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Dual Regulation of NMDA Receptor Functions by Direct Protein-Protein Interactions with the Dopamine D1 Receptor

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Summary

Dopamine D1-like receptors, composed of D1 and D5 receptors, have been documented to modulate glutamate-mediated fast excitatory synaptic neurotransmission. Here, we report that dopamine D1 receptors modulate NMDA glutamate receptor-mediated functions through direct protein-protein interactions. Two regions in the D1 receptor carboxyl tail can directly and selectively couple to NMDA glutamate receptor subunits NR1-1a and NR2A. While one interaction is involved in the inhibition of NMDA receptor-gated currents, the other is implicated in the attenuation of NMDA receptor-mediated excitotoxicity through a PI-3 kinase-dependent pathway.

Introduction

NMDA (N-methyl-D-aspartate) type glutamate receptors are highly permeable to calcium (Ca^{2+}) and play an important role in the regulation of activity-dependent neuroplasticity and excitotoxicity, which underlie many physiological and pathological processes including learning and memory, ethanol sensitivity, epilepsy, neuronal death, and mental disorders (reviewed in Dingledine et al., 1999).

NMDA receptor activation facilitates Ca^{2+} influx, which under pathological conditions can result in excitotoxicity. The "calcium overload" hypothesis is the prominent theory explaining NMDA-mediated excitotoxicity (Choi, 1995). The molecular mechanisms underlying NMDA-mediated excitotoxicity involve many Ca^{2+}-regulated processes in the cell including activations of proteases (Brorson et al., 1995), endonucleases (Zeevalk et al., 1993), nitric oxide synthase (Sattler et al., 1999), the production of free radicals (Lafon-Cazal et al., 1993), and mitochondrial membrane permeability (Duchen, 2000). In addition, NMDA-mediated excitotoxicity may depend on the local cellular environment (Sattler et al., 1998) and localization of the NMDA receptor (Hardingham et al., 2002).

In addition to a wide array of extracellular agents including Mg^{2+}, glycine, Zn^{2+}, and protons (reviewed in Dingledine et al., 1999), as well as intracellular agents such as Ca^{2+} (Rosenmund and Westbrook, 1993), Na^{+} (Yu and Salter 1998) and calmodulin (Ehlers et al., 1996; Wyszynski et al., 1997), NMDA receptor can also be regulated by protein kinases and phosphatases, which include tyrosine kinase/phosphatases (e.g., Src, PTPα, and STEP) (Yu et al., 1997; Lei et al., 2002; Pelkey et al., 2002) and serine/threonine kinases/phosphotases (i.e., protein kinase A (PKA), protein kinase C (PKC), and phosphatases 1/2) (Lieberman and Mody, 1994; Roche et al., 1994; Wang et al., 1994; Westphal et al., 1999; Greengard, 2001). Furthermore, numerous studies have demonstrated that NMDA receptor function may be regulated by G protein-coupled receptors, including D1-like receptors, through the activation of PKA/PKC dependent pathways (reviewed in Greengard, 2001).

Dopamine D1-like receptors contain seven transmembrane domains and preferentially couple to G_s proteins that can activate adenylate cyclase and PKA-dependent pathways (Missale et al., 1998). Previous studies have demonstrated the functional modulation of ligand-gated NMDA receptors by G protein-coupled D1-like receptors through classical pathways involving one or more intracellular second messengers and their downstream effectors such as adenylate cyclase and the phosphoprotein DARPP-32 (Cepeda et al., 1993; Levine et al., 1996; Harvey and Lacey, 1997; Blank et al., 1997; Snyder et al., 1998; Cepeda and Levine, 1998). Recently, direct protein-protein coupling has also been shown to enable functional crosstalk between ligand-gated $GABA_A$ and G protein-coupled dopamine D5 receptors (Liu et al., 2000), opening the possibility that other neurotransmitter receptors, including NMDA receptors, may form functional crosstalk through similar mechanisms. Here, we report two distinct direct protein-protein interactions between the D1 and NMDA receptor and the functional implications of these interactions.

Results

D1 and NMDA Receptors Form a Complex through the Carboxyl Tails

To determine the existence of D1:NMDA receptor complexes, we examined if NMDA receptors can coimmunoprecipitate with D1 receptors in rat hippocampal tissue. As depicted in Figure 1A, NMDA receptor NR1 and NR2A subunits coimmunoprecipitate with D1 receptors suggesting a physical interaction between D1 and NMDA receptors. In contrast, the α1 subunit of $GABA_A$ receptor, which has been shown to coimmunoprecipitate with the

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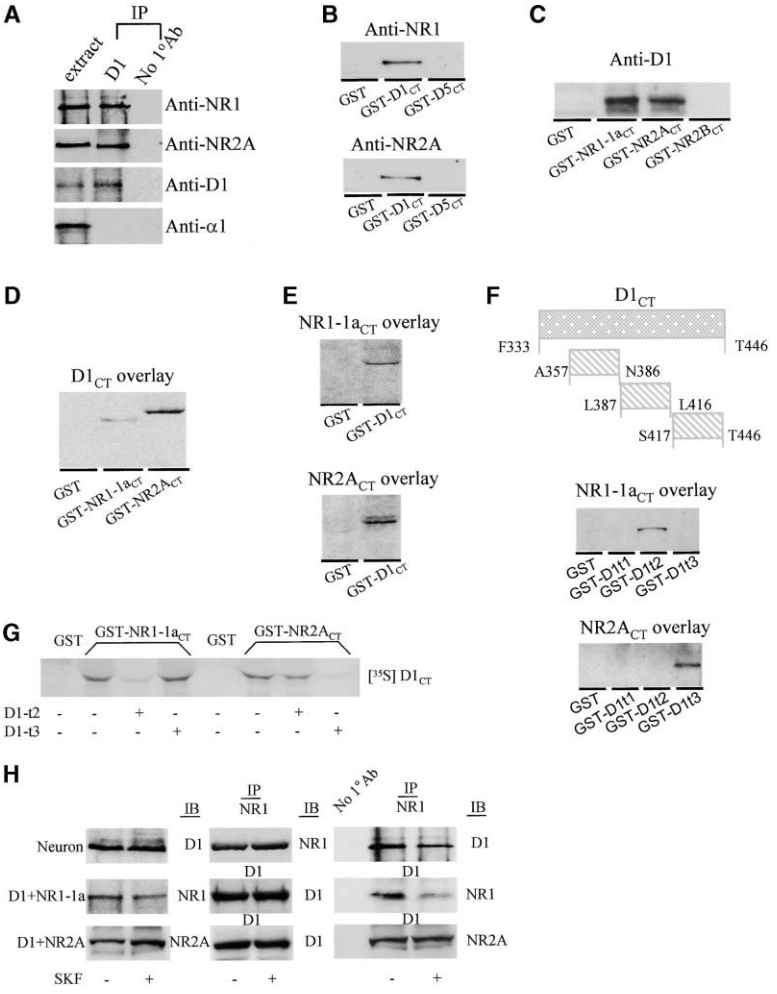

Figure 1. Association of D1 and NMDA Receptors in Rat Brain and In Vitro

(A) Coimmunoprecipitation of hippocampal NMDA receptor NR1 and NR2A subunits, but not the $GABA_A$ receptor α1 subunit, by D1 Ab (antibody).

(B) Western blots of hippocampal NR1 subunit (top), NR2A subunit (bottom) after affinity precipitation by GST-$D1_{CT}$, but not by GST-$D5_{CT}$ or GST alone.

(C) Western blots of hippocampal D1 receptors after affinity precipitation by GST-NR1-$1a_{CT}$, GST-$NR2A_{CT}$, but not by GST-$NR2B_{CT}$ or GST alone.

(D) Blot overlay assay depicting the direct binding of [^{35}S]-$D1_{CT}$ to the GST-NR1-$1a_{CT}$, GST-$NR2A_{CT}$.

(E) Direct binding of the [^{35}S]-NR1-$1a_{CT}$ (top), [^{35}S]-$NR2A_{CT}$ (bottom) to GST-$D1_{CT}$. GST was used as control.

(F) Top: schematic representation of the generated D1-t1; D1-t2; D1-t3 mini-genes. Amino acid sequence D1-t2 was critical for direct binding to NR1-1a subunit (middle); D1-t3 was critical for direct binding to NR2A subunit (bottom).

(G) In vitro binding assay showing the blockade of direct binding of NR1-1a to D1 CT upon the addition of D1-t2, but not D1-t3 peptide (left). Blockade of direct binding of NR2A to D1 CT upon the addition of D1-t3, but not D1-t2 peptide (right).

(H) Coimmunoprecipitation of D1 with NR1 subunit in hippocampal neurons (top), cells expressing D1 and NR1-1a subunit (middle) and coimmunoprecipitation of D1 with NR2A subunit in cells expressing D1 and NR2A subunit (bottom) with or without SKF 81297 pretreatment. Each coimmunoprecipitation was in parallel with Western blot analysis of the initial levels of solubilized protein and the directly immunoprecipitated proteins. The intensity of each protein band was quantified by densitometry (software: AIS from Imaging Research Inc). Data are representative of at least 3 independent experiments.

D5 receptor (Liu et al., 2000), did not coimmunoprecipitate with the D1 receptor. The carboxyl tail (CT) of both the D1 receptor and NR1/NR2 subunits contain putative consensus sequences for receptor phosphorylation and potential binding sites for various proteins important for signaling (e.g., calcyon, PSD-95, and calmodulin; Missale et al., 1998; Lezcano et al., 2000; Lamey et al., 2002; Sheng, 2001). To determine if the CT regions of both the D1 and NMDA receptors are involved in the formation of D1:NMDA receptor complex, various glutathione-S-transferase (GST) fusion proteins, encoding the CTs of either the D1-like receptors (GST-$D1_{CT}$:A_{332}-T_{446}, GST-$D5_{CT}$:A_{360}-H_{477}) or the NR1-1a (GST-NR1-$1a_{CT}$:E_{834}-S_{938}), NR2A (GST-$NR2A_{CT}$:D_{1350}-V_{1464}), NR2B (GST-$NR2B_{CT}$:P_{1365}-V_{1482}) subunits, were prepared and utilized in affinity purification assays. As shown in Figure 1B, GST-$D1_{CT}$, but not GST-$D5_{CT}$ or GST alone, precipitated solubilized hippocampal NR1 and NR2A subunits indicating that the D1 receptor can interact with NMDA receptors through its carboxyl tail. Similarly, both the GST-NR1-$1a_{CT}$ and GST-$NR2A_{CT}$, but not GST-$NR2B_{CT}$ or GST alone, could precipitate solubilized hippocampal D1 receptors, as illustrated in the D1 antibody immunolabeled Western blot (Figure 1C).

Direct Protein-Protein Interactions between D1 and NMDA Receptors

While these results demonstrate the presence of the D1:NMDA receptor complex in rat hippocampal tissue, it does not clarify whether the D1:NMDA receptor complex is formed through a direct interaction between D1 and NMDA receptors or mediated by an indirect interaction involving an accessory binding protein. Blot overlay experiments provided in vitro evidence that D1 and NMDA receptors can directly interact with each other. GST-NR1-$1a_{CT}$ and GST-$NR2A_{CT}$ were probed with in vitro translated [^{35}S]-methionine labeled peptides encoding D1 CT sequences ([^{35}S]-$D1_{CT}$). As shown in Figure 1D, the [^{35}S]-$D1_{CT}$ probe bound with both GST-NR1-$1a_{CT}$ and GST-$NR2A_{CT}$. The binding of [^{35}S]-$D1_{CT}$ was specific, as it did not bind with GST. Conversely, as represented in Figure 1E, [^{35}S]-NR1-$1a_{CT}$ (top) and [^{35}S]-$NR2A_{CT}$ (bottom) probes bound to GST-$D1_{CT}$, but failed to bind to GST. All blots were probed in parallel with GST antibody to

confirm equivalent protein loading (data not shown). In order to confirm these results and to delineate the region of the D1 CT involved in the interaction with the NR1-1a/2A subunits, three D1 CT GST-fusion proteins and mini-genes (D1-t1:A_{357}-N_{386}, D1-t2:L_{387}-L_{416}, and D1-t3:S_{417}-T_{446}) were constructed (Figure 1F, top). In vitro translated [^{35}S]-NR1-1a$_{CT}$ probe bound to GST-D1-t2, but not GST-D1-t1, GST-D1-t3, or GST (Figure 1F, middle) while [^{35}S]-NR2A$_{CT}$ probes bound to GST-D1-t3, but not GST-D1-t1, GST-D1-t2, or GST (Figure 1F, bottom). Therefore, it appears that the CT of the D1 receptor contains two separate domains that can mediate direct interactions with distinct subunits of the NMDA receptor.

To explore whether these motifs are sufficient for the interaction between the D1 and NMDA receptors, we examined the ability of purified peptides encoding D1-t2 and D1-t3 to block the direct binding of NR1-1a:D1-t2 and NR2A:D1-t3. As demonstrated by in vitro binding assays (Figure 1G), the ability of the GST-NR1-1a$_{CT}$ to hybridize with [^{35}S]-D1$_{CT}$ was almost completely blocked by preincubation with the D1-t2 peptide, but not with the D1-t3 peptide. Similarly, preincubation with the D1-t3 peptide, but not with the D1-t2 peptide, blocked the interaction of GST-NR2A$_{CT}$ with [^{35}S]-D1$_{CT}$. These data further support the existence of two distinct protein-protein interactions occurring between D1 and NMDA receptors.

Agonist Regulation of D1-NMDA Protein-Protein Interactions

Before investigating whether the direct protein-protein interactions between D1 and NMDA receptors have functional implications, we tested if D1 activation affects the observed protein-protein interactions. As shown in Figure 1H (top), in cultured rat hippocampal neurons, D1 and NMDA receptors could associate without exogenous D1 receptor agonist stimulation. Interestingly, stimulation of D1 with the agonist SKF 81297 resulted in a 37 $\pm$ 5% (mean $\pm$ SE, n = 3) decrease in the coimmunoprecipitation of NMDA receptors by the D1 receptor antibody. SKF 81297 stimulation did not significantly alter either the initial levels of solubilized protein or the levels of directly immunoprecipitated proteins. Since the associations between D1 and NMDA receptors are mediated by two separate protein-protein interactions, we next examined which interaction was affected by D1 stimulation. After SKF 81297 treatment, we found 37 $\pm$ 2% (mean $\pm$ SE, n = 3) decrease in the coimmunoprecipitation of NR1-1a subunits with the D1 receptor antibody in Cos-7 cells coexpressing D1 and NR1-1a subunits (Figure 1H, middle). However, the coimmunoprecipitation of the NR2A subunit with the D1 receptor antibody, in Cos-7 cells coexpressing D1 and NR2A subunits, was not affected (Figure 1H, bottom).

D1 Modulation of NMDA Currents through D1-t3:NR2A Interaction in HEK-293 Cells

To date, the functional interaction between D1 and NMDA receptors is largely thought to be mediated via the activation of D1 receptor-mediated intracellular cAMP/PKA dependent pathways (reviewed in Greengard, 2001). Therefore, to determine whether the observed direct protein-protein interactions between D1 and NMDA receptors confer functional relevance, we tested if D1 receptors can modulate NMDA currents independent of PKA and/or PKC activation. As illustrated in Figure 2A, in HEK-293 cells coexpressing D1 and NR1-1a/2A subunits, bath application of 10 μM SKF 81297, in the presence of PKA (Rp-cAMPs, 10 μM) and PKC (staurosporine, 1 μM) inhibitors, significantly reduced the peak and steady-state amplitudes of NMDA receptor currents by 28 $\pm$ 3% and 26 $\pm$ 6% (n = 11, $P < 0.01$), while the decay time constant was not changed. Control cells expressing only NMDA receptors were not responsive to SKF 81297 (data not shown). Moreover, SKF produced a decrease in the slope of the current-voltage curve with no change in the reversal potential (Figure 2B), suggesting that SKF-induced inhibition of the NMDA currents were due to reductions in NMDA receptor-activated whole-cell membrane conductance. These data provided evidence that D1 receptor activation modulates NMDA currents through a PKA/PKC independent pathway.

In addition, we determined that the modulation of NMDA currents by the D1 receptor was dependent on the identified direct protein-protein interaction between D1-t3 and the NR2A subunit. As illustrated in Figure 2C, in HEK-293 cells coexpressing both D1 receptor and NR1-1a/NR2A subunits, intracellular application of D1-t3 peptide significantly blocked the inhibition of NMDA currents induced by D1 receptor activation in a concentration-dependent manner. In contrast, the D1-t2 peptide had no such effects, suggesting the specific involvement of the D1-t3:NR2A interaction in the D1 receptor modulation of NMDA currents. The specificity of the D1-t3 peptide was further confirmed in cells coexpressing NMDA and D5 receptors, a D1-like receptor that can also be activated by SKF 81297 (Sunahara et al., 1991). The activation of D5 receptors also exerted an inhibitory effect on NMDA currents; however, the D5 inhibition of NMDA currents was not significantly affected by the intracellular application of the D1-t3 peptide (Figure 2C).

Furthermore, we found that SKF 81297 stimulation decreased the number of NMDA receptors expressed on cell surface, and that the SKF effect could be blocked by coexpressing the D1-t3 mini-gene, but not by the D1-t2 mini-gene (Figure 2D), suggesting that the observed D1 modulation of NMDA currents may be due to a decrease of NMDA receptor number on the cell surface.

D1 Modulation of NMDA Current in Hippocampal Culture Neurons

To examine the D1:NMDA receptor interactions in a relevant cellular milieu, primary rat hippocampal and striatal neurons were utilized in parallel experiments. As shown in Figure 3A, application of SKF 81297 significantly inhibited the peak amplitude of NMDA receptor currents by 22 $\pm$ 5% (n = 13, $p < 0.01$) in rat hippocampal neurons. This effect can be fully blocked by the D1-like receptor specific antagonist SCH-23390 (10 μM), but not by intracellular application of GTPγS (200 μM), a non-hydrolyzable analog of GTP that inhibits receptor-mediated activation of G proteins, suggesting the D1 effects on NMDA currents are not mediated through D1 receptors functionally coupling to endogenous G proteins (Figure 3C).

Consistent with the data from HEK-293 cells, intracel-

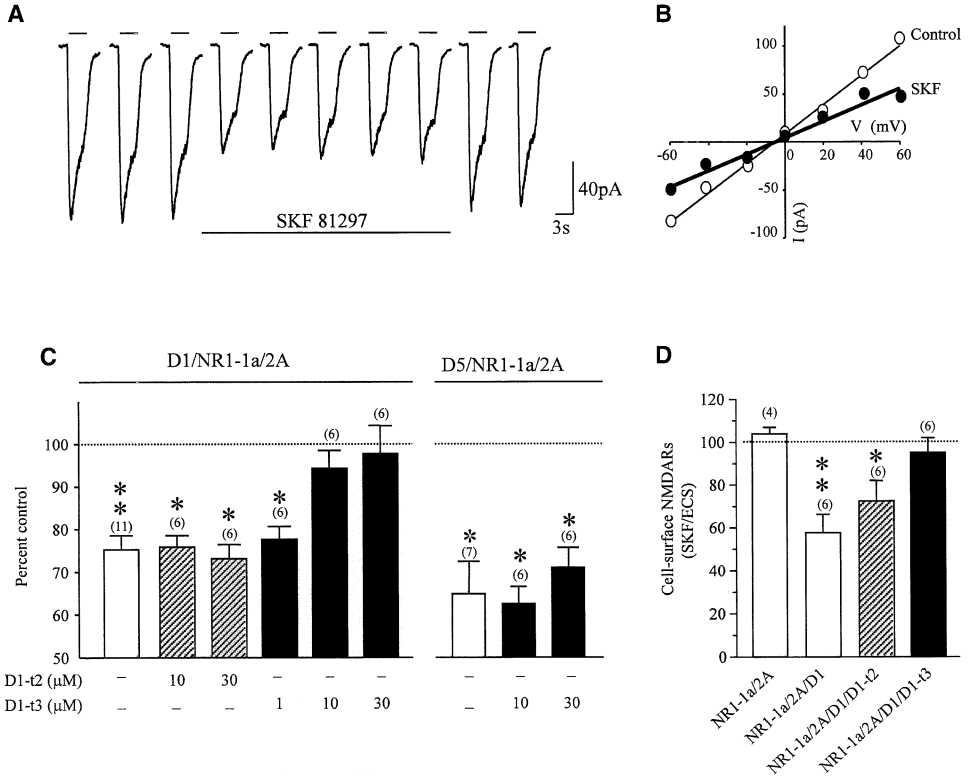

Figure 2. D1 Receptor Modulation of NMDA (NR1-1a/NR2A) Receptors-Mediated Whole-Cell Currents in HEK-293 Cells

(A) An example of L-aspartate evoked whole-cell responses in cells coexpressed with D1 and NMDA receptors before and during application of SKF 81297.

(B) Current-voltage (I/V) relationship before (open circles) and during the application of SKF 81297 (filled circles).

(C) Summarized data indicating effects of SKF 81297 application on NMDA currents in cells coexpressed with D1 or D5 receptors without (open bar) or with the intracellular application of D1-t2 (shaded bars) or D1-t3 (filled bars) peptides. Bars show the mean (± SE) of peak amplitudes of NMDA currents normalized with those before SKF 81297 applications. Dashed line indicates the control level of the currents before SKF 81297 application. *, **: $P < 0.05$, $P < 0.01$ (Wilcoxon test). Values in brackets indicate the number of cells tested.

(D) Summarized data indicating effects of SKF 81297 application on NMDA receptor number detected in the surface of cells without or with coexpression of D1 receptor, D1-t2 peptide (shaded bar), or D1-t3 peptide (filled bar). Bars show means (± SE) of the ratios of colorimetric readings under non-permeabilized conditions versus those under permeabilized conditions from cells treated with SKF 81297, normalized to their respective control groups (dash line) treated with extracellular solution (ECS). *, **: $P < 0.05$, $P < 0.01$ (t test). Values in brackets indicate the times of experiments conducted.

lular application of the D1-t3 peptide into hippocampal or striatal neurons from rat or wild-type mice ($D1^{+/+}$) significantly reduced the observed D1 inhibition of NMDA currents (Figures 3D–3F), while the D1-t2 peptide did not produce any effects on the D1 modulation of NMDA currents (data not shown). In contract, no significant effects of D1-t3 peptide could be found in cultured hippocampal neurons from D1 receptor-knock out ($D1^{-/-}$) mice (Figure 3F). The lingering SKF effects on NMDA currents still observed in hippocampal neurons with intracellular application of D1-t3 peptide or in $D1^{-/-}$ neurons may be due to the activation of other D1-like receptors expressed in hippocampus, such as D5 receptors. Therefore, these results strongly suggest that the interaction between D1-t3 and NR2A is critical for the D1 mediated modulation of NMDA currents.

D1 Modulation of NMDA-Mediated Excitotoxicity through D1-t2:NR1-1a Interaction in HEK-293 Cells

Excessive NMDA receptor activation can lead to excitotoxicity by inducing Ca^{2+} overload, potentially increasing Ca^{2+} mitochondrial influx and affecting the mitochondrial permeability transition pore, which is a catastrophic event for the cells that can initiate cell death pathways (reviewed in Choi, 1995; Lipton and Nicotera, 1998; Duchen, 2000). Therefore, attenuation of NMDA currents may lead to protection against NMDA receptor-mediated excitotoxicity by reducing Ca^{2+} influx. In HEK-293 cells cotransfected with D1 and NMDA receptors, apoptosis was induced by incubation with 100 μM NMDA and 10 μM glycine for 1 hr followed by 10 μM MK-801 for 24 hr as previously described (Sattler et al., 1999). As illustrated in Figure 4A (top), while less than 3% of sham-treated cells exhibited apoptosis, pretreatment with 10 μM SKF 81297 significantly reduced NMDA-induced apoptosis (NMDA: 78 ± 3% apoptotic, SKF/NMDA: 37 ± 4% apoptotic, $p < 0.01$). There was no significant difference in the number of apoptotic cells between SKF-treated and non-treated cells expressing only NMDA or D1 receptors (Figure 4B). Less than 3% of non-transfected cells exhibited apoptosis upon exposure to NMDA (data not shown). Subsequently, we tested whether the observed D1 receptor protective ef-

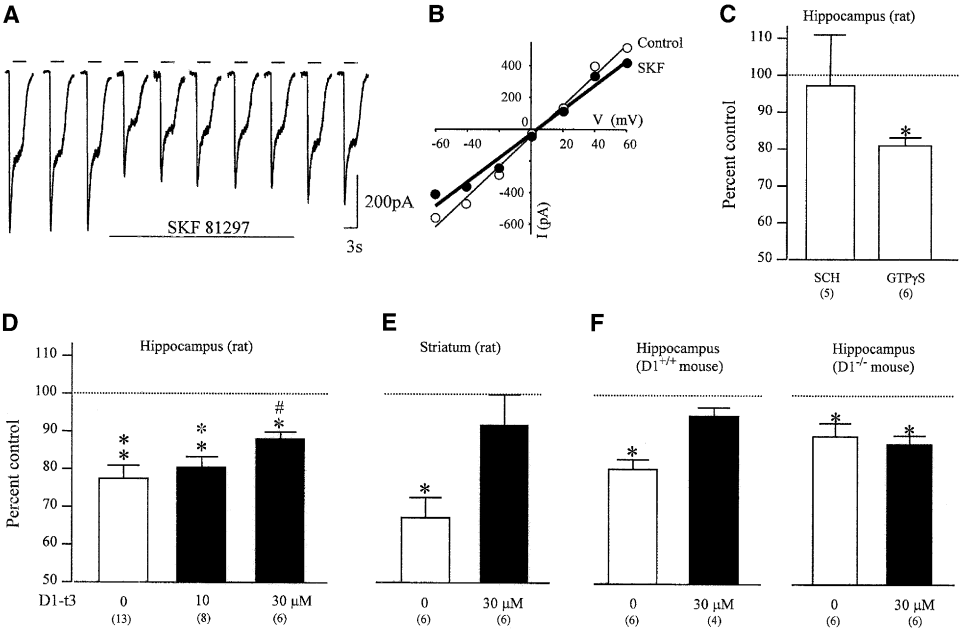

Figure 3. D1 Receptor Regulation of NMDA Receptor-Mediated Whole-Cell Currents in Cultured Neurons

(A) An example of whole-cell responses mediated by NMDA receptors in hippocampal neurons before and during the application of SKF 81297.

(B) Current-voltage (I/V) relationships before (open circles) and during the application of SKF 81297 (filled circles). Bars in (C), (D), (E), and (F) show peak NMDA current amplitudes (mean ± SE) normalized by those before SKF 81297 applications.

(C) The effect of SKF 81297 application of on NMDA currents with coapplication of SCH23390 (10 μM) or GTPγS (200 μM).

(D) The effects of SKF 81297 on NMDA currents recorded from hippocampal neurons with (filled bars) or without (open bar) intracellular application of D1-t3 peptide. The effects of SKF 81297 application on NMDA currents recorded with (filled bar) or without (open bar) intracellular application of D1-t3 peptide from striatal neurons (E), wild-type ($D1^{+/+}$) or D1 receptor-deficient ($D1^{-/-}$) mice (F). Dashed line in (C), (D), (E), and (F) indicate the control level of NMDA currents before SKF 81297 application. *, *: $P < 0.05$, $P < 0.01$ (Wilcoxon test); #: $P < 0.05$ (t test). Values in brackets indicate the number of cells tested.

fect on NMDA receptor-mediated excitotoxicity is actually a direct consequence of the inhibition of NMDA currents. Surprisingly, the attenuation of NMDA-induced apoptosis by activation of the D1 receptor was not effectively blocked by the coexpression of the D1-t3 mini-gene (NMDA: 88 ± 3% apoptotic, SKF/NMDA: 46.5 ± 8% apoptotic, $p < 0.01$, Figure 4A, bottom). However, the D1-t2 mini-gene completely abolished the D1 receptor's ability to attenuate NMDA receptor-mediated excitotoxicity (NMDA: 79 ± 2% apoptotic, SKF/NMDA: 85 ± 2% apoptotic, Figure 4A, middle), suggesting that the D1-t2:NR1-1a interaction is specifically involved in the protective effects mediated by D1 receptor activation.

To further eliminate the possibility that the observed D1 protective effect may be the consequence of the activation of the D1-mediated cAMP dependent signaling, we constructed a mini-gene encoding the third intracellular loop of the D1 receptor ($D1_{i3}$), which is involved in the physical coupling to G proteins (Bourne, 1997; Missale et al., 1998). Previous studies have used a polypeptide derived from $D1_{i3}$ to uncouple the interaction between D1 and Gds subunit to effectively antagonize D1 receptor signaling, as indexed by D1-mediated cAMP accumulation (Hawes et al., 1994; Feldman et al., 2002). Therefore, we examined the effect of $D1_{i3}$ mini-gene on D1 receptor modulation of NMDA toxicity. As shown in Figure 4B, the D1 receptor protective effects on NMDA induced apoptosis could not be blocked by $D1_{i3}$ mini-gene in HEK-293 cell coexpressing both D1 and NMDA receptors. The $D1_{i3}$ inhibitory effect on D1-mediated cAMP accumulation was tested in parallel experiments (data not shown). This data further supports our contention that G proteins do not mediate the SKF protective effects.

D1 Modulation of NMDA-Mediated Excitotoxicity in Hippocampal Culture Neurons

The protective effect of D1 receptor activation on NMDA-induced apoptosis was also seen in cultured rat hippocampal (NMDA: 92 ± 1% apoptotic, SKF/NMDA: 62 ± 2% apoptotic, $p < 0.01$; Figure 5A, top), striatal neurons and hippocampal neurons from wild-type mice (Figure 5B), but not in hippocampal neurons from $D1^{-/-}$ mice (Figure 5B). Hippocampal neurons infected with a recombinant adenovirus expressing the D1-t2 peptide resulted in a loss of the observed SKF 81297 protective effect (NMDA: 93 ± 0.5% apoptotic, SKF/NMDA: 93 ± 6% apoptotic, Figure 5A, middle), while neurons infected with a D1-t3 expressing adenovirus (NMDA: 98 ± 1% apoptotic, SKF/NMDA: 43 ± 4% apoptotic, $p < 0.01$, Figure 5A, bottom) or the wild-type adenovirus (data not shown) did not differ from non-infected neurons and retained the SKF 81297 protective effect. The inability of D1-t3 peptide to block the D1 protective effects on NMDA induced apoptosis delineates different pathways

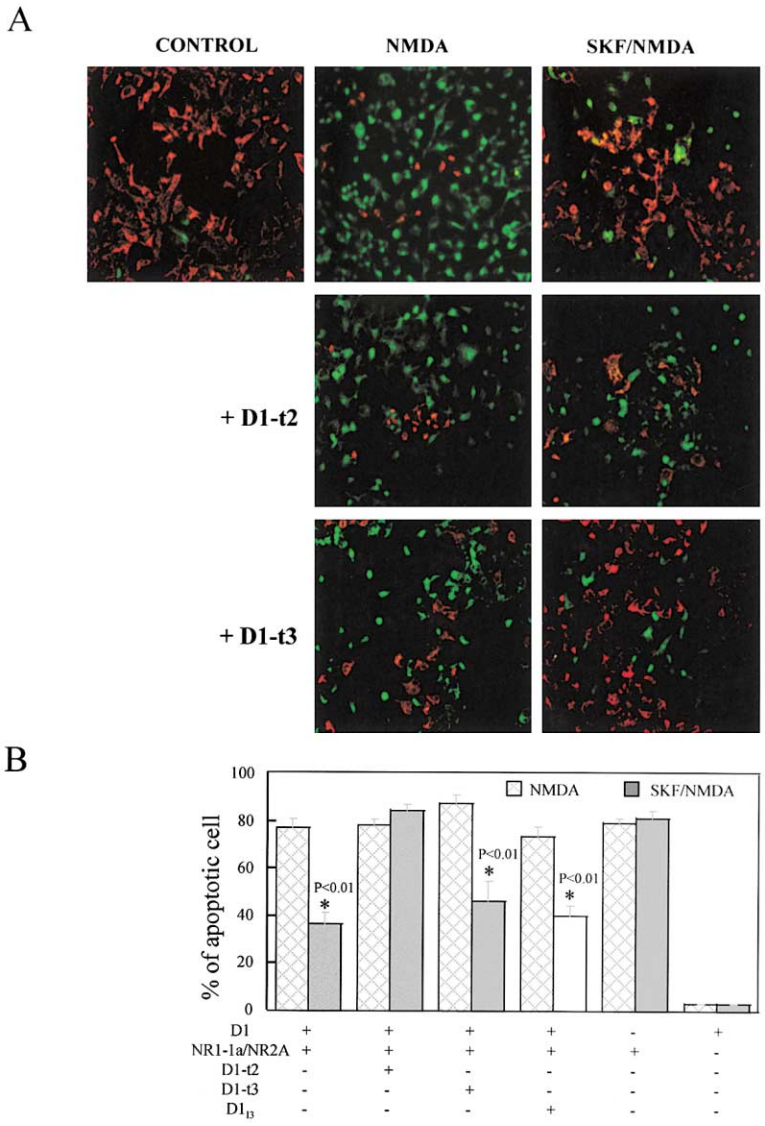

Figure 4. D1 Receptor Modulation of NMDA Receptor-Mediated Excitotoxicity in Cotransfected HEK-293 Cells

(A) Immunofluorescence imaging of the cotransfected HEK-293 cells with or without the D1-t2 (middle) or D1-t3 (lower image) minigenes respectively. Cells exhibit red fluorescence indicating healthy cells while the apoptotic cells exert green fluorescence.

(B) Bar graph summarizing the apoptosis data (with or without SKF 81297 treatment) obtained from HEK-293 cells with D1-t2, D1-t3 or $D1_{i3}$ mini-genes. Data are representative of at least 3 independent experiments. Apoptotic cells were counted in 15–20 fields in each experiment with a total cell sampling of 3000–6000.

for D1-mediated inhibition of NMDA currents and D1-mediated protection of NMDA induced excitotoxicity.

D1 Modulation of NMDA-Mediated Excitotoxicity through a PI-3 Kinase Dependent Mechanism

PI-3 kinases have been implicated in the regulation of many fundamental cellular responses including proliferation, transformation, protection from apoptosis, superoxide production, cell migration, and adhesion (reviewed in Toker and Cantley, 1997). We found that preincubation with the PI-3 kinase inhibitor wortmannin (100 nM, 30 min) completely abolished the ability of the D1 receptor to modulate NMDA-induced apoptosis in HEK-293 cells cotransfected with D1 and NMDA receptors (NMDA: 80 $\pm$ 5% apoptotic, SKF/NMDA: 78 $\pm$ 2% apoptotic) and hippocampal neurons (NMDA: 98 $\pm$ 3% apoptotic, SKF/NMDA: 98 $\pm$ 2% apoptotic, Figure 6A, a, b), indicating that the D1 receptor modulates NMDA-induced apoptosis may be through a PI-3 kinase dependent mechanism. Less than 1% of the cotransfected HEK-293 cells and the hippocampal neurons pretreated with wortmannin (100 nM, 30 min) displayed apoptosis. Furthermore, in fibroblast lines derived from wild-type and PI-3 kinase $p85\alpha^{-/-}$ embryos (Fruman et al., 1999) cotransfected with D1 and NMDA receptors, activation of D1 receptors exhibited no protective effects on NMDA-induced apoptosis in $p85\alpha^{-/-}$ cells (NMDA: 99 $\pm$ 3% apoptotic, SKF/NMDA: 97 $\pm$ 2% apoptotic, Figure 6A, c). In contrast, D1 receptor activation effectively protected cells from NMDA toxicity in wild-type cells (NMDA: 96 $\pm$ 1% apoptotic, SKF/NMDA: 46 $\pm$ 6% apoptotic, $P < 0.01$, Figure 6A, d). Untreated wild-type and $p85\alpha^{-/-}$ fibroblasts cotransfected with both D1 and NMDA receptors showed less than 3% apoptosis. Thus, these data demonstrated that the modulation of NMDA-mediated neurotoxicity by D1 receptors is through a PI-3 kinase-dependent mechanism.

It has been shown that calmodulin (CaM), which directly binds to PI-3 kinase, plays an important role in the activation and accumulation of the PI-3 kinase (Joyal et al., 1997; Fischer et al., 1998; Egea et al., 2001). We found that CaM acted as a competitive inhibitor to the

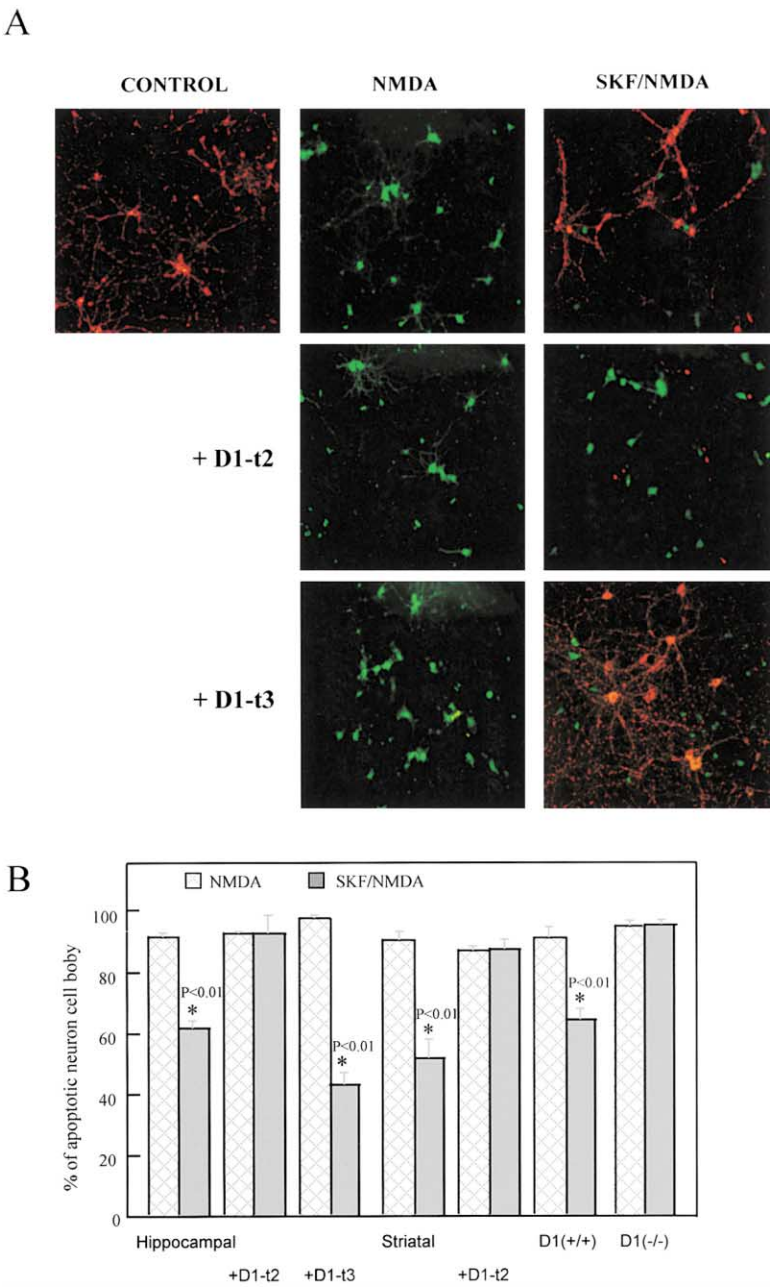

Figure 5. D1 Receptor Modulation of NMDA Receptor-Mediated Excitotoxicity in Neurons

(A) Immunofluorescence imaging of both non-infected hippocampal neurons and neurons infected with recombinant D1-t2 or D1-t3 adenovirus.

(B) Bar graph summarizing the apoptosis data (with or without SKF 81297) obtained from rat hippicampal and striatal neurons infected with recombinant D1-t2 or D1-t3 adenovirus and hippocampal neurons from D1 (+/+), D1 (−/−) mice. Data are representative of at least 3 independent experiments. Apoptotic neurons (cell body) were counted in 15–20 fields in each experiment with a total cell sampling of 3000–6000.

observed NR1-1a:D1-t2 coupling in a concentration and calcium-dependent manner (Figure 6B). The agonist stimulation of D1 receptors resulted in a dissociation of the D1:NR1 complex (Figure 1H) and promoted the formation of NR1-1a:CaM complex. As shown in Figure 6C (top) in Cos-7 cells coexpressing D1 and NR1-1a subunits, SKF 81297 stimulation of the D1 receptor led to an increased coimmunoprecipitation of the NR1-1a subunit with CaM by 57 ± 2% (mean ± SE, n = 3). Surprisingly, we also found that SKF 81297 applications not only significantly increased the association of PI-3 kinase with NR1 subunits by 62 ± 5% (mean ± SE, n = 3) in cotransfected Cos-7 cells and by 50 ± 7% (mean ± SE, n = 3) in hippocampal neurons (Figure 6, middle and bottom), but also increased PI-3 kinase activity, which could be blocked by CaM antagonist W-13 or by coexpressing the D1-t2 mini-gene, but not by the D1-t3 mini-gene (Figures 6D and 6E). Taken together, these data provide evidence for the modulation of NMDA-mediated neurotoxicity by D1 receptors through a PI-3 kinase dependent pathway.

Discussion

The regulation of NMDA receptors has important functional consequences given the role of NMDA receptors in synaptic plasticity and excitotoxicity. As illustrated in Figure 7, we have now identified two distinct protein-protein interactions between D1 and NMDA receptors that regulate NMDA functions. Two regions of the D1

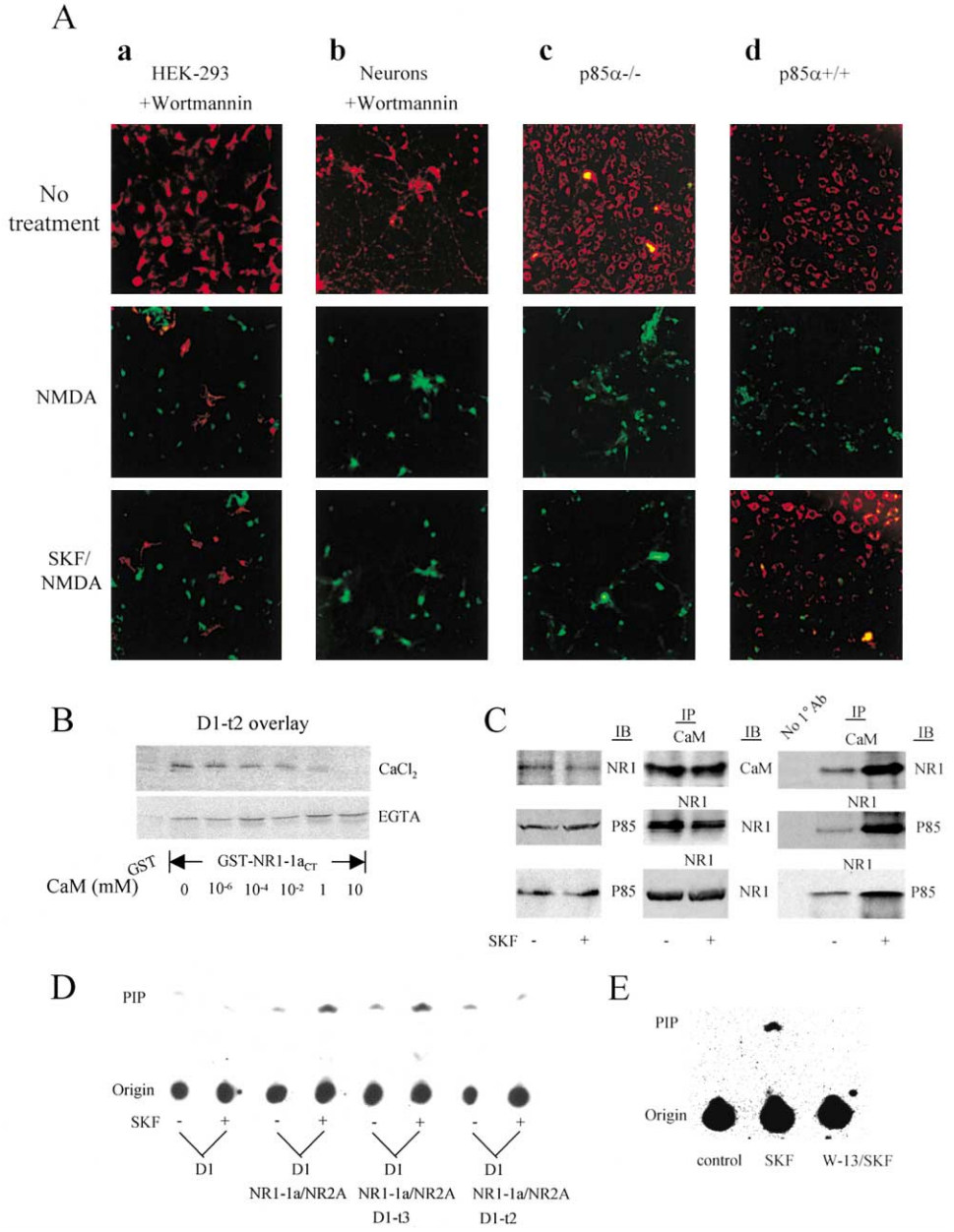

Figure 6. D1 Receptor Modulates NMDA-Mediated Excitotoxicity through a PI-3 Kinase Dependent Pathway

(A) Immunofluorescence imaging of cotransfected HEK-293 cells (a), hippocampal neurons (b) pretreated with 100 nM wortmannin, fibroblast derived from p85$^{\alpha\ -/-}$ (c) and wild-type (d) embryos. Data are representative of at least 3 independent experiments. Apoptotic cells (neuron cell body) were counted in 15–20 fields in each experiment with a total cell sampling of 3000–6000.

(B) In vitro binding assay demonstrating the competition between D1-t2 and CaM for binding to the C-terminal tail of NR1-1a subunit. GST-NR1-1a$_{CT}$ (10 μg) was incubated with [^{35}S]-D1-t2 in the presence of Ca^{2+} (top) or EGTA (bottom) with increasing concentrations of CaM.

(C) Coimmunoprecipitation of CaM with NR1-1a subunit (top); NR1-1a with p85 subunit of PI-3 kinase in Cos-7 cells coexpressing D1 and NR1-1a subunit (middle) and hippocampal neurons (bottom) with or without SKF pre-treatment. Each coimmunoprecipitation was in parallel with Western blot analysis of the initial levels of solubilized protein and the directly immunoprecipitated proteins. The intensity of each protein band was quantified by densitometry (software: AIS from Imaging Research, Inc). Data are representative of three independent experiments.

(D) PI-3 kinase activities in anti-p85 immunoprecipitates from Cos-7 cells expressing D1 receptor only, coexpressing D1 and NR1-1a2A subunits, D1/NMDA receptors with D1-t2 or D1-t3 mini gene (with or without pretreatment with 10 μM SKF 81297) were assayed using phosphatidylinositol as a substrate. Lipid products were extracted and separated by TLC. Position of phosphatidylinositol 3-phosphate (PIP) and origin of migration are indicated.

(E) D1 activation induced PI-3 kinase activity enhancement was blocked by pretreatment with calmodulin antagonist W-13 (10 μM) from Cos-7 cells coexpressing D1 and NR1-1a/2A subunits.

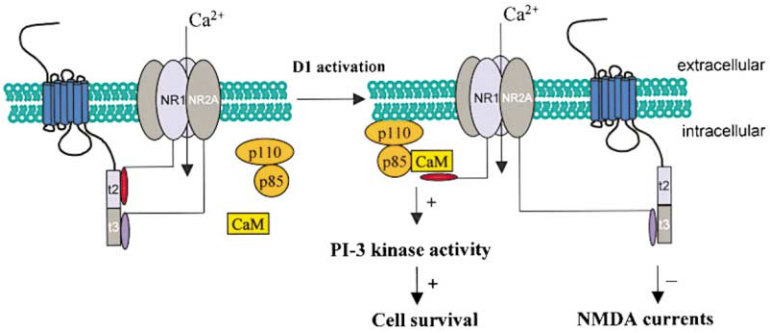

Figure 7. A Schematic of Direct Interactions between NMDA and D1 Receptors

Agonist activation of D1 receptors results in the dissociation of the D1-t2 region of the D1 receptor from the NR1 CT, allowing for the recruitment of CaM and PI-3 kinase (p85/p110 subunits) to the NR1 CT and subsequent activation of PI-3 kinase dependent cell survival mechanism(s). D1 activation inhibits NMDA currents through the D1-t3:NR2A interaction by reducing the receptor membrane expression.

receptor carboxyl tail (D1-t2, D1-t3) are associated with a specific subunit of the NMDA receptor (NR1-1a, NR2A). More importantly, by activating a single dopamine receptor subtype D1, each interaction can modulate one of two primary functional outcomes of NMDA receptor activation: NMDA-gated currents and excitotoxicity.

Whole-cell patch clamp recordings from hippocampal, striatal neurons, and HEK-293 cells coexpressing D1 and NMDA receptors exhibited an inhibition of NMDA-mediated currents upon D1 receptor activation by SKF 81297. The SKF effect could not be attributed to either PKC or PKA activation due to the application of inhibitors throughout the recordings. The inability of GTPγS to inhibit the SKF effect strongly suggests that D1 receptors modulate NMDA currents through a pathway independent from G protein-mediated D1 receptor signaling. Furthermore, we discovered that the SKF effect was significantly reduced by the D1-t3 peptide, but not with the D1-t2 peptide. Therefore, the inhibition of NMDA-mediated currents appeared to be specifically mediated by the D1-t3:NR2A interaction. In investigating possible mechanisms that may underlie the effects of D1 receptor agonist induced reduction of NMDA currents, we have found that SKF 81297 treatment significantly reduced the number of NMDA receptors on the cell surface, which was prevented by cotransfecting the D1-t3 mini-gene. Thus, the D1 modulation of NMDA receptors may be mediated through the regulation of NMDA receptor numbers on the cell surface, and that the D1:NR2A coupling may play a role in the regulation of NMDA receptor expression on the cell surface.

It has been generally acknowledged that excessive NMDA receptor activation can lead to excitotoxicity by inducing Ca^{2+} overload, potentially increasing Ca^{2+} mitochondrial influx and affecting the mitochondrial permeability transition pore which can lead to cell death (reviewed in Choi, 1995; Duchen, 2000). Therefore, attenuation of NMDA receptor currents may lead to protection against NMDA receptor-mediated excitotoxicity by reducing Ca^{2+} influx. However, in our study, it appears that the observed dopamine D1 receptor attenuation of NMDA receptor-induced toxicity was not dependent on the reduction of Ca^{2+} influx but by the activation of the PI-3 kinase. Although D1 receptor activation appears to reduce NMDA whole-cell currents by approximately 30%, comparable inhibition of NMDA whole-cell currents by application of 10 μM AP-5 (data not shown) does not reverse the excitotoxicity induced by NMDA treatment, which is in sharp contrast to the anti-apoptotic effects of PI-3 kinase dependent pathway upon activation of D1 receptors. Furthermore, neither D1 nor NMDA receptors (NR1-1a/NR2A) alone was capable of enhancing PI-3 kinase activity with or without agonist stimulation. Taken together with the observation that the D1-t2 mini-gene can block the enhancement of PI-3 kinase activity, the data suggest the critical role of the D1-t2:NR1-1a interaction in this process. We have demonstrated the competitive binding of D1-t2 and CaM to the NR1-1a$_{CT}$ in a concentration and calcium-dependent manner. Interestingly, agonist stimulation of D1 receptors, which protects cells from NMDA-mediated excitotoxicity, leads to the uncoupling of the D1-t2:NR1-1a complex, thereby leaving NR1-1a subunits more accessible to promote the formation of the NR1-1a:CaM:PI-3 kinase complex.

Previous studies have shown that the activation of CaM and PI-3 kinase depends upon Ca^{2+} availability (Fischer et al., 1998, Egea et al., 2001); therefore, the NR1-1a:CaM:PI-3 kinase complex formation could ideally place PI-3 kinase in close proximity to NMDA receptor-mediated Ca^{2+} influx, facilitated by the Ca^{2+} sensor, CaM. Furthermore, the NR1-1a:CaM:PI-3 kinase complex formation would recruit PI-3 kinases to membranes where it can phosphorylate phosphoinositides. Thus, Ca^{2+} influx during NMDA receptor activation would sequentially activate CaM and NMDA receptor-associated PI-3 kinase, and thereby activate PI-3 kinase-dependent cell survival mechanism(s) to reduce NMDA-induced excitotoxicity.

Recent evidence suggests that G protein-coupled receptor function may be more elaborate and involve direct protein coupling to form "intra-receptor" or "inter-receptor" classes of complexes. The dimerization of $GABA_BR1$ with $GABA_BR2$, which is necessary for the membrane expression of the functional receptor, along with the dimerization of δ-opioid receptors, are examples of intra-receptor oligomerization. Inter-receptor oligomerization can also occur between different types of G protein-coupled receptors, illustrated by interactions between the somatostatin receptor SSTR5 with the dopamine D2 receptor (Rocheville et al., 2000) and the adenosine A1 receptor with the dopamine D1 receptors (Gines et al., 2000). Moreover, receptor dimerization has not only been demonstrated within a certain family of receptors, such as the G protein-coupled receptor family, but has also been demonstrated between different receptor families. This report, taken together with the

identification of the direct interaction of the G protein-coupled dopamine D5 receptor to the ligand gated ion channel $GABA_A$ receptor (Liu et al., 2000), provides examples of dimerization between different families of receptors and an alternative mechanism for receptor crosstalk. Thus, the formation of "macro-receptor" complexes may facilitate both direct and indirect means to regulate receptor signaling.

In summary, our data provides further evidence that G protein-coupled receptors can modulate ligand-gated ion channels through the direct protein-protein interactions. The two independent direct protein-protein interactions between D1 and NMDA receptors provide a mechanism by which the activation of the dopamine D1 receptor selectively regulates multiple NMDA receptor-mediated functions. The observed subunit specific interactions allow for functional differentiation of heteromeric ligand-gated ion channels that are composed of multiple subunits. Specifically, we have shown the delineation between D1 receptor-modulation of NMDA receptor currents from the protection of NMDA-mediated excitotoxicity mediated by the recruitment of PI-3 kinase and the subsequent activation of downstream PI-3 kinase dependent anti-apoptotic mechanisms.

Experimental Procedures

GST Fusion Proteins and Minigenes

$D1_{CT}$, D1-t1, D1-t2, D1-t3, NR1-$1a_{CT}$, and $NR2A_{CT}$ cDNA-encoding fragments were amplified by PCR from full-length cDNA clones. All 5′ and 3′ oligo-nucleotides incorporated BamHI and EcoRI sites respectively to facilitate subcloning into pcDNA3 or pGEX4T-2. Initiation methionine residues and stop codons were also incorporated where appropriate. GST-fusion proteins were prepared from bacterial lysates as described by the manufacturer (Amersham). To confirm appropriate splice fusion and the absence of spurious PCR generated nucleotide errors, all constructs were resequenced.

In Vitro Binding Assays

GST-NR1-$1a_{CT}$, GST-$NR2A_{CT}$, or GST (10 μg) alone was incubated with [^{35}S]-$D1_{CT}$ probe in the presence or absence of D1-t2 or D1-t3 peptide for 4 hr at RT. The beads were then washed six times with tissue homogenizing buffer and eluted with 10 mM glutathione. Eluates were separated by SDS-PAGE and visualized by autoradiography.

Apoptosis Detection and Fluorescence Microscopy

Apoptosis detection and fluorescence microscopy were essentially performed as previously described (Lee et al., 2001). Briefly, HEK-293 cells coexpressing D1, NR1-1a/2A subunits, or where indicated, were treated for 20 min with either 10 μM SKF 81297 or control vehicle followed by the induction of apoptosis as previously described (Sattler et al., 1999). Apoptotic cells were detected using the ApoAlert MitoSensor Kit (Clontech), which detects alterations in mitochondrial membrane potentials upon induction of apoptosis (Green and Reed, 1998). After treatment with the ApoAlert MitoSensor reagent for 20 min at 37°C, cells were subsequently examined under a fluorescence microscope (Leica DM1RB) with computer image capture capability. The proportion of healthy to apoptotic cells was quantified using image capture software (MCID 5.1, Imaging Research Inc.) with the capacity for automatic target detection, identifying fluorescing images through defined optical density and spatial criteria parameters.

Whole-Cell Recordings in Both Cultured Hippocampal Neurons and HEK-293 Cells

Whole-cell recordings were conducted as previously described (Yu and Salter 1998; Lei et al., 2002). In brief, cells were bathed in a standard extracellular solution containing (mM): NaCl: 140, KCl: 5.4, glucose: 33, $CaCl_2$: 1.3, HEPES: 25, TTX: 0.001, glycine: 0.003 (for neurons) or 0.03 (for transfected cells), [pH 7.35], and osmolarity 310–320 mosM. Recording pipettes were made from thin-walled borosilicate glass capillaries (WPI, Sarasota, FL), pulled to a diameter of 1–2 μm at the tip with a resistance of 4–7 MΩ, and filled with intracellular solution composed of (mM): CsCl: 140, BAPTA: 1, HEPES: 10, $MgCl_2$: 2, K-ATP: 4, staurosporine: 0.001, 10 μM Rp-cAMP: 0.01; [pH 7.25], and osmolarity 290–300 mOsm. Whole-cell currents were evoked by application of L-aspartate or NMDA (250 μM) via a multi-barrel fast-step perfusion system, recoded in the voltage-clamp model using Axopatch 200B amplifiers (Axon Instruments, Foster City, CA) at a holding potential of −60 mV except where indicated and analyzed with pClamp6 software (Axon Instruments).

Recombinant Adenovirus Construction and Infection

Recombinant adenoviruses were formed by cotransfecting cDNAs encoding the D1-t2, D1-t3 in the shuttle vector pDC315 (Microbix) with replication-deficient adenovirus type 5 DNA into HEK-293 cells. The recombinant adenoviruses containing the D1-t2, D1-t3 cDNAs were isolated, confirmed by restriction analysis, plaque-purified, expanded, and titered. For infection, primary hippocampal cultures were infected with approximately 10 to 20 plaque-forming units per neuron [multiplicity of infection (moi)] of recombinant adenovirus in 500 μl culture medium. Cultures were supplemented with 1.5 ml of fresh medium 1 hr after infection.

PI-3 Kinase Assay

Coimmunoprecipitations were carried out with polyclonal antibodies to the p85 subunit of PI-3 kinase (2 μg/sample; Upstate Biotech) from Cos-7 cells expressing D1 or coexpressing D1 and NMDA receptors. The agarose-antigen-antibody complex pellets were then washed 3× each with buffers (buffer I: PBS containing 1% NP 40; buffer II: 0.5 M LiCl, 0.1 M Tris [pH 7.5]; buffer III: 10 mM Tris [pH 7.5], 100 mM NaCl). After washing, the complex was resuspended in 70 μl buffer III containing 14.3 mM $MgCl_2$ and 100 μg phosphoinositides (Sigma). The reaction was initiated by the addition of 0.88 mM ATP and 5 μCi [^{32}P] ATP. Incubation was performed for 10 min at 37°C and the reaction was stopped by the addition of 160 μl of $CHCl_3$: MeOH (1:1) and 20 μl of 6 M HCl. Lipids were extracted and spotted onto silica gel TLC plates. Spots corresponding to phosphatidylinositol 3-phosphate were detected by autoradiography and identified based on their comigration with a known standard.

Cell-ELISA Assays

Cell-ELISA assays (colorimetric assays) were done essentially as previously described (Man et al., 2000). In brief, the same density of HEK-293 cells cotransfected with cDNAs encoding various receptor constructs were treated with SKF-81297 and fixed in 4% paraformaldehyde for 10 min in the absence (non-permeant conditions) or the presence (permeant conditions) of 1% Triton X-100. Cells were incubated with a monoclonal antibody against Flag epitope (Sigma; 1 μg/ml to detect the Flag epitope inserted into the N terminus of NR1 subunit) for the purpose of labeling the receptors on the cell surface under non-permeabilized conditions or the entire receptor pool under permeabilized conditions. After incubation with corresponding HRP-conjugated secondary antibodies, HRP substrate OPD was added to produce a color reaction that was stopped with 3N HCl. The rate of cell surface expression of NR1 was presented as the ratio of colorimetric readings under non-permeabilized conditions to those under permeabilized conditions and then normalized to their respective control groups. Analysis was done using at least 6–12 separate dishes in each group.

Coimmunoprecipitation, affinity pull-down, blot overlay assay, and Western blot analyses were performed as previously described in our group (Liu et al., 2000).

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