII), although GluR1 is not the relevant substrate (Hayashi et al., 2000).

In addition, GluR1 phosphorylation by protein kinase A (PKA) is necessary, but not sufficient, for synaptic delivery (Ehlers, 2000; Esteban et al., 2003; Lee et al., 2003; Lu et al., 2003). D1-class DA receptors are positively coupled to PKA and also regulate Ca2+ signaling (Neve et al., 2004), suggesting cellular mechanisms by which DA may influence LTP. Indeed, the D1 receptor–PKA pathway is important in modulating LTP and memory processes in many brain regions (Jay, 2003). The PFC plays a well established role in working memory (Goldman-Rakic, 1987), but is also important for addiction-related behaviors such as impulsivity (Jentsch and Taylor, 1999) and reward-related learning (Cardinal et al., 2002). Pyramidal neurons are the most common target of DA terminals in the PFC (Verney et al., 1990) and D1-class DA receptors play a particularly important role in modulating pyramidal cell excitability and plasticity (Seamans and Yang, 2004). This occurs through D1 receptor modulation of synaptic transmission and voltage-sensitive conductances. However, given the convergence of DA and glutamate inputs onto the spines of pyramidal neurons (Sesack et al., 2003) and evidence from studies of primates PFC for proximity of postsynaptic D1 receptors to glutamate synapses (Goldman-Rakic et al., 2000), we hypothesized that DA may also influence plasticity by regulating AMPA receptor trafficking at nearby glutamate synapses. This would be consistent with the key role of AMPA receptor trafficking in LTP and LTD (see above), the importance of the D1 receptor–PKA pathway for regulating LTP and LTD in the PFC (Jay et al., 1998; Gurden et al., 2000; Huang et al., 2004), and the demonstration that D1 receptor–PKA signaling regulates AMPA receptor trafficking in other brain regions (Wolf et al., 2004).

We tested this hypothesis in dissociated cultures prepared...
from postnatal rat PFC. We found that previous D1 receptor stimulation facilitates AMPA receptor synaptic insertion during NMDA receptor activation, whereas D2 receptors exert opposite effects.

Materials and Methods

Prefrontal cortex cultures. Postnatal day 1 rats were anesthetized by hypothermia on ice. The medial prefrontal cortex was isolated and dissociated with papain (20–25 U/ml; Worthington Biochemical, Lakewood, NJ) at 37°C. Cells were plated onto coverslips coated with poly-D-lysine (100 μg/ml; Sigma, St. Louis, MO) in 24-well culture plates at a density of 20,000 cells per well and grown in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine, 0.5% gentamicine, and 2% B27. 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.

Immunochemistry. Cell-surface GluR1 was labeled by incubating live cultures with polyclonal antibody recognizing the extracellular N-terminal domain of GluR1 (amino acids 271–285; RTSDK-SRHDTRVVDKR; 1:15; Oncogene, Carpinetaria, CA) in Neurobasal growth medium for 30 min. 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. Then, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked with 5% donkey serum in PBS for 60 min, and incubated with monoclonal antibody to the synaptic marker synaptobrevin (SB; vesicle-associated membrane protein 2 [VAMP-2] (1:2000; overnight at 4°C; Synaptic Systems, Goettingen, Germany) followed by donkey anti-mouse secondary antibody conjugated to Alexa 488 (1:2000; 1 h at room temperature; Molecular Probes, Eugene, OR). A punctate pattern of staining was observed for both GluR1 and synaptobrevin. Methods for quantifying total surface GluR1 expression and synaptic GluR1 expression are described below (Data analysis). For cell-surface D1 and D2 receptor staining, live cells were incubated for 30 min with antibody recognizing the extracellular second loop of the D1 receptor or antibody recognizing the extracellular N-terminal domain of the D2 receptor (gifts from Dr. Marjorie Ariano, Rosalind Franklin University of Medicine and Science/The Chicago Medical School). After fixation, donkey anti-rabbit Cy3 antibody (1:500; Jackson ImmunoResearch) was incubated with the cells at room temperature for 1 h.

In experiments with glycine, cultured neurons were treated with glycine for 3 min at room temperature in a bathing solution (140 mM NaCl, 1.3 mM CaCl2, 5.0 mM KCl, 25 mM HEPES, 10 mM glucose, 0.5 mM TTX, pH 7.4) and then transferred to the same solution without any added glycine for 15 min at room temperature (Lu et al., 2001). Cells were then immunostained for GluR1 and synaptobrevin as described above.

In some experiments, the rate of GluR1 externalization was determined by preblocking existing surface receptors with primary antibody and nonconjugated secondary antibody, bringing cells to room temperature to allow receptor externalization, and then detecting newly externalized receptors with a second round of immunostaining (Lu et al., 2001). According to the original method, preblocking steps are conducted at 4°C to minimize GluR1 trafficking (Lu et al., 2001). However, we found that PFC neurons cannot tolerate long incubations (>1 h) at this temperature. They exhibit dendritic blebbing, cell rupture, and nucleus loss (data not shown). Therefore, we modified the method by conducting preblocking steps at a slightly higher temperature (15°C) in a 5% CO2 incubator. Others have used a similar temperature (17°C) to limit AMPA receptor trafficking (Sekine-Aizawa and Huganir, 2004). According to our modified method, live cells were first incubated with the GluR1 antibody (1:15 in Neurobasal growth medium) for 30 min at 15°C in a 5% CO2 incubator (Tritech Research, Los Angeles, CA). Cells were then rinsed twice with Neurobasal medium (preequilibrated to 15°C) and then incubated with nonconjugated goat anti-rabbit antibody (5 μg/ml; Sigma) for 30 min at 15°C in a 5% CO2 incubator. Then, cells were incubated at room temperature or 37°C, either in control medium or medium containing test drugs, to allow the insertion of new GluR1 subunits into the cell membrane. After this incubation, cultures were rinsed, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and rinsed twice with PBS. To detect the newly externalized GluR1, cultures were incubated with N-GluR1 antibody (1:100) at room temperature for 1 h followed by incubation with 5% donkey serum in PBS for 1 h. After three washes with PBS, cells were incubated with Cy3-conjugated donkey anti-rabbit secondary antibody (1:500) at room temperature for 1 h. Because cells are not permeabilized, the second round of immunostaining detects only newly externalized GluR1 subunits. To determine the location of newly externalized GluR1, synapses were immunostained with mouse monoclonal antibody to synaptobrevin/VAMP-2 (1:2000) and Alexa 488 donkey anti-mouse IgG (1:2000) after permeabilization with 0.1% Triton X-100 in PBS for 15 min and incubation with 5% donkey serum in PBS for 1 h.

Data analysis. 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 run 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. Processes located about one soma diameter from the soma were selected for analysis under phase contrast imaging to avoid the possibility of experimenter bias based on the intensity of fluorescence staining. The soma was excluded in all measurements. Image analysis was performed using methods similar to those described previously and was based on measuring the area of labeled puncta rather than counting the number of labeled puncta (Beattie et al., 2002). For each image, the total area of fluorescent GluR1-stained puncta was measured automatically using a threshold that was set at least two times higher than the average background fluorescence in processes of untreated control cells. This value was then divided by the total area of the measured processes, which was determined by setting a lower threshold level to measure background fluorescence produced by the fixed cells. The same approach was used for defining the area of SB staining and the area of GluR1/synaptobrevin colocalization (SB + GluR1 area). Synaptic GluR1 incorporation was expressed as the fraction of total SB staining that overlapped with GluR1 staining (SB + GluR1).
area/total SB area). For each experimental group, results were normalized to a control group run simultaneously. All values in figures and text refer to mean ± SEM. Independent group t tests were used for comparisons between two experimental groups, and ANOVA was used to compare several groups. When ANOVA indicated significant group differences, a post hoc Dunn’s test was used to compare experimental groups to the control group (n, number of fields analyzed).

Results
Prefrontal cortex neurons express cell-surface AMPA receptors, D1 receptors, and D2 receptors
Prefrontal cortex cultures were prepared from rats on postnatal day 1. We focused on pyramidal neurons because they receive convergent DA and glutamate inputs and undergo DA-modulated synaptic plasticity (see Introduction). Experiments were performed after 2–3 weeks in culture, at which time pyramidal neurons show extensive cell-surface staining for AMPA receptors (Fig. 1A), D1 receptors (Fig. 1B), and D2 receptors (Fig. 1C). In the experiments described below, we use DA agonists that stimulate either D1-class (D1/D5) or D2-class (D2/D3/D4) DA receptors but do not distinguish between members of each class. For simplicity, we will use the terms “D1” and “D2” to refer to effects that may involve the D1-class or D2-class of DA receptors, although D1 and D2 receptors are also highly expressed in the rat PFC (Bentivoglio and Morelli, 2005) and may be involved in our observed effects.

D1 receptor stimulation increases GluR1 surface expression but not synaptic incorporation
To examine the effect of D1 receptor stimulation on cell-surface and synaptic GluR1 expression, PFC neurons were incubated with medium (control group) or the D1 agonist (2-carboxypiperazine-4-yl)三亚甲基-7,8-二氢-1-苯基-2,3,4,5-四氢-1H-3-苯并azines hydrobromide (SKF 81297) (SKF) for 5 min. Then, live cultures were incubated with antibody recognizing the extracellular N terminus of GluR1, fixed, permeabilized, and incubated with antibody to the synaptic marker synaptobrevin/VAMP-2. GluR1 and synaptobrevin staining were visualized using secondary antibodies conjugated to Cy3 and Alexa 488, respectively. Total surface GluR1 staining was determined by measuring the area of GluR1-positive puncta on processes of pyramidal neurons. Synaptic GluR1 incorporation was defined as the fraction of total synaptobrevin area that overlapped with GluR1 area. Both synaptic and nonsynaptic GluR1 staining had a punctate appearance, consistent with results in hippocampal neurons (Carroll et al., 1999).

Incubation with the D1 agonist SKF 81297 for 5 min significantly increased GluR1 surface expression in PFC neurons (Fig. 2A, C). This effect was significantly attenuated if the D1 receptor antagonist R-(-)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH 23390) (SCH) (10 μM) was added 5 min before SKF 81297 (Fig. 2A, C).

However, SKF 81297 did not produce a significant increase in overlap of synaptobrevin and GluR1 staining (Fig. 2B, D). These results indicate that SKF 81297 increased surface expression of GluR1 but not its synaptic incorporation.

The D1 agonist-induced increase in GluR1 cell-surface expression might arise from accelerated membrane insertion, or, alternatively, the same change could be achieved by slowing the rate of receptor internalization. To distinguish between these mechanisms, we selectively labeled newly inserted GluR1 using a preblocking method modified from Lu et al. (2001). Briefly, preexisting cell-surface GluR1 is masked by preblocking at 15°C with primary antibody and nonconjugated secondary antibody. Then, cells are brought to room temperature for 5 min to enable externalization of new GluR1 subunits. Newly externalized GluR1 is detected with a second round of immunostaining under nonpermeant conditions, this time using a Cy3-conjugated secondary antibody. Finally, cells are stained for synaptobrevin, under permeant conditions, to determine whether the insertion of GluR1 occurred at synapses. Cells treated with SKF 81297 (1 μM) for 5 min showed much higher levels of new surface GluR1 than control cultures, demonstrating that D1 receptor stimulation increases the rate of GluR1 externalization (Fig. 3A; quantitative analysis shown on left side of Fig. 3B). However, very little of this newly externalized GluR1 was colocalized with synaptobrevin (3.7 ± 0.5%). To better quantify synaptic GluR1 incorporation, we determined the percentage of total synaptobrevin area that...
with a D₁ agonist at 37°C for 5 min, washed to remove the D₁ agonist, and then returned to normal medium in a 37°C CO₂ incubator for 5, 25, or 55 min (the latter two time points were adjusted so SKF-treated cultures would have the same total incubation time at 37°C as control cultures). Cultures were then stained for cell-surface GluR1, permeabilized, and stained for synaptobrevin.

First, we analyzed the total amount of new cell-surface GluR1 staining (Fig. 3C). Compared with a “0 min control group,” which was fixed immediately after preblocking, surface GluR1 staining increased gradually in control cultures incubated at 37°C in normal medium (~150% of 0 min control group at 60 min). Exposure to SKF 81297 markedly accelerated GluR1 externalization. When analyzed 5 min after SKF washout, the SKF-treated cultures exhibited significantly higher surface GluR1 staining than control cultures examined after 5 min at 37°C (255 ± 22 and 103 ± 17% of 0 min control group, respectively). At 25 min after SKF washout, surface GluR1 staining had declined but was still significantly greater than in the 30 min control group. At 55 min after SKF washout, the staining was only slightly higher than the 60 min control group (Fig. 3C).

Next, we analyzed the location of newly externalized GluR1 at each time point by quantifying the fraction of total synaptobrevin area that overlapped with GluR1 area. This value was very low for the 0 min control group (2.5 ± 0.5%). However, although total surface GluR1 increased substantially during incubation in control medium at 37°C (Fig. 3C), there was very little translocation to synapses (Fig. 3D). Similarly, although synaptic GluR1 incorporation started at a slightly higher level in the SKF-treated cultures, there was very little translocation to synapses during the remainder of the 37°C incubation and no significant difference in synaptic GluR1 incorporation between the SKF-treated group and the control group at any time point (Fig. 3D). These results indicate that newly externalized GluR1, whether inserted into the membrane under basal conditions or inserted in an accelerated manner in response to D₁ receptor stimulation, remains primarily extrasynaptic for at least 1 h. This is consistent with evidence that synaptic insertion of GluR1 requires LTP or CaMKII activation (Shi et al., 1999; Hayashi et al., 2000). The existence of synaptic GluR1 in neurons stained after 2–3 weeks in culture (Fig. 2B) may be explained as the cumulative effect of a slow rate of synaptic insertion during the period in culture.

**Increased GluR1 surface expression induced by D₁ receptor stimulation requires PKA activation**

We hypothesized that D₁ receptors modulate AMPA receptor trafficking because they are positively coupled to PKA. If this is true, PKA activation should exert the same effects as D₁ receptor stimulation on GluR1 surface expression and synaptic expression. To test this, cultures were incubated for 5 min with Sp-
adrenochrome 3',5'-cyclic monophosphorothioate triethylammonium salt (SpcAMPS) (10 μM), a membrane-permeable PKA activator. As observed for the D₁ agonist, SpcAMPS produced a significant increase in GluR1 surface expression by pyramidal neurons (Fig. 4A, C) but did not produce a significant increase in overlap of GluR1 and synaptobrevin staining (Fig. 4B, D). Thus, PKA activation is sufficient for surface expression of GluR1-containing AMPA receptors but not for synaptic expression.

To verify that the PKA pathway is responsible for the D₁ receptor-induced increase in GluR1 surface expression, we first examined the ability of PKA inhibitors to prevent this effect. Cultures were incubated for 10 min with Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt (RpcAMPS) (10 μM), a membrane-permeable PKA inhibitor, and the D₁ agonist SKF 81297 (1 μM) was added for the last 5 min of the incubation. RpcAMPS blocked the D₁ agonist-induced increase in GluR1 surface expression, whereas incubation with RpcAMPS alone had no effect (Fig. 4C). Next, we examined whether PKA stimulation occluded the effect of D₁ receptor stimulation. Cultures were incubated for 10 min with a maximally effective concentration of the PKA activator SpcAMPS (10 μM), and SKF 81297 (1 μM) was added for the final 5 min of the incubation. After PKA activation, SKF 81297 was unable to further increase GluR1 surface expression (Fig. 4C), confirming that D₁ receptors influence AMPA receptor trafficking via the PKA pathway. We also examined the effect of these experimental manipulations on GluR1 synaptic expression. As expected from results shown in Figures 2 and 3, synaptic GluR1 expression was not altered by SpcAMPS and RpcAMPS, which indicates that D₁ receptor stimulation increases the extrasynaptic GluR1 pool available for synaptic insertion during LTP. To test this, we adapted methods from Lu et al. (2001), who used brief exposure to glycine (an obligatory coagonist of the NMDA receptor) to selectively activate synaptic NMDA receptors, leading to LTP of AMPA receptor-mediated miniature EPSCs and increased synaptic GluR1 expression in cultured hippocampal neurons. If our hypothesis regarding D₁ receptor activation was correct, we anticipated that previous exposure to a D₁ agonist would enable a subthreshold concentration of glycine to produce synaptic GluR1 incorporation.

D₁ receptor stimulation facilitates synaptic GluR1 insertion during NMDA receptor stimulation

Several studies have found that D₁ receptor activation facilitates LTP in the rat PFC through a PKA-dependent mechanism (Jay et al., 1998; Gurden et al., 2000; Huang et al., 2004). We hypothesized that D₁ receptor stimulation increases the extrasynaptic cell-surface pool of GluR1 in PFC neurons and thus increases the GluR1 pool available for synaptic insertion during LTP. To test this, we first adapted methods from Lu et al. (2001), who used brief exposure to glycine (an obligatory coagonist of the NMDA receptor), to selectively activate synaptic NMDA receptors, leading to LTP of AMPA receptor-mediated miniature EPSCs and increased synaptic GluR1 expression in cultured hippocampal neurons. If our hypothesis regarding D₁ receptor activation was correct, we anticipated that previous exposure to a D₁ agonist would enable a subthreshold concentration of glycine to produce synaptic GluR1 incorporation.

To identify an appropriate concentration of glycine, PFC cultures were incubated with glycine (1, 10, or 100 μM) in bathing solution (see Materials and Methods) for 3 min, rinsed, transferred to the bathing solution without glycine for 15 min, incubated for 30 min with N-terminal GluR1 antibody to label surface GluR1, and then stained for synaptobrevin after fixation and permeabilization. Glycine produced a concentration-dependent increase in both surface GluR1 expression (Fig. 5A) and synaptic GluR1 incorporation (Fig. 5B), with significant increases pro-
and synaptobrevin colocalization, whereas D1 agonist pretreatment enabled glycine to produce a significant increase in colocalization (Fig. 6B, C). This was not observed if glycine was applied before the D1 agonist (Fig. 6B, C). Facilitation also failed to occur if the D1 agonist and glycine were applied together, perhaps because D1 agonists have multiple effects on PFC excitability, some of which oppose the D1 receptor-mediated facilitation of synaptic GluR1 incorporation. For example, there is evidence that D1 agonists decrease glutamate transmission through presynaptic mechanisms in the PFC (Gao et al., 2001; Seamans et al., 2001). This would reduce NMDA receptor transmission, despite the presence of glycine, preventing newly externalized AMPA receptors from being inserted into synaptic sites.

These results show that GluR1 externalized at extrasynaptic sites by D1 receptor stimulation can be translocated to synapses by subsequent NMDA receptor activation. The slight, nonsignificant increase in synaptic GluR1 incorporation produced by SKF alone (Fig. 6B, C) may be attributable to the use of a low Mg2+ bathing solution in these experiments, which would increase NMDA receptor activity to some extent even in the absence of glycine. This might enable a small amount of the GluR1 externalized by the D1 agonist to be translocated to synaptic sites. In support of this interpretation, chemical LTP can be induced by lowering the Mg2+ concentration of the artificial CSF during forskolin/rolipram or SpcAMPS application (Otmakhov et al., 2004).

D2 receptor activation attenuates the D1 agonist-induced increase in surface GluR1 expression

To determine whether D2 receptors also modulate AMPA receptor trafficking, PFC cultures were incubated with the D2 agonist quinpirole (0.1 or 1 μM; 10 min). Quinpirole produced a trend toward decreased surface expression of GluR1 (Fig. 7A). When quinpirole (1 μM) was applied with SKF 81297 (1 μM), quinpirole significantly attenuated the increase in GluR1 surface expression produced by the D1 agonist (Fig. 7A). To further study D1 and D2 receptor interactions, we used DA and the nonselective DA receptor agonist apomorphine. Both DA (1 or 10 μM; 10 min) and apomorphine (1 or 10 μM; 10 min) significantly decreased surface GluR1 expression (Fig. 7B, C). To determine the role of D1 and D2 receptors in the effect of DA, selective D1 and D2 receptor antagonists were applied. The D1 receptor antagonist raclopride, applied 5 min before DA, reversed the inhibitory effects of DA such that GluR1 surface expression was increased over control levels (Fig. 7D). We interpret this to indicate that blocking D2 receptors unmasked the effect of D1 receptor stimulation. When the D1 receptor antagonist SCH 23390 was added 5 min before DA, the inhibitory effect of DA was greater than when DA was added alone (Fig. 7D), presumably because the D2 receptor-mediated effect was no longer opposed by D1 receptor activation. Coapplication of raclopride and SCH 23390 fully blocked the effect of DA on GluR1 surface expression (Fig. 7D). When the antagonists were administered in the absence of DA, they did not significantly influence GluR1 surface expression (Fig. 7D). Together, these results demonstrate that DA decreases GluR1 surface expression by activating D2 receptors, and that D2 receptor activation occludes the effect of D1 receptor activation in dissociated PFC cultures.

Dopamine attenuates synaptic GluR1 expression during NMDA receptor stimulation

Using a subthreshold concentration of glycine (1 μM), we demonstrated that activation of D1 receptors potentiated synaptic...
Figure 7. Dopamine decreases surface GluR1 expression on PFC neurons via D1 receptor activation. A, The D1 agonist quinpirole blocks the increased surface GluR1 expression induced by D1 receptor stimulation. Quantification of surface GluR1 expression in control neurons (medium; 10 min) and neurons treated with quinpirole (0.1 or 1 μM; 10 min), SKF 81297 (0.1 or 1 μM; 10 min), or quinpirole (1 μM) plus SKF 81297 (1 μM) (a, p < 0.05 compared with control group; b, p < 0.05 compared with SKF 81297 group; Dunn’s test; n = 24–39). B, C, Dopamine and apomorphine decrease surface GluR1 expression. Quantification of cell-surface GluR1 staining in control neurons (medium; 10 min) and neurons treated with DA (0.1, 1, or 10 μM; 10 min; n = 28–43) or apomorphine (0.1, 1, or 10 μM; 10 min; n = 26–31). Both drugs significantly decreased surface GluR1 expression (*p < 0.05; Dunn’s test). D, Quantification of cell-surface GluR1 staining in control neurons and neurons treated with raclopride (1 μM), raclopride (1 μM) plus DA (1 μM), SCH 23390 (10 μM), SCH 23390 (10 μM) plus DA (1 μM), and raclopride (1 μM) plus SCH 23390 (10 μM) plus DA (1 μM). Cells were incubated with antagonists for 5 min, and then DA was added for another 10 min (a, p < 0.05 compared with control group; b, p < 0.05 compared with raclopride plus DA group; c, p < 0.05 compared with SCH plus DA group; n = 20–37). Error bars indicate SEM. Quin, Quinpirole; Rac, raclopride; Con, control.

Discussion

Interaction of D1 and D2 DA receptors in the modulation of AMPA receptor trafficking in the PFC

Activation of the D1 receptor–PKA signaling pathway increased GluR1 surface expression in pyramidal neurons of the PFC by increasing the rate of GluR1 externalization. The new receptors were found at extrasynaptic sites, indicating that D1 receptor stimulation is not sufficient for synaptic GluR1 insertion. However, they could be translocated into synapses by subsequent activation of synaptic NMDA receptors. These results provide direct support for a two-step process of GluR1 synaptic incorporation consisting of insertion into extrasynaptic sites followed by lateral movement into synapses (Passafaro et al., 2001; Bredt and Nicoll, 2003). Furthermore, they support and extend results in organotypic hippocampal slices demonstrating that PKA phosphorylation is necessary but not sufficient to drive GluR1 into synapses; CaMKII must also be activated (Esteban et al., 2003). Other findings also support a role for PKA phosphorylation in AMPA receptor trafficking during LTP (Ehlers, 2000; Lee et al., 2003; Lu et al., 2003). Together, these results suggest that PKA phosphorylation of GluR1 sets the number of AMPA receptors available for synaptic delivery. CaMKII activates the cellular machinery that results in GluR1 synaptic delivery (Lisman et al., 2002).

D2 receptor stimulation exerted opposite effects on GluR1 trafficking to those of D1 agonists, and when both receptors were activated by DA, the D2 effect occluded the D1 effect. Does this mean that the net effect of endogenous DA transmission on AMPA receptor externalization in the intact PFC is inhibitory and that observed effects of D1 receptor signaling are therefore not important? On the contrary, D1 receptor signaling plays a dominant role in the intact PFC, as evidenced by studies showing that optimal working memory performance, the best studied measure of PFC function, depends mainly on D1 receptor activation both in primates (Sawaguchi and Goldman-Rakic, 1991) and rodents (Zahrt et al., 1997; Seamans et al., 1998). Similarly, DA regulates synaptic plasticity in the PFC of the intact rat primarily through activation of D1 receptors (Jay, 2003).

Figure 8. Dopamine attenuates synaptic GluR1 expression during NMDA receptor stimulation. A, DA attenuates the increase in GluR1 surface expression produced by glycine (10 μM). Cultures were treated with medium, DA (1 μM), or glycine (10 μM). Cultures in the DA–glycine group were treated with DA for 10 min, rinsed, and treated for 3 min with glycine. The order of drug exposure was reversed in the glycine→DA group (a, p < 0.05 compared with control group; b, p < 0.05 compared with glycine group; Dunn’s test; n = 21–34). B, DA attenuates the increase in GluR1 synaptic incorporation produced by glycine (10 μM). Data are from the same experimental groups as in A. Results are presented as the percentage of the total SB area that overlaps with GluR1 area, normalized to the control group (a, p < 0.05 compared with control group; b, p < 0.05 compared with glycine group; Dunn’s test; n = 21–34). Error bars indicate SEM.
How can the dominant role for D₁ receptors be reconciled with the ability of D₂ agonists to occlude the effect of D₁ agonists in PFC cultures? A solution is suggested by evidence that D₁ and D₂ receptors signal independently, rather than antagonistically, in the intact PFC. One reason is that low concentrations of DA (nanomolar) selectively activate D₁ receptors in the PFC, whereas higher concentrations (micromolar) are required to activate D₂ receptors (Zheng et al., 1999; Trantham-Davidson et al., 2004). The lower, D₁-preferring, concentrations of DA correspond to those measured in the PFC during working memory tasks in rats (Phillips et al., 2004). DA levels may reach micromolar concentrations near release sites before diffusing away to nanomolar concentrations in the extracellular space (Kawagoe et al., 1992; Garris and Wightman, 1994). High micromolar concentrations associated with particularly alerting stimuli or stress may selectively activate D₂ receptors closer to release sites, rather than the predominantly extrasynaptic D₁ receptors (Smiley et al., 1994; Yung et al., 1995; Caille et al., 1996). Independent D₁ and D₂ receptor signaling may also reflect the abundance of D₁-like receptors in the PFC (Bentivoglio and Morelli, 2005) and different patterns of cellular localization for D₁ and D₂ receptors in the PFC (Vincent et al., 1993; Gaspar et al., 1995). The important point is that physiological DA levels in the PFC exert important functional effects by activating D₁ receptor signaling in isolation from D₂ receptor signaling, justifying consideration of the functional consequences of independently activating the D₁ receptor signaling pathway.

**D₁ receptors and plasticity in the PFC**

Experiments in anesthetized rats have found that tetanic stimulation of hippocampal projections to the PFC produces NMDA receptor-dependent LTP that is enhanced by locally applied DA or by stimulation of the ventral tegmental area (VTA) and reduced by VTA lesions (Gurden et al., 1999). This effect of DA is mediated via activation of D₁ receptors through a PKA-dependent mechanism (Jay et al., 1998; Gurden et al., 2000). Our results suggest an explanation for these observations, that is, D₁ receptor stimulation increases the extrasynaptic pool of AMPA receptors available for synaptic insertion in response to NMDA receptor stimulation during the tetanus. This mechanism may also underlie a different type of DA-induced plasticity in the PFC. In PFC slices, bath application of DA normally favors the emergence of LTD (Law-Tho et al., 1995; Otani et al., 1998). However, if DA is applied, washed out, and then added to the bath again in conjunction with high-frequency stimulation, LTP is induced instead of LTD (Blond et al., 2002). This may reflect a priming effect of D₁ receptor stimulation during the first bath application of DA on AMPA receptor synaptic insertion during the second stimulation period. Other results support the idea that D₁ receptors can facilitate both LTD and LTP in PFC neurons under appropriate experimental conditions (Huang et al., 2004).

Extrapolating to the behavioral level, our results help explain the requirement for coordinated D₁–PKA signaling and NMDA receptor activation in the PFC during appetitive learning (Baldwin et al., 2002) and perhaps in working memory (Jay, 2003; Seamans and Yang, 2004), although it should be noted that the D₁–PKA pathway has other important cellular targets in the PFC. The D₁–PKA signaling pathway also contributes to learning and memory processes in other brain regions, including the hippocampus and the striatal complex (Berke and Hyman, 2000; Jay, 2003; Beninger and Gerdjikov, 2004; Kelley, 2004). Facilitation of synaptic GluR1 insertion may contribute to D₁ receptor effects in these regions, based on the PKA dependence of AMPA receptor externalization in hippocampus (see above) and our results showing that D₁ receptor stimulation accelerates AMPA receptor externalization in nucleus accumbens neurons (Chao et al., 2002; Mangiavacchi and Wolf, 2004).

Our results may also be relevant to the role of DA in regulating the excitability of PFC pyramidal neurons. This role is very complex, because DA receptors are located postsynaptically but also on terminals and interneurons, and have many effects on voltage-gated and synaptic currents (Seamans and Yang, 2004). One important variable in determining the effect of DA is the time elapsed between applying DA and the electrophysiological test (Otani et al., 2003; Seamans and Yang, 2004). Experiments using intracellular current injection to assess the excitability of deep-layer pyramidal neurons in rodent PFC have found a transient depression in excitability that is primarily D₂ receptor-mediated and a delayed but prolonged increase in excitability that is mediated by D₁ receptors (Yang and Seamans, 1996; Gulledge and Jaffe, 1998, 2001; Gorelova and Yang, 2000; Seamans and Yang, 2004) (for review, see Otani et al., 2003). Similarly, D₁ receptor agonists reduce NMDA receptor currents in PFC neurons, whereas D₂ agonists increase them (Zheng et al., 1999; Seamans et al., 2001). The prolonged increase in excitability produced by D₁ agonists helps explain their role in maintaining the depolarized up state in pyramidal neurons, a state that facilitates action potentials and plasticity (O’Donnell, 2003), and may contribute to self-sustaining activity in PFC neurons during working memory (Durstewitz and Seamans, 2002; Seamans and Yang, 2004).

D₁ receptors increase the excitability of PFC pyramidal neurons by modulating voltage-sensitive conductances (Seamans and Yang, 2004). However, D₁ receptor facilitation of AMPA receptor externalization may also contribute. By increasing the number of AMPA receptors on the cell surface, D₁ receptor stimulation would ultimately lead to increased excitatory transmission, provided that NMDA receptor stimulation is sufficient to produce synaptic insertion of AMPA receptors once they have been externalized by D₁ receptor stimulation. This mechanism predicts an activity-dependent increase in excitability that would manifest slowly, consistent with electrophysiological studies of D₁ receptor function (see above). When levels of glutamate transmission rise, this same mechanism may help to facilitate the induction of LTP, as discussed above. It is interesting to speculate that synaptic targeting of AMPA receptors after D₁ receptor stimulation may be facilitated by D₁ receptor-induced increases in the sensitivity of postsynaptic NMDA receptors (Seamans et al., 2001; Wang and O’Donnell, 2001).

The ability of D₁ receptors to facilitate AMPA receptor synaptic targeting in PFC cultures was dependent on the temporal relationship between activation of D₁ and NMDA receptors. Facilitation occurred when D₁ receptor stimulation preceded NMDA receptor stimulation, but not when NMDA receptor stimulation occurred first, presumably because the increase in the extrasynaptic pool of AMPA receptors must precede NMDA receptor stimulation. Strikingly similar temporal requirements were observed in experiments showing that brief exposure to a novel environment enhanced the ability of a weak tetanus to induce LTP in hippocampal CA1 neurons (Li et al., 2003). Novelty-induced facilitation of LTP was mediated by D₁ receptor–PKA signaling and occurred only if exposure to the novel environment preceded the weak tetanus, not if the order was reversed, and could be abolished by introducing a delay between novelty and the weak tetanus. Summing up, there appears to be a temporal window shortly after D₁ receptor stimulation during
which neuronal activation is more likely to produce synaptic insertion of AMPA receptors leading to LTP.

**D2 receptors and plasticity in the PFC**

In contrast to D1 receptors, the role of D2 receptors in PFC function is less clear. Recent evidence suggests that they modulate specific components of working memory (Wang et al., 2004). As mentioned above, one consequence of activating D2 receptors in the PFC is a rapid depression of the excitability of PFC pyramidal neurons that results from activation of GABA transmission and modulation of a Na+ conductance (Gulledge and Jaffe, 1998, 2001). These effects may contribute to the important role of D2 receptors in facilitating LTD induction in PFC slices (Law-Tho et al., 1995; Otani et al., 1998, 1999). Our results, showing that D2 receptors decrease AMPA receptor surface and synaptic expression, suggest an additional mechanism by which D2 receptors may depress neuronal excitability and plasticity. Inhibition of PKA is unlikely to account for this effect of D2 receptor activation, because a PKA inhibitor did not reproduce the effect of a D2 agonist on AMPA receptor trafficking (Fig. 4). D2-class receptors influence many signaling molecules in addition to adenylyl cyclase, including ion channels, MAP (mitogen-activated protein) kinases, and phospholipases (Neve et al., 2004).

**Conclusions**

Our results suggest that DA receptors may regulate synaptic plasticity by modulating AMPA receptor surface and synaptic expression. Under normal circumstances, this may contribute to the role of DA in learning adaptive behaviors important for survival (Kelley and Berridge, 2002). During repeated cocaine exposure, unregulated DA receptor signaling may lead to inappropriate modulation of AMPA receptor trafficking and abnormal synaptic plasticity, which in turn may trigger inappropriate synaptic re-modeling (Robinson and Kolb, 1999). These processes may contribute to the rewiring of neuronal circuits that underlies the transition from casual to compulsive drug use.

**References**


