Greengard 2001; Flores-Hernandez *et al.* 2002; Seamans and Yang 2004). However, emerging evidence indicates that D_1 can also activate other types of G proteins, thereby activating Ca^{2+} and protein kinase C (PKC) (Chergui and Lacey 1999; Chen *et al.* 2004; Neve *et al.* 2004). In addition, dynamic trafficking of NMDA receptors to and from the synaptic sites is increasingly appreciated as a key determinant in synaptic transmission and plasticity (Wenthold *et al.* 2003; Nong *et al.* 2004), as well as in pathologic states such as schizophrenia (Lau and Zukin 2007). NMDA receptor subunits can be directly phosphorylated by serine/threonine kinases PKA and PKC (Scott *et al.* 2003; Chen and Roche 2007) or Src family kinases (Salter and Kalia 2004). Therefore, an important question is how NMDA receptor trafficking is regulated by DA and what are the roles of PKA and PKC in the D_1-mediated regulation of NMDA receptor trafficking. In this study, we hypothesized that D_1 activation enhances NMDA receptor trafficking through rapid synaptic insertion of NMDA receptors, which might be differentially regulated by PKA and PKC. We tested the effects of PKA and PKC inhibitors on D_1-induced trafficking processes of NMDA receptors, including total expression, surface expression, synaptic insertion, and synaptic function. We found that although both PKA and PKC mediate the D_1-induced NMDA receptor trafficking, the phospholipase C (PLC)-PKC-Src pathway is only required for the surface expression and synaptic insertion of NMDA receptors.

Experimental procedures

Detailed experimental protocols can be found in the Appendix S1.

Electrophysiology in PFC slices

Rats at P12–30 were deeply anesthetized and the brains were sliced at 300 μm (Gao *et al.* 2001). Whole-cell recordings were conducted in medial PFC and the recordings were conducted at ~35°C. To record NMDAR mEPSCs, cells were held at −60 mV in Mg^{2+}-free solution with perfusion of tetrodotoxin (TTX, 0.5 μM), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, 20 μM), and picrotoxin (50–100 μM, see Figure S1). The NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) were evoked by stimulating layer 2/3 in the presence of Mg^{2+} (1 mM), picrotoxin (50–100 μM) and NBQX (20 μM) at +40 mV. In some cells, NMDA (100 μM) was puff-applied from a glass pipette (~2 MΩ) and NMDA currents were recorded at −60 mV under same conditions. The electric signals were amplified and acquired with pCLAMP 9.2. A typical NMDA mEPSC with 20–80% rise time 7 ms and 63% decay time 240 ms was selected to create a sample template for detecting mEPSCs. The frequency (event number) and amplitude of individual mEPSC events were automatically detected with Clampfit by using the template. The amplitudes of the evoked EPSCs were measured by averaging 30 traces from the onset to peak of EPSC.

Neuronal cultures and treatment schedules

Neurons at 16–18 days *in vitro* were treated for 10 min with SKF-81297 (1 μM). For inhibitor and antagonist experiments, cells were pre-incubated in the following drugs (Sigma, St. Louis, MO, USA) and then co-applied with SKF-81297 at the indicated times and concentrations: KT5720 (20 min, 5 μM), Go6983 (10 min, 20 μM), SCH-23390 (10 min, 10 μM), U73122 (10 min, 10 μM), 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (10 min, 10 μM) and inactive structural analogue PP3 (10 min, 10 μM).

Immunocytochemical analysis

(i) *Immunostaining of total protein:* Immunocytochemical analysis was performed according to established protocols (Glynn and McAllister 2006). Briefly, coverslips were fixed with methanol for 10 min followed by permeabilization for 5 min in 0.25% Triton X-100. Primary antibodies were visualized with FITC- or Cy3-conjugated secondary antibodies (1 : 500–1 : 1000). The specificity of all of these antibodies has been demonstrated previously and was rechecked (see Figure S2). (ii) *Surface labeling:* We labeled surface NMDA receptor (NR) 2B using an NR2B *N*-terminal antibody (Zymed Laboratories Inc., South San Francisco, CA, USA). The labeling was performed as previously described (Lu *et al.* 2001). Live cultures were incubated with antibody for 30 min. Cells were then fixed for 15 min, blocked with 5% donkey serum for 60 min, and incubated with secondary antibody conjugated to FITC for 60 min under non-permeant conditions. (iii) *Receptor insertion assay:* The rate of NR2B insertion is determined by pre-blocking existing surface receptors with primary antibody and non-conjugated secondary antibody, bringing cells to 20–22°C to allow receptor insertion, and then detecting newly inserted receptors with a second round of immunostaining (Lu *et al.* 2001).

Image analysis

All groups were analyzed simultaneously using cells from the same culture preparation. For each experimental group, cells from at least three different wells were used, and approximately five cells from each well were analyzed. Processes located about 1 soma diameter from the soma were selected for analysis. Immunocytochemical quantification was performed using ImageJ software (Glynn and McAllister 2006).

Results

D_1 activation potentiates NMDA receptor-mediated mEPSCs in PFC pyramidal neurons

We first recorded NMDA mEPSCs from layer V pyramidal neurons in medial PFC in the presence of the sodium channel blocker TTX (0.5–1 μM), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor antagonist NBQX (20 μM), and $GABA_A$ receptor antagonist picrotoxin (50–100 μM) under conditions of zero-Mg^{2+} with membrane potential held at −60 mV. After baseline recording for 5–10 min (Fig. 1), we tested the effect of dopamine D_1 agonist SKF-81297 on the mEPSCs. Bath application of SKF-81297 (1 μM) resulted in a significant potentiation of mEPSCs. As

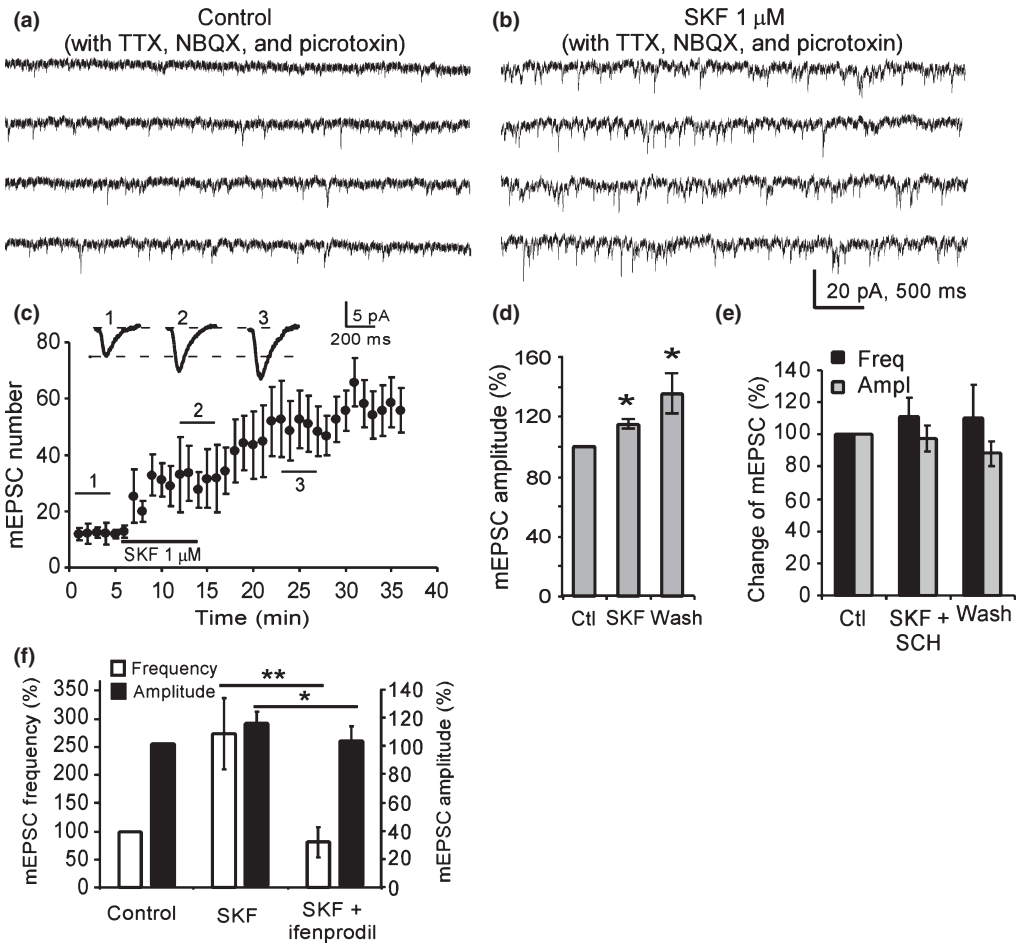

Fig. 1 D_1 activation enhances NMDA receptor-mediated synaptic responses. (a) An example of NMDA mEPSC recorded at −60 mV in zero-Mg^{2+} solution. The NMDA mEPSCs were clearly detectable under the control condition. (b) The NMDAR mEPSCs from the same cell in panel (a) were significantly increased by D_1 agonist SKF-81297 (1 μM). (c) Time course showing the significant increase of NMDA mEPSC numbers ($n = 6$, $p < 0.01$). Insets denote the average traces in the marked periods. (d) Summary graphs show that the amplitudes of NMDAR mEPSCs were significantly increased during and after washing out of SKF-81297 ($n = 6$, $^*p > 0.05$). (e) SKF-81297-induced changes in mEPSC frequency and amplitude were prevented by pre-treatment with D_1 antagonist SCH-23390 (10 μM, $n = 6$, $p > 0.05$). (f) Bath application of specific NR2B antagonist ifenprodil (3 μM) effectively blocked the potentiation of NMDA mEPSCs induced by SKF-81297 ($n = 6$; $^{**}p < 0.01$).

shown in Fig. 1(a–d), both frequency and amplitude of NMDAR mEPSCs were significantly increased during and after washing out of SKF-81297 ($n = 6$, $p < 0.05$ for both). The NMDA mEPSCs were effectively blocked by NMDA receptor antagonist (2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate (APV, 50 μM, data not shown). Under conditions of zero-Mg^{2+}, both frequency and amplitude of mEPSCs appeared to be stable, without significance, in the absence of SKF-81297 ($n = 4$; $p > 0.05$, see Figure S1). To confirm the D_1 action, D_1 antagonist SCH-23390 (10 μM) was administered for 5 min and then SCH-23390 and SKF-81297 were co-applied for 10 min. SCH-23390 itself exhibited no significant effects on the NMDA mEPSCs (data not shown). Under this condition, SCH-23390 effectively blocked the potentiation of NMDA mEPSCs caused by SKF-81297 in both frequency and amplitude ($n = 6$; $p > 0.05$, Fig. 1e). In another set of experiments, we recorded the AMPA receptor-mediated mEPSCs at −60 mV in the presence of (2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate (APV), TTX, and picrotoxin. Under this condition, we found the neither frequency nor amplitude of the AMPA mEPSCs was changed by D_1 agonist SKF-81297 (1 μM) ($n = 6$, $p > 0.05$, Figure S3).

Next, we determined which NMDA receptor subunits are involved in D_1-mediated regulation of NMDA mEPSCs. Bath application of specific NR2B antagonist ifenprodil (3 μM), which preferentially inhibits NR1/

NR2B channels (IC_{50} = 0.34 μM) (Williams 1993; Tovar and Westbrook 1999), effectively blocked the potentiation of NMDA mEPSCs caused by SKF-81297 (Fig. 1f, $n = 6$; $p < 0.05$). These data suggest that D_1-mediated potentiation of NMDA mEPSCs mainly depends on NR1/NR2B, although NR1/NR2A cannot be excluded. Taken together, these data indicate that D_1 activation potentiates NMDA mEPSCs in PFC and the D_1 agonist is likely to initiate an intracellular signal pathway to strengthen synaptic transmission.

D_1 activation enhances NMDA-induced current and NMDA receptor-mediated EPSCs in PFC slices

Either pre- or post-synaptic mechanisms could be responsible for the potentiation of NMDA mEPSCs by SKF-81297. To differentiate between these possibilities, we directly examined SKF-81297 effect on NMDA-induced current. NMDA (100 μM) was applied every 90 s for 2 s through a glass pipette (~2 MΩ) to elicit NMDA current in the presence of Mg^{2+} (1 mM), TTX (0.5 μM), NBQX (20 μM), and picrotoxin (50 μM) at −60 mV. After application of SKF-81297 (1 μM), NMDA-induced current was gradually increased (Fig. 2a). The maximal amplitude of NMDA current increased by 50.4 ± 11.41% in SKF-81297 ($n = 5$; $p < 0.01$; Fig. 2b). Furthermore, we examined the EPSC response to paired pulses, a measure that is exquisitely sensitive to transmitter release. As shown in Fig. 2(c), NMDA EPSCs were elicited by stimulating intracortical fibers in layer 2/3 with two successive stimuli of identical strength at an interval of 100 ms (10 Hz) before and after application of SKF-81297. Although the amplitudes of both the first and the second EPSCs were increased by SKF-81297 (Fig. 2d), the ratio of paired pulses was slightly decreased without significant difference ($n = 7$, $p > 0.05$; Fig. 2e). In addition, no significant difference in amplitude variability was found, as shown in analysis of coefficiency of variation (Fig. 2f). These results suggest that the effect of D_1 on NMDA mEPSCs is very likely attributable to the changes of post-synaptic NMDA receptors, although altered pre-synaptic glutamate release cannot be completely excluded.

D_1 regulation of NMDA mEPSCs involves both PKA and PKC signaling pathways

D_1 activation-induced enhancement of NMDA responses can be mediated by a number of redundant and cooperative signaling cascades (Seamans and Yang 2004; Cepeda and Levine 2006). The most prominent action is through the activation of D_1 receptor-mediated intracellular cAMP/PKA/DARPP-32 cascade (Blank *et al.* 1997; Cepeda *et al.* 1998; Snyder *et al.* 1998; Flores-Hernandez *et al.* 2002). Previous studies have also shown that PKC plays critical roles in the D_1-NMDA interactions (Chergui and Lacey 1999; Chen *et al.* 2004). In order to explore the signaling pathway involved in the D_1-mediated enhancement of NMDA currents, we first bath-applied PKA or PKC inhibitor to examine its effect in NMDA mEPSCs. After baseline recording, we pre-treated with PKA inhibitor KT5720 (5 μM) or H89 (20 μM), or PKC inhibitor Go6983 (20 μM), respectively, for 5 min, and then administered one of these drugs with SKF-81297 (1 μM) for 10 min. As shown in Fig. 3(a), the D_1-mediated effects on the frequency of NMDA mEPSCs were partially but nonetheless significantly blocked by either PKA or PKC inhibitor ($p < 0.05$ vs. control and $p < 0.01$ vs. SKF only). On the other hand, these drugs not only completely blocked the effects of SKF-81297 ($p < 0.01$ for all) but also induced slight decrease of mEPSC amplitudes in PKA inhibitor KT5720 (but not H89) and PKC inhibitor Go6983. The

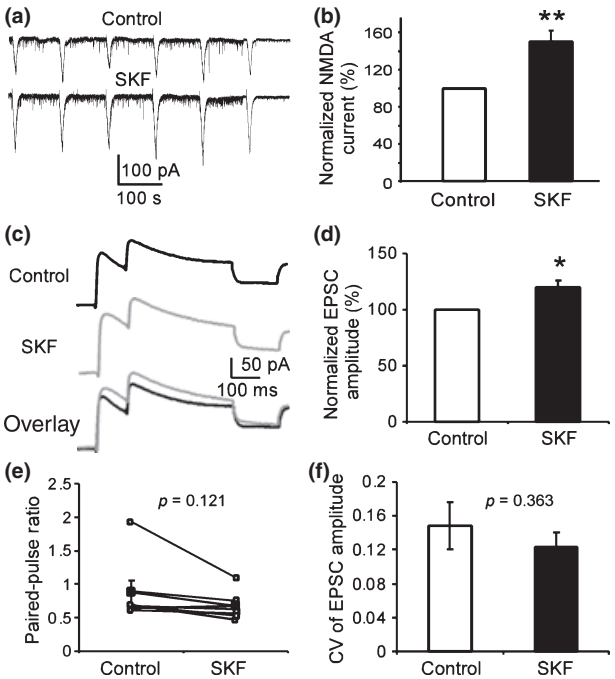

Fig. 2 SKF-81297 potentiates the mEPSC through effects on the NMDA receptors in the post-synaptic site. (a and b) Representative traces of the inward current (at −60 mV) induced by puff-applied NMDA (100 μM, every 2 s for 90 s) in the presence of TTX (0.5 μM), NBQX (20 μM) and picrotoxin (100 μM). The NMDA-induced currents were significantly potentiated by bath-applied SKF-81297 (1 μM), with maximum amplitude increased by 50.4 ± 11.4% ($n = 5$, $^{**}p < 0.01$). (c) Sample traces showing the effects of D_1 on NMDA EPSCs evoked by stimulating layer 2/3 with a paired-pulse stimulation (inter-stimulus interval = 100 ms or 10 Hz) in the presence of picrotoxin (100 μM) and NBQX (20 μM) with membrane potential held at +40 mV. After 5 min stable baseline recording, SKF-81297 (1 μM) was bath-applied to the PFC slices. (d) Summary histogram showing that the amplitude of the first NMDA EPSC was significantly increased by SKF-81297 application ($n = 7$, $^{*}p < 0.05$). (e and f) The paired-pulse ratio and coefficient of variation of the NMDAR EPSCs were unaltered by SKF-81297 ($p > 0.05$ for both).

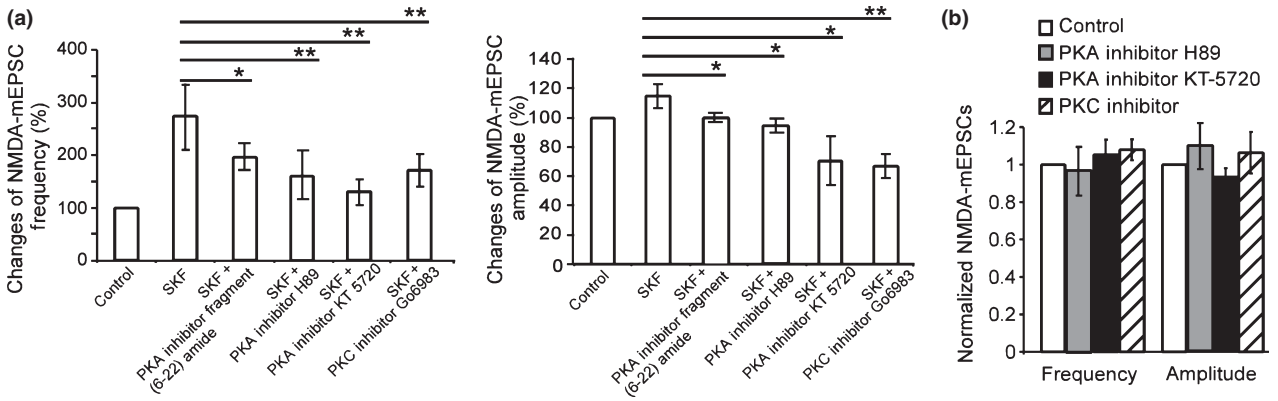

Fig. 3 D_1 agonist-induced increase of NMDA-mEPSCs was attenuated by PKA inhibitor and PKC inhibitor. (a) The SKF-81297-induced potentiation of both NMDA mEPSC frequency and amplitude was significantly decreased by co-application of PKA inhibitor H89 (20 μM), KT5720 (5 μM), or PKC inhibitor Go6983 (20 μM) with SKF-81297 (1 μM) ($n = 6$, $^*p < 0.05$, $^{**}p < 0.01$). Dialysis with PKA inhibitory peptide PKI6–22 also significantly blocked the SKF-induced changes in the NMDA mEPSC frequency and amplitude ($n = 6$, $p < 0.05$). (b) Neither frequency nor amplitude of NMDA mEPSCs was affected by PKA inhibitor H89, KT5720, or PKC inhibitor Go6983 administered alone without SKF ($n = 6$, $p > 0.05$).

reason for this discrepancy is unknown. The mEPSC frequency changes do not follow the same pattern with the changes in amplitudes, but were consistent with several previous studies that have also reported a slight decrease of EPSC amplitude under similar conditions (Huang and Kandel 1995; Chergui and Lacey 1999; Smith *et al.* 2005). To further investigate the role of PKA in the enhancement of NMDA currents, we included a membrane-impermeant PKA inhibitor peptide fragment (PKI6–22) amide (20 μM) in the recording pipette in one set of experiments, Dialysis with PKI6–22 significantly blocked the SKF-induced changes in the NMDA mEPSC frequency and amplitude ($n = 6$, $p < 0.05$, Fig. 3a). This result was consistent with a previous study (Smith *et al.* 2005) but differed from another report (Chen *et al.* 2004). In addition, neither frequency nor amplitude of NMDA mEPSCs was affected by PKC inhibitor or PKA inhibitor administered alone without SKF ($n = 6$, $p > 0.05$, Fig. 3b). These data suggested that not only PKA, PKC is also involved in the D_1-induced regulation of NMDA currents, consistent with previous reports (Neve *et al.* 2004; Seamans and Yang 2004; Gu *et al.* 2007). It is therefore possible that PKA and PKC coordinately mediate the D_1 effects on NMDA receptors (Scott *et al.* 2003; Chen and Roche 2007).

D_1-mediated increase of dendritic localization of NMDA receptors depends on PKA but not PKC signaling

To directly address the role of D_1 activation on NMDA receptor trafficking, we treated cultured PFC neurons with or without D_1 agonist SKF-81297 and measured the cluster number and fluorescence intensity of NR2B subunits. Because efforts to identify antibodies that are capable of labeling surface NR1 and NR2A subunits were unsuccessful (Hallett *et al.* 2006) (Li *et al.*, unpublished observations) and NR2B subunits are mainly regulated by D_1 activation (see Fig. 1f) (Gao and Wolf 2008), we focused our effort on NR2B. Treatment with SKF-81297 (1 μM) for 10 min led to significant increase of dendritic localization of NR2B subunits (Fig. 4(a–l) and m) (cluster numbers: 39.1 ± 1.09 in control vs. 65.4 ± 1.07 in SKF-81297, $n = 20$, $p < 0.05$). D_1 antagonist SCH-23390 (10 μM) blocked the effect of SKF-81297 on NMDA receptors (cluster number: 32.44 ± 1.32, $n = 20$; $p < 0.01$ compared with the SKF-81297 group). D_1 activation by SKF-81297, however, exhibited no alterations in cluster number or fluorescence intensity of postsynaptic density-95 (PSD-95), a scaffold post-synaptic protein closely associated with NMDA receptors (see Figure S4). Interestingly, inclusion of PKA inhibitor KT5720 (5 μM), but not PKC inhibitor Go6983 (20 μM), effectively blocked the D_1 effect on total NR2B expression (Fig. 4m, cluster numbers: SKF + PKA inhibitor, 42.7 ± 6.1, $n = 20$, $p > 0.05$; SKF + PKC inhibitor, 54.2 ± 9.8, $n = 20$, $p < 0.05$). These data suggest that PKA, but not PKC, mediated the D_1 activation-induced increase of total NR2B subunits at the neuronal dendrites.

Both PKA and PKC mediate D_1-induced increase of surface expression of NMDA receptors

To directly measure the effect of D_1 activation on the surface expression of NMDA receptors, neurons treated with SKF-81297 were labeled with an antibody against the extracellular *N*-terminal region of NR2B. Using the surface labeling method (Hallett *et al.* 2006), control neurons stained under non-permeabilizing conditions showed surface staining for endogenous NR2B in a punctate pattern that was expressed on the dendrites (Fig. 5a,b,a' and b'). Treatment with SKF-81297 increased the NR2B surface labeling on PFC neuronal dendrites, measured by either the NR2B cluster number

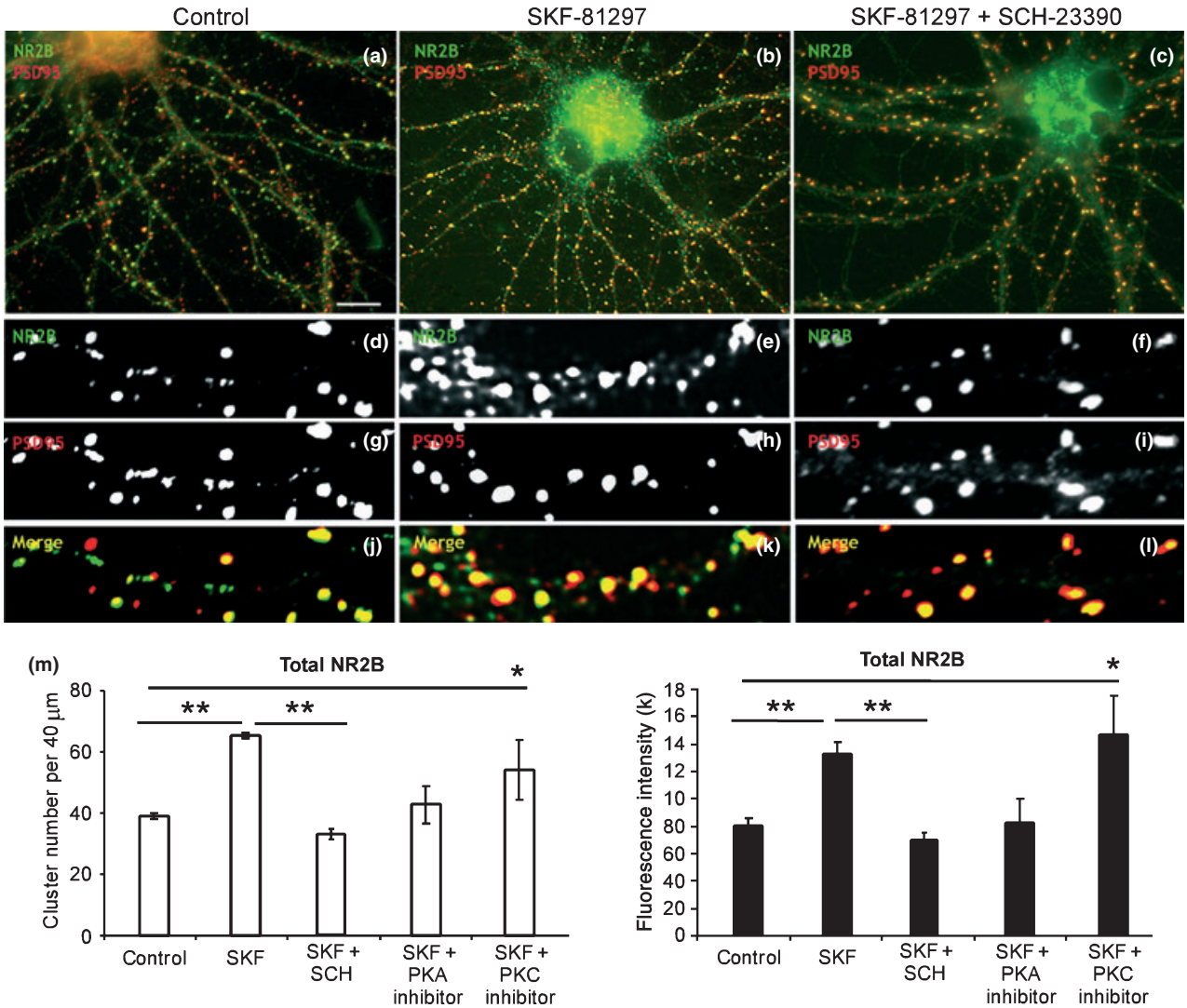

Fig. 4 D_1 activation increases total NR2B clusters in permeabilizing conditions. (a–l), PFC neurons at 16 days *in vitro* (DIV 16) were treated with vehicle (a, d, g, j), SKF-81297 (1 μM) (b, e, h, k) or SKF-81297 in the presence of SCH (10 μM) (c, f, i, l), respectively, and double labeled for endogenous NR2B (green) and PSD-95 (red) (scale bar = 5 μm). (m) Quantification of total NR2B immunofluorescence staining. SKF-81297 significantly increased total NR2B puncta number and fluorescence intensity compared with control ($n = 20$, $^*p < 0.05$, $^{**}p < 0.01$). PKA inhibitor KT5720 (5 μM), but not PKC inhibitor Go6983 (20 μM), completely blocked D_1 effect on NR2B cluster numbers and fluorescence intensity ($n = 20$, $^{**}p < 0.01$).

(control: 15.2 ± 0.6, $n = 12$; SKF: 20.6 ± 0.9, $n = 12$, $p < 0.01$) or fluorescence intensity ($p < 0.01$) (Fig. 5c). We further tested the signaling pathways required in the increased NR2B cell surface labeling induced by D_1 activation. Previous studies suggested that PKA mediates GluR1 trafficking/surface expression (Snyder *et al.* 2005; Gao *et al.* 2006; Man *et al.* 2007; Serulle *et al.* 2007) but not NMDA receptors (Gao and Wolf 2008). In contrast, long-term potentiation leads to rapid surface expression of NMDARs but not AMPA receptors in a PKC- and Src-family-dependent manner (Grosshans *et al.* 2002). We therefore wondered whether PKA and PKC play different roles in the D_1-mediated trafficking of NMDA receptors. Consistent with a role of cAMP/PKA in D_1 receptor signaling, inclusion of PKA inhibitor KT5720 (5 μM) effectively blocked the effect of SKF-81297 (1 μM) on surface NR2B expression (cluster numbers: control 16.6 ± 1.2; SKF 22.4 ± 1.8; SKF + SCH 17.6 ± 2.1; SKF + PKA inhibitor 17.8 ± 0.5; $n = 12$; $p > 0.05$ for all comparisons, Fig. 5c). In the cortical and hippocampal neurons, the PKC pathway is known to regulate NMDA receptor function (Lan *et al.* 2001; Grosshans *et al.* 2002; Salter and Kalia 2004; Lin *et al.* 2006), although it is unknown whether this pathway also plays a role in D_1 modulation of NMDA

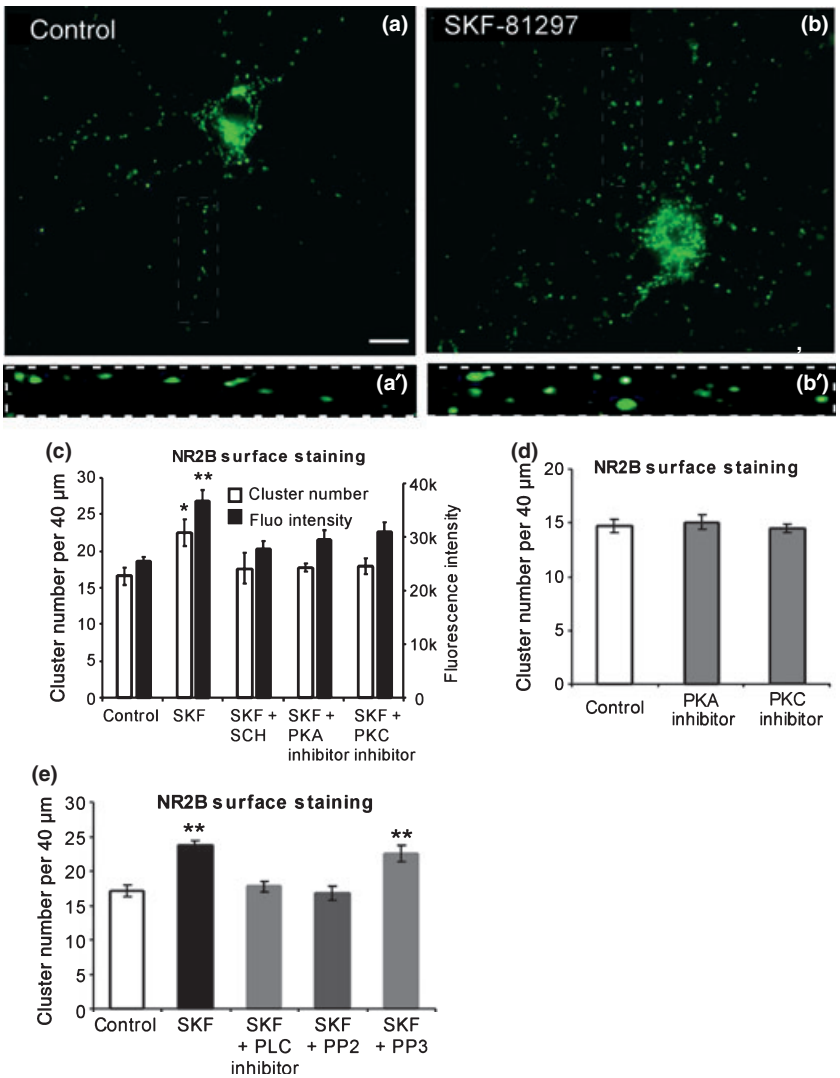

Fig. 5 D_1-mediated increase in cell surface expression of NR2B is mediated by both PKA and PLC-PKC-Src pathways. (a and b, a' and b') Neurons treated with various chemicals were stained with anti-*N*-terminal NR2B under non-permeabilized conditions to label cell surface NR2B (scale bar = 10 μm). (c) Quantitative analysis from 40-μm dendrites of immunostained neurons showing a significant increase in surface NR2B clusters and fluorescence intensity in SKF-81297-treated neurons ($n = 12$, $^*p < 0.05$, $^{**}p < 0.01$). The significant increase in surface NR2B clusters and fluorescence intensity in SKF-81297-treated neurons was blocked by D_1 antagonist SCH-23390 (10 μM), PKA inhibitor KT5720 (5 μM), and PKC inhibitor Go6983 (20 μM), respectively ($n = 12$, $p > 0.05$). (d) In contrast, neither PKA nor PKC inhibitor administered alone affected cell surface NR2B expression ($n = 6$ for each treatment, $p > 0.05$). (e) D_1-mediated increase in NR2B surface expression was blocked by PLC inhibitor (U73122 10 μM, $n = 12$, $p > 0.05$) and Src kinase inhibitor (PP2, 10 μM, $n = 12$, $p > 0.05$) but not by inactive structural analogue PP3 (10 μM, $n = 12$, $^{**}p < 0.01$).

receptors because D_1 signaling is traditionally coupled to cAMP/PKA, not PKC. When PKC inhibitor Go6983 (20 μM) was included in the SKF-81297 treatment, D_1 activation no longer enhanced the surface labeling of NR2B clusters (17.9 ± 1.1, $n = 12$, $p > 0.05$; Fig. 5c). Neither PKA inhibitor KT5720 nor PKC inhibitor Go6983 administered alone affected the cell surface expression of NR2B (Fig. 5d). PLC can activate PKC, which in turn activates Src in hippocampal neurons, so we further tested the requirement of PLC and Src family kinases. In these experiments, the cluster numbers increased from 17.1 ± 0.8 in control to 23.7 ± 0.8 in SKF-81297 ($n = 12$; Fig. 5e). Both the PLC inhibitor U73122 (10 μM) and Src family kinase inhibitor PP2 (10 μM) blocked the D_1 effect (SKF + PLC inhibitor, 17.7 ± 0.7, $p > 0.05$; SKF + PP2, 16.8 ± 0.9, $p > 0.05$, $n = 12$). Conversely, the inactive structural analogue PP3 (10 μM) did not block the D_1 effect (22.5 ± 1.1, $n = 12$, $p < 0.01$).

Both PKA and PKC mediate D_1-induced increase of new insertion of NMDA receptors

An increase in surface expression of NMDA receptors could be because of increased insertion or decreased internalization of NMDA receptors or both. To directly address whether D_1 activation affects the rate of NMDA receptor insertion, we selectively labeled newly inserted NR2B using a pre-blocking method (see Appendix S1) (Lu *et al.* 2001). Neurons treated with SKF-81297 (1 μM) showed significantly higher levels of newly inserted NR2B than control cultures, demonstrating that D_1 receptor stimulation increased the rate of NR2B insertion (Fig. 6). Using this approach, we examined the role of PKA and PKC signaling in D_1 activation-induced insertion of NR2B receptors. We observed that both PKA and PKC inhibitors (5 μM KT5720 and 20 μM Go6983, respectively) significantly blocked the D_1 effect on NR2B insertion ($n = 12$, cluster numbers: control, 10.3 ± 0.4; SKF, 14.4 ± 0.6, $p < 0.01$; SKF + PKA inhibitor, 10.7 ± 0.2, $p > 0.05$; SKF

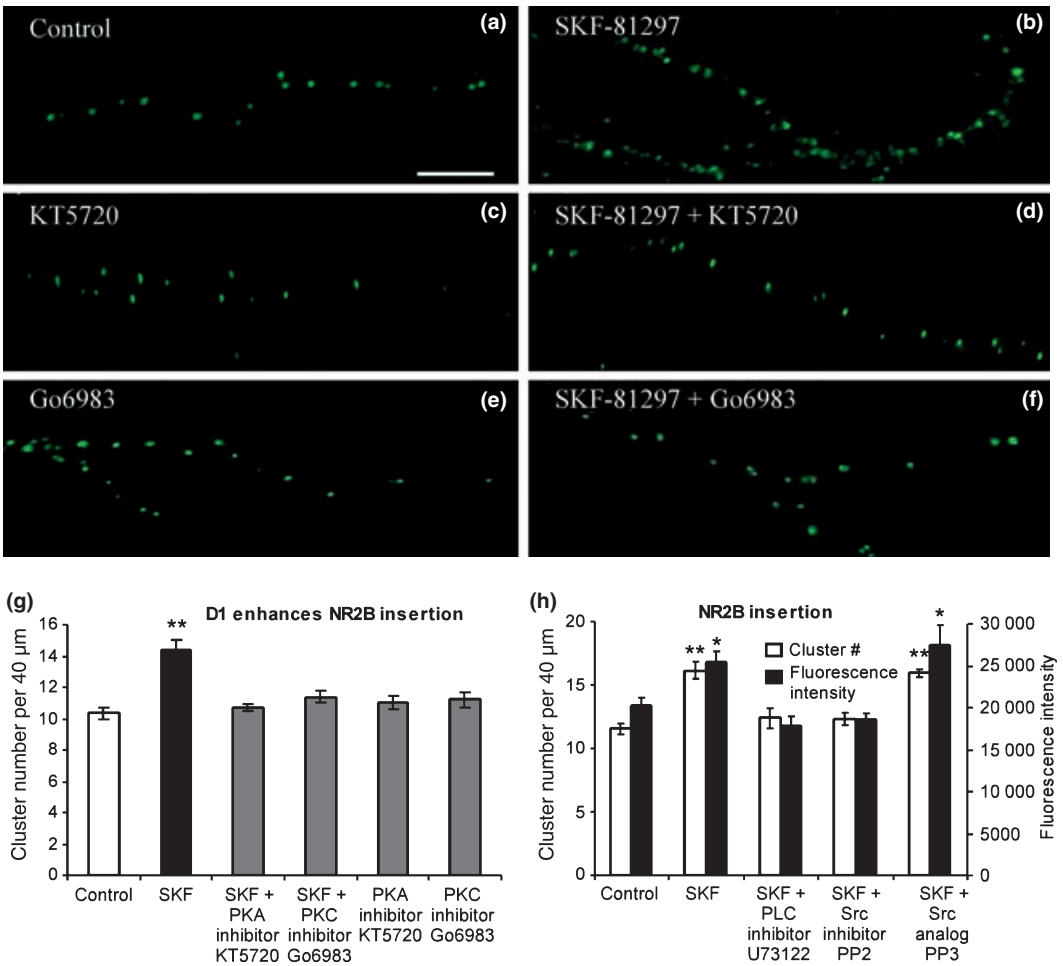

Fig. 6 D_1-mediated NR2B insertion to plasma membrane is PLC-PKC-Src pathway dependent. (a–f) NR2B newly inserted into cell membrane under various conditions (scale bar = 10 μm). D_1 enhancement of NR2B insertion into cell membrane appeared to depend on PKA (d) and PKC (f). (g) Summary histogram showing that D_1 agonist SKF-81297 significantly increased NR2B insertion into cell membrane ($n = 12$, $^{**}p < 0.01$). PKA inhibitor KT5720 (5 μM) and PKC inhibitor (Go6983, 20 μM) blocked the effects of SKF-81297 on NR2B expression ($n = 12$ in each condition, $p < 0.05$; g). (h) D_1 receptor-mediated NR2B insertion was also obstructed by coapplication of PLC inhibitor U73122 (10 μM, $n = 12$, $p > 0.05$) and Src inhibitor PP2 (10 μM, $n = 12$, $p > 0.05$) but not by inactive analogue PP3 (10 μM, $n = 12$, $^{*}p < 0.05$, $^{**}p < 0.01$).

+ PKC inhibitor, 11.4 ± 0.4, $p > 0.05$) (Fig. 6g). Interestingly, neither PKA inhibitor (11.0 ± 0.4, $n = 12$) nor PKC inhibitor Go6983 (11.2 ± 0.5, $n = 12$) used alone affected NR2B insertion at the basal level (Fig. 6g). Furthermore, PLC inhibitor U73122 (10 μM) or Src family kinase inhibitor PP2 (10 μM), but not its inactive analogue PP3 (10 μM), abolished the enhancement of NR2B insertion induced by D_1 activation ($n = 12$, cluster numbers: control, 11.5 ± 0.5; SKF, 16.1 ± 0.6, $p < 0.01$; SKF + PLC inhibitor, 12.4 ± 0.8, $p > 0.05$; SKF + PP2, 12.3 ± 0.5, $p > 0.05$; SKF + PP3, 15.9 ± 0.3, $p < 0.01$) (Fig. 6h). This finding suggests that the D_1-mediated synaptic insertion of NR2B subunits requires both PKA and PLC-PKC-Src pathways and the trafficking of NR2B is responsible to the D_1 enhancement of NMDA receptor function.

The newly inserted NMDA receptors induced by D_1 activation are localized on synaptic site and are functional

NMDA receptors need to be on the cell surface to receive the glutamate binding. Our data, as well as others (Hallett *et al.* 2006; Gao and Wolf 2008), indicated that D_1 receptor activation leads to rapid trafficking of NMDA receptor subunits and increases surface expression of both NR1 and NR2B subunits in the dendrites of cultured neurons. It remains, however, unknown whether the increased surface receptors are functional or not because increase of NMDA receptors on the cell surface can be caused by increased receptors on synaptic sites or extrasynaptic sites. Only an increase in NMDA receptors on synaptic sites can strengthen the function of newly inserted receptors. Therefore, D_1-mediated potentiation of NMDA mEPSCs could be because

of NMDA receptors undergoing translocation from an intracellular location to the synapses. We tested this assumption by using MK-801, an activity-dependent and irreversible NMDA receptor antagonist (Rosenmund *et al.* 1993). Previous studies showed that MK-801 could only block the synaptic NMDA receptors that opened in response to synaptically released glutamate and once the receptors are blocked, they become dysfunctional and the channel properties are irreversible (Tovar and Westbrook 2002). We took the advantage of these properties to examine whether D_1 strengthens NMDA current by translocating NMDA receptors to synapses. The NMDAR-mediated EPSCs were evoked by stimulating layer 2/3 with a single pulse (0.1 ms, 10–100 μA, 0.1 Hz) in the presence of picrotoxin (50–100 μM) and NBQX (20 μM) at +40 mV. After 5 min stable recording, MK-801 (20 μM) was bath-applied to the PFC slices. As shown in Fig. 7(a), the NMDA EPSCs were progressively and completely blocked after ~10 min. The NMDA EPSCs were at an almost undetectable level in the control group even 20 min after washout of the unbound MK-801 in the absence of stimulation. In contrast, when SKF-81297 (1 μM, 10 min) was bath-applied during the washing period, a clear recovery of NMDA EPSCs in response to the 0.1-Hz stimulation was observed (Fig. 7a). The amplitude recovery of NMDA EPSCs in the SKF-81297-treated neurons was significantly greater than that in controls (6.54 ± 6.89% in control vs. 43.4 ± 2.55% in SKF-81297, $n = 6$, $p < 0.01$, Fig. 7b). Consistently, the recovery of NMDA EPSCs induced by SKF-81297 was blocked by co-application of SKF with either PKA inhibitor KT5720 or NR2B inhibitor ifenprodil (recovered only by 17.1 ± 2.16% and 15.1 ± 3.62%, respectively, $n = 6$ for each drug and $p < 0.01$ for both, Fig. 7b). These data further suggest that the newly inserted NR2B-containing NMDA receptors induced by D_1 activation are likely to be translocated from intracellular or extrasynaptic sites to the synaptic membrane where they become functional.

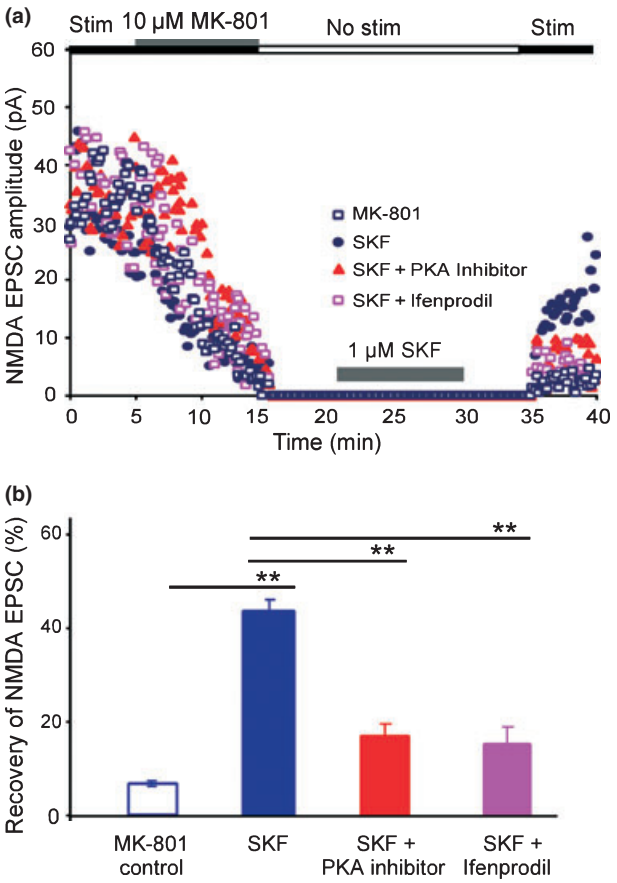

Fig. 7 D_1 promotes the recovery of NMDAR currents by translocating intracellular NMDA receptors to the synapse. (a) The NMDA EPSCs were evoked by stimulating layer 2/3 in the presence of picrotoxin (100 μM) and NBQX (20 μM) at +40 mV. After 5 min stable recording, MK-801 (20 μM) was bath-applied to PFC slices for 10 min to progressively block the NMDA EPSCs. The stimulation was stopped for 20 min to wash out the residual MK-801 after the EPSCs were completely and irreversibly blocked. The NMDA EPSCs were at an almost undetectable level under control condition but were significantly increased when SKF-81297 (1 μM) was bath-applied for 10 min during the washout period. Co-application SKF-81297 (1 μM) with either PKA inhibitor KT5720 (5 μM) or NR2B antagonist ifenprodil (3 μM) blocked the recovery of NMDA EPSCs induced by SKF-81297. (b) Summary histogram showing that SKF-81297 significantly promoted the recovery of NMDA EPSCs ($n = 6$, $^{**}p < 0.01$) and this effect was blocked by either PKA inhibitor or NR2B antagonist.

Discussion

This study has identified that rapid NMDA trafficking and synaptic insertion underlie the D_1-induced enhancement of NMDA receptors. Importantly, we show that both PKA and PKC signaling pathways play important roles in the regulatory process. However, it appears that, although both PKA and PKC mediate the D_1 activation-induced enhancement of NMDA receptors, the PLC-PKC-Src pathway is only required for surface expression and new synaptic insertion of NMDA receptors.

We found that D_1 receptor activation enhanced both frequency and amplitude of NMDA mEPSCs in PFC pyramidal neurons. Although the frequency of mEPSCs was previously interpreted as being of pre-synaptic origin, the change of mEPSC frequency could be directly attributable to alterations of post-synaptic receptors (Smith *et al.* 2005). In fact, after washing D_1 agonist, the potentiated mEPSCs did not return to baseline and, instead, were even further facilitated. One possibility is that SKF-81297 primed the intracellular signal pathway(s) to potentiate receptor trafficking, and once it was triggered, it would not depend on agonist itself (Seamans *et al.* 2001). This assumption, however, depends on the D_1 action on post-synaptic receptors. Indeed, D_1 agonist SKF-81297 enhanced puff

NMDA-induced current and evoked NMDA EPSCs, but did not change the paired-pulse ratio and coefficient of variation of the evoked NMDA-EPSCs (Zheng *et al.* 1999; Chen *et al.* 2004), indicating a direct post-synaptic effect. The increased total NR2B subunits and clusters of both NR1 and NR2B in the neuronal dendrites also provided direct evidence of post-synaptic receptor change. Furthermore, the AMPA mEPSCs were unchanged by low concentration of SKF-81297 (1 μM) under our recording conditions. At relative high concentrations, dopamine through D_1 activation actually produced a decrease in AMPA EPSCs, indicating a reduction of glutamate release at pre-synaptic site (Nicola and Malenka 1997; Gao *et al.* 2001; Seamans *et al.* 2001; Ding *et al.* 2003). Although it remains unclear how pre-synaptic reduction of glutamate release would reconcile with post-synaptic trafficking of AMPA and NMDA receptors, it is proposed that by increasing the NMDA EPSCs, yet slightly reducing release, D_1 activation may selectively enhance sustained synaptic inputs (Seamans *et al.* 2001).

NMDAR trafficking presents a dynamic and powerful mechanism for the regulation of synaptic transmission and plasticity (Wenthold *et al.* 2003; Lau and Zukin 2007). Previous studies indicated that the D_1-induced increase of NR2B expressions is possibly initiated by phosphorylation of existing receptors (Dunah and Standaert 2001; Hallett *et al.* 2006; Gao and Wolf 2008; Tong and Gibb 2008). In the PFC neurons, D_4 activation decreased NR1 surface expression (Wang *et al.* 2003), whereas D_1 receptors facilitated the trafficking of NMDA receptors (Chen *et al.* 2004; Gao and Wolf 2008). Our data provided novel evidence that D_1-induced enhancement of NMDA receptor function depends on rapid insertion of NMDA receptors to the synaptic surface. Most importantly, we found that the D_1 receptor-mediated increase in NMDA receptor trafficking depends on both PKA and PKC/Src but their roles appear to be different in the regulatory process.

Although both serine kinases PKA and PKC and tyrosine kinase Src/Fyn can regulate NMDA receptor trafficking (Lan *et al.* 2001; Scott *et al.* 2003; Salter and Kalia 2004; Chen and Roche 2007), it is unknown how PKA and PKC regulate the trafficking of NMDA receptors induced by D_1 activation. In this study, we defined a signaling cascade from PLC to PKC to Src family kinase that is important for the trafficking of NMDA receptors after D_1 activation. The requirement of PLC/PKC/Src signaling cascade in the enhanced trafficking of NMDA receptors after D_1 activation is intriguing because D_1 receptor is well known to activate the classical D_1/cAMP/PKA/DARPP-32 pathway (Blank *et al.* 1997; Cepeda *et al.* 1998; Snyder *et al.* 1998; Flores-Hernandez *et al.* 2002). In addition, although our results showed that PKA inhibitor blocked the D_1-induced changes of surface expression and synaptic insertion of NR2B subunits, its role in these trafficking processes could be not be confirmed or excluded because PKA inhibitor also blocked the D_1-induced changes in total NR2B expression. As PKC inhibition did not block the D_1-induced increase in total NR2B at dendritic spines, but nonetheless blocked the surface insertion of NMDA receptors, we speculate a two step process: first is the increase of NMDA receptors underneath the dendritic spines (which is dependent on PKA) followed by the insertion of NMDA receptors into the spines (which is dependent on PKC). The first step could involve local protein synthesis, and/or PKA-dependent NMDA receptor re-distribution (Crump *et al.* 2001; Smith *et al.* 2005). This assumption, however, needs further exploration. Indeed, it has been shown that although PKA mediates the effect of D_1 stimulation on the surface expression of AMPA receptors (Mangiavacchi and Wolf 2004; Smith *et al.* 2005; Sun *et al.* 2005, 2008; Gao *et al.* 2006), PKA activation was not sufficient to alter the surface expression of NMDA receptors in the cultured PFC neurons (Chen *et al.* 2004; Gao and Wolf 2008). In contrast, our data suggest that the D_1/PLC/PKC pathway may mediate the delivery of NMDA receptors, likely involved in surface expression and new insertion of NMDA receptors only, in consistence with previous studies (Chergui and Lacey 1999; Chen *et al.* 2004; Gao and Wolf 2008). Therefore, it is possible that PKA activation targets NMDA receptors to synapses (Crump *et al.* 2001), whereas PKC activation delivers NMDA receptors to the neuronal surface (Lan *et al.* 2001; Fong *et al.* 2002; Grosshans *et al.* 2002).

Furthermore, we have provided evidence that the process of new synaptic insertion of NR2B subunits on the cell surface triggered by D_1 stimulation appeared to be very rapid. Indeed, a recent study showed that subunit composition of synaptic NMDA receptors was extremely fast at neonatal synapses (Bellone and Nicoll 2007), as rapid as that seen with AMPA receptors (Smith *et al.* 2005; Bellone and Nicoll 2007). This rapid potentiation of NMDA receptor trafficking in the dendrites of prefrontal neurons by D_1 activation at very low (presumably optimal) concentration will have immediate effects on the integrative capacity of the synapse and can lead to important functional outcomes (Bellone and Nicoll 2007), particularly for working memory and other cognitive functions in the PFC. Indeed, D_1 antagonists have deleterious effects on the performance of working memory tasks in the primate PFC (Williams and Goldman-Rakic 1995). The lack of D_1 receptor sequestration in response to agonists because of its heteromerization with NMDA receptors would prolong dopamine's action on synaptic strength (Fiorentini and Missale 2004) and cognitive functions. In addition, both dopaminergic dysfunction and glutamatergic hypofunction are important for the development and symptomatology of some mental diseases such as schizophrenia and drug addiction. The novel NMDA trafficking pathway identified in the D_1 and NMDA receptor interaction is therefore important for understanding the interaction between dopamine and glutamate in these psychiatric disorders.

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Conflict of interest

The authors claim no conflict of interest.

Supporting information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Supplementary Materials and Methods.

Figure S1. Control experiments for recording of NMDA mEPSCs under zero-Mg^{2+} condition.

Figure S2. Non-specificity because of secondary antibodies is negligible in immunostaining processes.

Figure S3. The AMPA receptor-mediated currents were not affected by D_1 agonist.

Figure S4. D_1 activation increases NMDA receptor NR1 clusters but not PSD-95 in total staining.

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