Cellular/Molecular

Protein–Protein Coupling/Uncoupling Enables Dopamine D_2 Receptor Regulation of AMPA Receptor-Mediated Excitotoxicity

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There is considerable evidence that dopamine D_2 receptors can modulate AMPA receptor-mediated neurotoxicity. However, the molecular mechanism underlying this process remains essentially unclear. Here we report that D_2 receptors inhibit AMPA-mediated neurotoxicity through two pathways: the activation of phosphoinositide-3 kinase (PI-3K) and downregulation of AMPA receptor plasma membrane expression, both involving a series of protein–protein coupling/uncoupling events. Agonist stimulation of D_2 receptors promotes the formation of the direct protein–protein interaction between the third intracellular loop of the D_2 receptor and the ATPase *N*-ethylmaleimide-sensitive factor (NSF) while uncoupling the NSF interaction with the carboxyl tail (CT) of the glutamate receptor GluR2 subunit of AMPA receptors. Previous studies have shown that full-length NSF directly couples to the $GluR2_{CT}$ and facilitates AMPA receptor plasma membrane expression. Furthermore, the CT region of GluR2 subunit is also responsible for several other intracellular protein couplings, including p85 subunit of PI-3K. Therefore, the direct coupling of D_2–NSF and concomitant decrease in the NSF–GluR2 interaction results in a decrease of AMPA receptor membrane expression and an increase in the interaction between GluR2 and the p85 and subsequent activation of PI-3K. Disruption of the D_2–NSF interaction abolished the ability of D_2 receptor to attenuate AMPA-mediated neurotoxicity by blocking the D_2 activation-induced changes in PI-3K activity and AMPA receptor plasma membrane expression. Furthermore, the D_2–NSF–GluR2–p85 interactions are also responsible for the D_2 inhibition of ischemia-induced cell death. These data may provide a new avenue to identify specific targets for therapeutics to modulate glutamate receptor-governed diseases, such as stroke.

Key words: **dopamine receptors; AMPA receptors; NSF; protein–protein interactions; cell death; PI-3 kinase; G-proteins**

Introduction

AMPA receptors are ligand-gated ion channels that are composed of different glutamate receptor subunits, termed GluR1, GluR2, GluR3, and GluR4 (Boulter et al., 1990; Keinanen et al., 1990; Nakanishi et al., 1990). Although the exact subunit composition of native AMPA receptors is unclear, immunoprecipitation strategies from rat hippocampus have shown that two major AMPA receptor complexes are composed of the GluR2 subunit in combination with either GluR1 or GluR3 subunit (Wenthold et al., 1996). Previous studies have shown that AMPA receptor phosphorylation/dephosphorylation, by PKA (cAMP-dependent protein kinase), protein kinase C (PKC), or CaM kinase (calcium–calmodulin kinase), can regulate synaptic glutamatergic activity (Greengard et al., 1991; Lau and Huganir, 1995; Yakel et al., 1995; Barria et al., 1997). Recently, studies have shown the direct binding of AMPA receptor subunit carboxyl tail (CT) regions to a variety of intracellular proteins, such as PKC-interacting protein (Pick 1), *N*-ethylmaleimide-sensitive factor (NSF), adapter protein-2 (AP-2), and Grip (glutamate receptor-interacting protein), plays an important role in receptor targeting, membrane expression, internalization, clustering, and in the modulation of receptor activity and activation of signaling pathways (Dong et al., 1997; Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Noel et al., 1999; Xia et al., 1999).

Numerous reports have documented that excessive glutamate, through NMDA/AMPA receptors, activates the excitotoxic process (Choi et al., 1995; Lipton and Nicotera, 1998; Duchen, 2000), which may play an important role in the pathogenesis of acute stroke, hypoglycemic brain damage, and Huntington's disease (Wieloch, 1985; Beal and Martin, 1986; Simon et al., 1986; Boast et al., 1988). Strong evidence that AMPA receptors may play a role in post-ischemic neurodegeneration comes from studies investigating the protective effects of AMPA receptor antagonists against ischemia-induced neuronal death (Pulsinelli et al., 1993; Sheardown et al., 1993; Shaw et al., 1999) and changes in AMPA receptor subunit expression in certain brain regions after ischemia (Pellegrini-Giampietro et al., 1994; Gorter et al., 1997;

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Ying et al., 1997), indicating a physiological role of AMPA receptors in post-ischemic neurodegeneration.

Dopamine D_2 receptors belong to a superfamily of single polypeptide seven transmembrane domain receptors that exert their biological effects via intracellular G_i/G_o-protein-coupled signaling cascades. Two D_2 receptor variants, termed D_2Long and D_2Short, arise from alternative splicing. Localization studies have shown that the D_2Short receptor is the predominant presynaptic D_2 receptor, whereas D_2Long is preferentially involved in postsynaptic signaling (Usiello et al., 2000; Lindgren et al., 2003). There is considerable evidence demonstrating that D_2 receptors modulate glutamate-induced neurotoxicity (Garside et al., 1996; Cepeda and Levine, 1998; Sawada et al., 1998). D_2 receptor agonists have shown a protective effect against both hippocampal and nigrostriatal damage after ischemia (Liu et al., 1995; Hall et al., 1996; O'Neill et al., 1998). Systemic administration of the glutamatergic agonist kainic acid results in hippocampal cell death in D_2 receptor knock-out mice but not wild-type mice (Bozzi et al., 2000; Bozzi and Borrelli, 2002). All of these results reveal a central role of D_2 receptors in the inhibitory control of glutamate-induced excitotoxicity; however, the mechanism underlying this process is essentially unclear. Recently, we have shown that direct protein–protein interaction can facilitate functional cross-talk between G-protein-coupled receptors (GPCRs) and ligand-gated ion channels (Liu et al., 2000; Lee et al., 2002a; Pei et al., 2004). These data offer a possible molecular basis for the previously observed functional interactions between D_2 and AMPA receptors. Therefore, our present proposal investigates the D_2 receptor modulation of AMPA receptor-mediated neurotoxicity and explores the potential role of protein–protein interactions as an underlying mechanism to this process.

Materials and Methods

Apoptosis detection and fluorescence microscopy. Apoptosis detection and fluorescence microscopy were essentially performed as described previously (Lee et al., 2001, 2002a). Briefly, human embryonic kidney-293T (HEK-293T) cells coexpressing D_2Long, GluR1/2 subunits, or when indicated, the $D_{2(IL3\text{-}2C)}$ were treated for 20 min with either 10 μM quinpirole or control vehicle, followed by exposing cells to AMPA (30 μM) for 24 h as described previously (Carriedo et al., 1998; Iihara et al., 2001). Apoptotic cells were detected using the ApoAlert MitoSensor kit (Clontech, Palo Alto, CA), which detects alterations in mitochondrial membrane potentials during induction of apoptosis (Green and Reed, 1998). After treatment with the ApoAlert MitoSensor reagent for 20 min at 37°C, cells were examined under a fluorescence microscope (DM1RB; Leica, Nussloch, Germany) with computer image-capture capability. The proportion of healthy to apoptotic cells was quantified using image-capture software (MCID 5.1; Imaging Research, St. Catharines, Ontario, Canada) with the capacity for automatic target detection, identifying fluorescing images through defined optical density and spatial criteria parameters that were consistent for all samples.

Quantification of AMPA-mediated excitotoxicity: use of multiwell fluorescence scanner with propidium iodide. AMPA receptor-mediated excitotoxicity was induced as described above in HEK-293T cells coexpressing both D_2 and GluR1/2 subunits with or without pretreatment with the D_2 receptor agonist quinpirole (10 μM). To quantify AMPA-mediated cell death, culture medium was replaced by extracellular solution containing propidium iodide (PI) (Molecular Probes, Eugene, OR) at a final concentration of 50 μg/ml. After 30 min incubation at 37°C, fluorescence intensity in each well was measured with a plate reader (HTS 7000; PerkinElmer, Wellesley, MA) as described previously (Sattler et al., 1997, 1998). The fraction of dead cells in each well was calculated as follows: fraction dead $= (F_t - F_0)/F_{NMDA}$, where F_t is PI fluorescence at time t, F_0 is initial PI fluorescence at time 0, and F_{NMDA} is background-subtracted PI fluorescence of identical cultures from the same dissection and plating, 24 h after a 60 min exposure to 1 mM NMDA at 37°C. Previous studies have proven that this NMDA exposure routinely produced near-complete neuronal death in each culture but had no effect on surrounding glia (Bruno et al., 1994; David et al., 1996; Sattler et al., 1997, 1998, 2000).

Glutathione S-transferase-fusion proteins and mini-genes. Dopamine $D_{2(CT)}$, $D_{2(IL3)}$, $D_{2(IL3\text{-}1)}$, $D_{2(IL3\text{-}2)}$, $D_{2(IL3\text{-}2A)}$, $D_{2(IL3\text{-}2B)}$, and $D_{2(IL3\text{-}2C)}$ cDNA-encoding fragments were amplified by PCR from full-length cDNA clones (supplemental Fig. B, available at www.jneurosci.org as supplemental material). All 5′ and 3′ oligonucleotides incorporated *Bam*HI and *Eco*RI sites, respectively, to facilitate subcloning into pcDNA3 or pGEX4T-3. Initiation methionine residues and stop codons were also incorporated when appropriate. Glutathione *S*-transferase (GST)-fusion proteins were prepared from bacterial lysates as described by the manufacturer (Amersham Biosciences, Arlington Heights, IL). To confirm appropriate splice fusion and the absence of spurious PCR-generated nucleotide errors, all constructs were resequenced. GST–$GluR2_{CT833\text{–}853}$ and GST–$GluR2_{CT833\text{–}843}$ were generous gifts from Dr. Y. T. Wang (University of British Columbia, Vancouver, British Columbia, Canada).

Coimmunoprecipitation, protein affinity purification (pull-down), and Western blotting. Coimmunoprecipitation, affinity pull-down, and Western blot analyses were performed as described previously (Liu et al., 2000; Lee et al., 2002a). Rat brain hippocampi (100 mg) were homogenized in buffer [containing 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% igepalCA630, 0.5–1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (5 μl/100 mg tissue; Sigma, St. Louis, MO)] and centrifuged at 10,000 × *g* at 4°C for 20 min. The supernatant was extracted, and protein concentrations were measured (Pierce, Rockford, IL). For coimmunoprecipitation experiments, solubilized hippocampal/cell extracts (500–700 μg of protein) were incubated in the presence of primary antibodies anti-D_1 (SC-1434; Santa Cruz Biotechnology, Santa Cruz, CA), anti-D_2 (AB1558; Chemicon, Temecula, CA), or IgG (1–2 μg) for 4 h at 4°C, followed by the addition of 20 μl of protein A/G agarose (Santa Cruz Biotechnology) for 12 h. Pellets were washed four times in buffer described above, boiled for 5 min in SDS sample buffer, and subjected to SDS-PAGE. Tissue-extracted protein (20–50 μg) was used as control in each experiment. For affinity purification experiments, solubilized hippocampal extracts (50–100 μg of protein) were incubated with glutathione-Sepharose beads (Amersham Biosciences) bound to the indicated GST-fusion proteins (50–100 μg) at room temperature for 1 h. Beads were washed three times with 600 μl of PBS containing 0.1–0.5% Triton X-100 before the bound proteins were eluted with glutathione elution buffer. Elutes were incubated in sample buffer and subjected to 10% SDS-PAGE for Western blot analysis. Blots were blocked with 5% nonfat dried milk dissolved in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, washed three times with TBST buffer, incubated with the appropriate primary antibody [anti-GluR2 (MAB397; Chemicon) or anti-NSF (AB1764; Chemicon), diluted in 1% milk in TBST] overnight at 4°C, and washed again with TBST buffer three times, and the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (diluted in 1% milk in TBST; Sigma) for 1.5 h at room temperate. The proteins were visualized with enhanced chemiluminescence reagents as described previously (Amersham Biosciences).

In vitro *binding assays.* Glutathione beads carrying GST-fusion proteins [$D_{2(IL3\text{-}2)}$, $D_{2(IL3\text{-}2A)}$, $D_{2(IL3\text{-}2B)}$, and $D_{2(IL3\text{-}2C)}$] or GST (10–20 μg of each) alone was incubated with [^{35}S]methionine-labeled NSF probe, respectively. The beads were then washed six times with PBS containing 0.5% Triton X-100 and eluted with 10 mM glutathione elution buffer. Eluates were separated by SDS-PAGE and visualized by autoradiography.

Laser confocal microscopy. As described previously, HEK-293T cells that were transiently transfected with GluR1, GluR2–hemagglutinin (HA), and D_2Long vectors were treated with 10 μM quinpirole 48 h after transfection. Briefly, cells were subsequently fixed with 4% paraformaldehyde, blocked with 10% normal goat serum in 1× PBS, and permeabilized with 0.2% Triton X-100. Afterward, cells were incubated with HA antibody (MMS-101P; Covance Research Products, Berkeley, CA) and monoclonal D_2 antibody (SC-5303; Santa Cruz Biotechnology) for 16–18 h, after which cells were incubated with anti-rabbit-FITC or anti-

mouse-cyanine 3 (Cy3) antibodies (Jackson ImmunoResearch, West Grove, PA) for 1–2 h. Mounted coverslips were then examined with a laser confocal microscope (LSM510; Zeiss, Oberkochen, Germany).

Cell ELISA. Cell ELISA (colorimetric assays) were done essentially as described previously (Lee et al., 2002a). HEK-293T cells were transiently transfected with the indicated cDNA constructs by the Lipofectamine (Invitrogen, San Diego, CA) method (6–10 μg of each indicated cDNA per 7.5×10^6 cells), equally distributed to two six-well plates (35 mm/well), and grown for 2–4 d. The same density of cotransfected cells was treated with 10 μM quinpirole or ECS before fixing in 4% paraformaldehyde for 10 min in the absence (nonpermeabilized conditions) or presence (permeabilized conditions) of 1% Triton X-100. Cells were incubated with a monoclonal antibody against the HA epitope (MMS-101P; Covance Research Products) (1 μg/ml to detect the HA epitope inserted into the extracellular N terminus of GluR2 subunit) for the purpose of labeling the receptors on the cell surface under nonpermeabilized conditions or the entire receptor pool under permeabilized conditions. After incubation with corresponding HRP-conjugated secondary antibodies (Sigma), HRP substrate *o*-phenylenediamine (Sigma) was added to produce a color reaction that was stopped with 3N HCl. The cell surface expression of HA–GluR2 after pretreatment with quinpirole was presented as the ratio of colorimetric readings under nonpermeabilized conditions to those under permeabilized conditions and then normalized to their respective control groups (pretreated with ECS). Analysis was done using at least 12 separate dishes in each group. Cell ELISA using primary hippocampal neurons were performed identically with assays using HEK-293T cells, with the exception that anti-GluR2 antibody (MAB397; Chemicon) instead of anti-HA was used as primary antibody.

Primary cultures, oxygen–glucose deprivation treatment, and TAT–$D_{2(IL3\text{-}2C)}$ peptide construction/purification. Primary cultures from hippocampus were prepared from fetal Wistar rats (embryonic day 17–19) on Cell$^+$ (Sarstedt, Nümbrecht, Germany) culture dishes as described previously (Liu et al., 2000). The cultures were used for experiments at 12–15 d after plating. The hippocampal cultures were treated with quinpirole (20 min, 10 μM) or human immunodeficiency virus-1 transactivating regulatory protein (TAT)–$D_{2(IL3\text{-}2C)}$ peptides (1 h, 10 μM) before oxygen–glucose deprivation (OGD) experiments. The OGD experiments were performed as described previously (Ning et al., 2004) using a specialized, humidified chamber (Plas-Labs, Lansing, MI) kept at 37°C, which contained an anaerobic gas mixture (85% N_2, 10% H_2, and 5% CO_2) (Goldberg and Choi, 1993; Ying et al., 1997). To initiate OGD, culture medium was replaced with deoxygenated, glucose-free extracellular solution (in mM: 140 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, and 10 HEPES). After 1 h challenge, cultures were removed from the anaerobic chamber, and the OGD solution in the cultures was replaced with maintenance medium. Cells were then allowed to recover for 24 h in a humidified incubator with 95% O_2 and 5% CO_2 at 37°C. TAT peptide was constructed by subcloning the $D_{2(IL3\text{-}2C)}$ phencyclidine fragment into pTAT vector received from Dr. S. Dowdey (University of California, San Diego, La Jolla, CA), which includes a His6 tag and an HA tag to facilitate the protein peptide purification and the visualization of the intraneuronal accumulation of the peptides. The TAT peptide was purified from bacterial lysates using an Ni-NTA spin column as described by the manufacturer (Invitrogen). The TAT-carried peptides were rendered cell permeant by fusing the $D_{2(IL3\text{-}2C)}$ PCR fragment to the cell-membrane transduction domain of the TAT protein (YGRKKRRQRRR) as described previously (Aarts et al., 2002). The TAT–$D_{2(IL3\text{-}2C)}$ was applied to primary cultures directly (10 μM) for 1 h. The primary culture will be examined by confocal microscopy for HA-peptide uptake using the anti-HA antibody and FITC-conjugated secondary antibody.

Phosphoinositide-3 kinase assay. Coimmunoprecipitations were performed with polyclonal antibodies to the p85 subunit of phosphoinositide-3 kinase (PI-3K) (2 μg/sample; Upstate Biotechnology, Lake Placid, NY) from Cos-7 cells expressing D_2Long and GluR1/2 subunits. The agarose–antigen antibody complex pellets were then washed three times each with buffers (buffer I: PBS containing 1% NP 40; buffer II: 0.5 M LiCl and 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 of buffer III containing 14.3 mM $MgCl_2$ and 100 μg of phosphoisitides (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 thin-layer chromatography (TLC) plates. Spots corresponding to phosphatidylinositol 3-phosphate were detected by autoradiography and identified based on their comigration with a known standard.

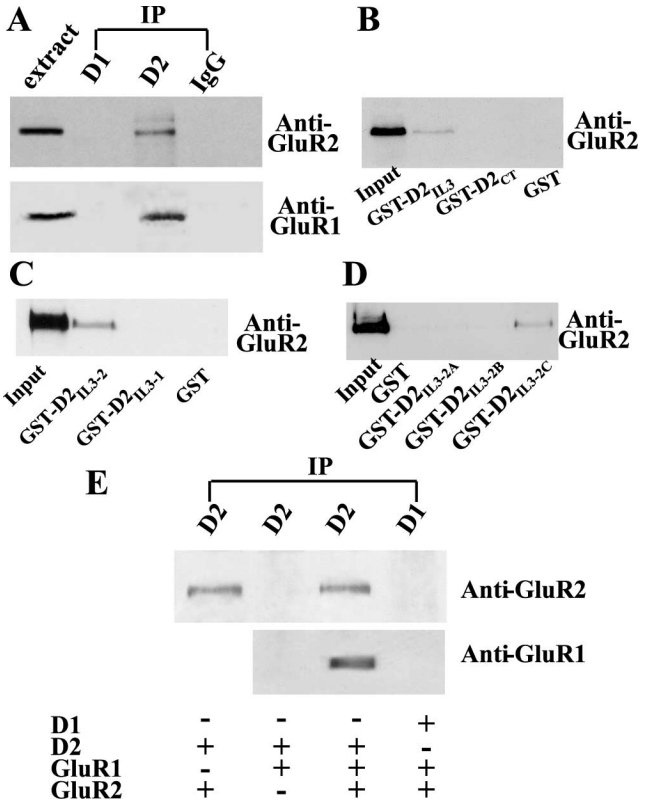

Figure 1. D_2 receptors exhibit a biochemical interaction with GluR2. ***A***, Coimmunoprecipitation of GluR2 subunit from solubilized rat hippocampal tissue with D_2, but not D_1, receptor antibody. ***B***, Western blots of GluR2 after affinity precipitation from solubilized rat hippocampal tissue by GST–$D_{2(IL3)}$ but not by GST–$D_{2(CT)}$ or GST alone. ***C***, Identification of the D_2 receptor region involved in the D_2–GluR2 interaction. GST-fusion proteins encoding regions within the D_2 third intracellular loop were used to affinity purify the GluR2 subunit. Only GST–$D_{2(IL3\text{-}2)}$ was able to affinity purify the GluR2 subunit. ***D***, Additional delineation of the D_2 receptor third intracellular loop involved in the D_2–GluR2 interaction. Western blot analysis reveals that GST–$D_{2(IL3\text{-}2C)}$, but not GST–$D_{2(IL3\text{-}2A)}$, GST–$D_{2(IL3\text{-}2B)}$, or GST alone, was able to pull down the GluR2 subunit from solubilized rat hippocampus. ***E***, Western blot analysis of D_1 and D_2 receptor antibody immunoprecipitates from HEK-293T cells transfected with D_1, D_2, GluR1, and/or GluR2 (as indicated) reveals that the D_2 receptor is capable of forming a complex with only the GluR2 and not the GluR1 subunit. Furthermore, the D_1 receptor does not coimmunoprecipitate the GluR1 or GluR2 AMPA receptor subunits. IP, Immunoprecipitation.

Results

Protein–protein interaction between AMPA receptor and dopamine D_2 receptors

In an attempt to define the physical basis and potential pathway for the functional interaction between D_2 and AMPA receptors, we first examined the existence of D_2–AMPA receptor complexes by determining whether AMPA receptors can coimmunoprecipitate with D_2 receptors in rat hippocampal tissue. As depicted in Figure 1*A*, the antibody against the D_2 receptor was able to coimmunoprecipitate AMPA receptor GluR2 and GluR1 subunits, suggesting that D_2 receptors can form a complex with AMPA receptors. In contrast, the D_1 receptor antibody, which has been shown to coimmunoprecipitate with NMDA receptors (Lee et al.,

2002a), did not coimmunoprecipitate the AMPA receptor subunits. To further characterize the specific region of D_2 receptors that may be responsible for D_2–AMPA complex formation, GST-fusion protein and mini-gene constructs were designed from sequences within two intracellular regions of the D_2 receptor ($D_{2(IL3)}$, K_{211}-Q_{373}; $D_{2(CT)}$, T_{428}-C_{443}). Using the affinity purification/pull-down method, we tested the ability of GST-fusion proteins encoding $D_{2(IL3)}$ and $D_{2(CT)}$ to recognize and precipitate AMPA receptor from solubilized rat hippocampal tissue. We determined the D_2 receptor region that allows D_2 receptors to complex with AMPA receptor is limited to the $D_{2(IL3)}$ region. This conclusion is based on the observation that only GST–$D_{2(IL3)}$, but not GST–$D_{2(CT)}$ or GST alone, recognizes and precipitates GluR2 subunit from solubilized hippocampal tissues (Fig. 1*B*). To confirm these results and to further delineate the region of the $D_{2(IL3)}$ involved in the D_2–AMPA receptor interaction, a series of affinity purification assays were performed that involved four $D_{2(IL3)}$ GST-fusion proteins ($D_{2(IL3-1)}$, I_{210}-V_{270}; $D_{2(IL3-2)}$, G_{242}-Q_{373}; $D_{2(IL3-2A)}$, D_{271}-P_{300}; $D_{2(IL3-2B)}$, S_{301}-I_{340}; $D_{2(IL3-2C)}$, F_{341}-Q_{373}) (for illustration, see supplemental Fig. B, available at www.jneurosci.org as supplemental material). Fusion proteins $D_{2(IL3-2A)}$, $D_{2(IL3-2B)}$, and $D_{2(IL3-2C)}$ are composed of sequences within $D_{2(IL3-2)}$. As illustrated in Figure 1, *C* and *D*, affinity purification assays showed that only GST–$D_{2(IL3-2)}$ and GST–$D_{2(IL3-2C)}$, but not $D_{2(IL3-1)}$, $D_{2(IL3-2A)}$, or $D_{2(IL3-2B)}$, were able to precipitate solubilized hippocampal GluR2 subunit, suggesting that F_{341}-Q_{373} fragment of the D_2 receptor is essential for the receptor to form protein complexes with AMPA receptors. We next examined which subunit of AMPA receptors interacts with D_2 receptors. Again, we used coimmunoprecipitation assays using lysates from HEK-293T cells cotransfected with the D_2Long receptor and GluR1/pcD, GluR2/pcD, or GluR1/2 subunits. We initially chose to use D_2Long instead of D_2Short based on the fact that D_2Long is preferentially involved in postsynaptic signaling (Usiello et al., 2000; Lindgren et al., 2003). As shown in Figure 1*E*, the D_2 receptor antibody is able to coimmunoprecipitate the GluR2 subunit without the existence of GluR1 subunit (top). However, D_2 receptors interact with the GluR1 subunit only in the presence of the GluR2 subunit (Fig. 1*E*, bottom), indicating that GluR2 subunit is responsible for D_2–AMPA receptor complex formation.

Although the preceding experiments addressed the presence of the D_2–AMPA complex in rat hippocampal tissue, it does not clarify whether the D_2–AMPA complex is formed exclusively with D_2 and AMPA receptors only or whether additional mediating proteins are involved. Therefore, we investigated whether D_2 receptors interact with AMPA receptors through direct physical coupling or by an indirect interaction involving an accessory binding protein. Results from both the *in vitro* binding assay and *in vitro* blot overlay were negative (data not shown) and suggest that D_2 and AMPA receptors are not able to directly interact with each other and require a mediating accessory protein.

D_2 receptors interact with GluR2 subunit through NSF

Based on the fact that (1) the GluR2 subunit is responsible for the formation of D_2–AMPA complex, (2) previous studies have revealed the direct interaction between the CT of the GluR2 subunit ($GluR2_{CT844-853}$) and NSF (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998), and most importantly, (3) the NSF and GluR2 interaction has been implicated in the AMPA-mediated neurotoxicity, we next examined whether NSF is the protein that mediates the D_2–GluR2 interaction. NSF is a homohexameric ATPase (Hanson et al., 1995; Fleming et al., 1998) that is an essential component of the protein machinery responsible for various membrane fusion events, including intercisternal Golgi protein transport and the exocytosis of synaptic vesicles (Rothman, 1994). To ascertain whether NSF is indeed the protein that facilitates the D_2–GluR2 interaction, we first established the existence of D_2–NSF complexes by coimmunoprecipitation experiments. As shown in Figure 2*A*, the D_2 receptor antibody was able to coimmunoprecipitate NSF, suggesting that NSF is a potential member of the D_2–AMPA complex. To determine whether NSF–D_2 interaction is mediated by the same motif of the D_2 receptor that is responsible for the formation of D_2–GluR2 complex, a series of affinity purification experiments were performed with GST-fusion proteins encoding the intracellular domains of the D_2 receptor as used in Figure 1, *C* and *D*. As expected, only GST–$D_{2(IL3-2C)}$ was able to recognize and precipitate NSF from solubilized rat hippocampal tissues (Fig. 2*B–D*), suggesting that F_{341}-Q_{373} fragment of D_2 receptor is also responsible in the formation of a protein complex with both NSF and the GluR2 subunit. Moreover, from the *in vitro* binding assay, D_2 receptors appear to interact directly with NSF. As shown in Figure 2*E*, ^{35}S-labeled NSF directly interacts with GST–$D_{2(IL3-2)}$ and GST–$D_{2(IL3-2C)}$. To investigate the absolute requirement of the $D_{2(IL3-2C)}$ sequence in the formation of D_2–NSF–GluR2 complex, GST–$D_{2(IL3-2)}$ was used in affinity purification experiments in which hippocampal lysates were preincubated with or without the purified $D_{2(IL3-2C)}$ peptide. As shown in Figure 2*F*, GST–$D_{2(IL3-2)}$ was able to precipitate both NSF and the GluR2 subunit, although equimolar amounts of GST–$D_{2(IL3-2)}$ appear to precipitate different levels of NSF and GluR2. However, pretreatment of $D_{2(IL3-2C)}$ peptide not only significantly decreased the amount of NSF that interacts with D_2 but also abolished the D_2–GluR2 interaction, indicating that $D_{2(IL3-2C)}$ is the region responsible for D_2–NSF direct coupling and that D_2 interacts with GluR2 indirectly through NSF. To further confirm that NSF mediates the D_2–GluR2 interaction, we applied a modified *in vitro* binding assay with the use of ^{35}S-labeled NSF and $D_{2(IL3-2)}$. As shown in Figure 2*G*, [^{35}S]NSF binds to GST–$GluR2_{CT}$ without the presence of [^{35}S]$D_{2(IL3-2)}$ (top), whereas [^{35}S]$D_{2(IL3-2)}$ displays a weak binding to GST–$GluR2_{CT}$ only at the presence of [^{35}S]NSF (bottom). Furthermore, when hippocampal lysates were incubated with either the GST–$GluR2_{CT833-853}$ (a portion of the C-terminus region of the GluR2 subunit containing the GluR2–NSF binding site) or GST–$GluR2_{CT833-843}$ (proximal partial of GST–$GluR2_{CT833-853}$ that lacks the GluR2–NSF binding site) (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998), only the GST–$GluR2_{CT833-853}$ peptide was capable of blocking the coimmunoprecipitation of the GluR2 with the D_2 receptor (Fig. 2*H*). Together, the data indicate that NSF is potentially the mediating protein that allows D_2 receptors to interact with AMPA receptors.

Activation of D_2 receptor inhibits AMPA receptor-mediated excitotoxicity in HEK-293T cells: implication of the D_2–NSF–GluR2 interaction

To investigate the functional implication of this biochemical interaction between dopamine D_2 and AMPA receptors, we tested the effects of D_2 receptor activation on AMPA receptor-mediated excitotoxicity in HEK-293T cells coexpressing D_2 and AMPA receptors. Based on previous reports, we focused on the GluR1/2 AMPA receptor combination, one of the two most common AMPA receptor subunit combinations in the hippocampus, and, in addition, these subunits have important defined roles in AMPA receptor trafficking and synaptic plasticity (Wenthold et al., 1996). AMPA receptor-mediated excitotoxicity was induced

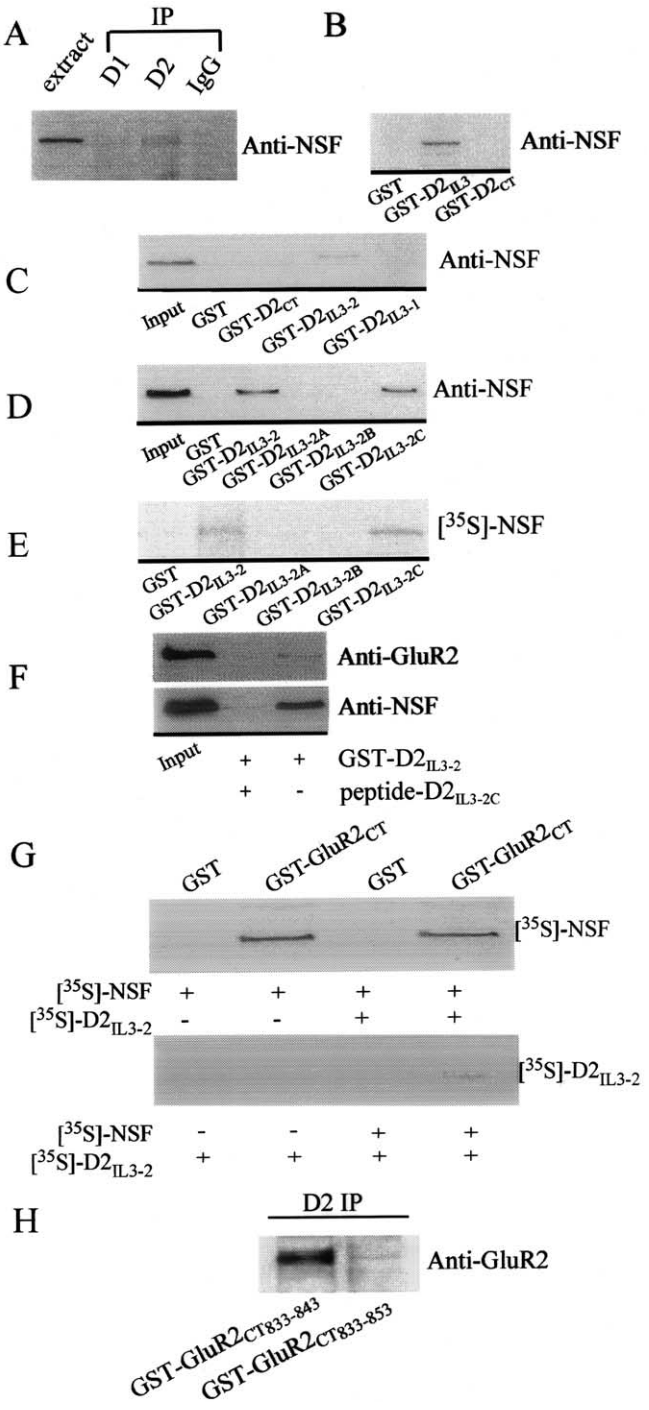

Figure 2. An interaction between the D_2 receptor and GluR2 is likely mediated by a direct physical interaction with NSF. ***A***, Western blot reveals that NSF is able to coimmunoprecipitate with D_2, but not D_1, receptors from rat hippocampal lysates. ***B***, The interaction between NSF and the D_2 receptor is mediated by the third intracellular loop, as shown by the anti-NSF Western blot in which GST–$D_{2(IL3)}$, but not GST–$D_{2(CT)}$ or GST alone, was able to pull down NSF. ***C***, The D_2–NSF interaction is mediated by the second half of the third intracellular loop, with only the GST–$D_{2(IL3-2)}$ being able to pull down NSF. ***D***, Identical to the D_2–GluR2 interaction, the D_2–NSF interaction appears to be mediated by the most distal region of the third intracellular loop, with only GST–$D_{2(IL3-2C)}$ being able to pull down NSF. ***E***, *In vitro* binding studies using ^{35}S-labeled NSF reveal that the interaction between the D_2 receptor and NSF can be mediated by a direct interaction between NSF and the $D_{2(IL3-2C)}$ region of the D_2 receptor. ***F***, Coincubation of rat hippocampal lysates with a $D_{2(IL3-2C)}$ peptide blocks the ability of GST–$D_{2(IL3-2)}$ to precipitate both NSF and GluR2. ***G***, *In vitro* binding assay in which GST or GST–$GluR_{2(CT)}$ are coincubated with [^{35}S]NSF, [^{35}S]$D_{2(IL3-2)}$, or both proteins to determine the necessity of NSF to mediate the D_2–GluR2 interaction. [^{35}S]NSF binds directly to GST–$GluR2_{CT}$ (top), but [^{35}S]$D_{2(IL3-2)}$ fails to bind to GST–$GluR2_{CT}$ directly and requires the presence of NSF to mediate the $D_{2(IL3-2)}$–$GluR2_{CT}$ interaction (bottom). ***H***, Anti-GluR2 Western blot analysis of coimmunoprecipitation samples from hippocampal lysates that were preincubated with either GST–$GluR2_{CT833-853}$ or GST–$GluR2_{CT833-843}$ before the addition of D_2 receptor antibody reveals a large decrease in the amount of GluR2 subunits that are coimmunoprecipitated with the D_2 receptor in the presence of the GST–$GluR2_{CT833-853}$. IP, Immunoprecipitation.

by incubation with 30 μM AMPA and 25 μM cyclothiazide (this was used to prevent AMPA receptors desensitization) for 24 h as described previously (Brorson et al., 1995; Jensen et al., 1998). Apoptotic cells were detected using the ApoAlert MitoSensor kit (Clontech) as described previously (Lee et al., 2002a). As illustrated in Figure 3*A* (top), pretreatment with 10 μM quinpirole greatly reduced AMPA receptor-mediated cell death. There was no difference in cell death number between quinpirole-treated and nontreated cells expressing only AMPA or D_2 receptors, and <3% of nontransfected or sham-treated cells exhibited apoptosis during exposure to AMPA (data not shown). Although we described the D_2–NSF–GluR2 protein–protein interaction with some detail, it is still unclear whether the observed D_2–NSF–GluR2 interaction is responsible for the observed D_2 receptor modulation of AMPA-mediated cell death. It should be noted that HEK-293T cells express endogenous NSF (supplemental Fig. C, top, available at www.jneurosci.org as supplemental material). To define the functional consequence of the observed D_2–NSF–GluR2 interaction, we examined whether the blockade of the D_2–NSF–AMPA interaction by using $D_{2(IL3-2C)}$ mini-gene would also abolish the protective effect on AMPA-mediated neurotoxicity by the activation of D_2 receptors. We measured cell toxicity using ApoAlert mitochondrial reagent (Clontech) as used previously (Lee et al., 2001, 2002). Briefly, the ApoAlert reagent, which is an index of mitochondrial transmembrane potential changes during apoptosis, in healthy cells is taken up by the mitochondria, form aggregates, and emits red fluorescence. However, in apoptotic cells, the reagents stays in the cytoplasm, remains as monomers, and emits green fluorescence. As illustrated in Figure 3*A* (bottom), the D_2-induced protective effect on AMPA-mediated excitotoxicity was attenuated by the overexpression of $D_{2(IL3-2C)}$ mini-gene in HEK-293T cells coexpressing D_2 and GluR1/2 subunits. Together with results that show coexpressing the $D_{2(IL3-2C)}$ mini-gene disrupts the D_2–NSF–GluR2 complex (Fig. 2*F*), these data strongly suggest that the interactions between the D_2 receptor, NSF, and GluR2 are essential for the quinpirole-mediated rescue from AMPA-induced toxicity.

We further quantified this D_2-induced decrease in AMPA-mediated excitotoxicity using a PI fluorescence assay (Sattler et al., 2000). Analogous to the ApoAlert MitoSensor results, the PI fluorescence assay revealed 45 $\pm$ 0.4% ($n = 6$; $p < 0.01$) (Fig. 3*B*) decrease in AMPA-mediated excitotoxicity by D_2 receptor activation. Furthermore, this process could be significantly blocked by pretreatment with D_2 receptor-specific antagonist raclopride in a concentration-dependent manner, indicating the requirement of D_2 activation in the process. Although in the absence of D_2 stimulation there is slight protective effect in cells coexpressing D_2 (Fig. 3*C*), this effect is small compared with the rescue seen during D_2 receptor activation seen in Figure 3*B*. As a member of the GPCR family, D_2 receptor activation initiates a biological response mediated by G-proteins, specifically through a $G_{i/o}$-dependent pathway. However, we observed that D_2 receptor modulation of AMPA receptor-mediated apoptosis does not appear to be dependent on the $G_{i/o}$-dependent pathways, because preincubating cells with pertussis toxin (PTX) (100–150 ng/ml),

which uncouples D_2 receptors from $G_{i/o}$-proteins, was not able to abolish the observed D_2 receptor modulation to AMPA receptor-mediated apoptosis (Fig. 3*B*). The effect of PTX to functionally block the ability of the D_2 receptor to inhibit cAMP accumulation was confirmed in parallel experiments on cells expressing both D_2 and AMPA receptors (data not shown). More importantly and similar to the ApoAlert assay, quinpirole pretreatment of cells coexpressing D_2/GluR1/2/$D_{2(IL3\text{-}2C)}$ did not rescue cells from AMPA-induced toxicity and exhibited virtually identical levels of toxicity compared with cells coexpressing D_2/GluR1/2 without quinpirole treatment (Fig. 3*B*). Again, these data provide more evidence that D_2 activation modulates AMPA-mediated excitotoxicity through the D_2–NSF–GluR2 protein–protein interaction.

Quinpirole treatment decreases GluR2 subunit plasma membrane expression

Previous studies have shown that the interaction between GluR2 and NSF is able to regulate the rapid turnover of the AMPA receptors at the synaptic membrane (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). Blocking the binding of GluR2 to NSF resulted in a rapid turnover of AMPA receptors at the synaptic membrane (Noel et al., 1999), which leads to a rapid and substantial decrease in AMPA receptor-mediated synaptic transmission and a significant reduction in the frequency of AMPA receptor-mediated spontaneous miniature EPSCs (Nishimune et al., 1998; Song et al., 1998). A recent study also demonstrated that NSF stabilizes the synaptic AMPA receptors by disruption of GluR2–Pick1 interaction (Hanley et al., 2002). Furthermore, disruption of the GluR2–NSF interaction protects hippocampal neurons from ischemic stress (Ralph et al., 2001). Therefore, we speculated that the D_2-induced protective effect on AMPA-mediated cell death may be attributable to decreased AMPA receptor plasma membrane expression. Immunocytofluorescence visualization of D_2 receptor and AMPA receptor GluR2 subunits coexpressed in HEK-293T cells revealed a significant amount of colocalization of the two receptors at the cell surface (Fig. 4*A*, top). However, quinpirole treatment induced an internalization of both receptors in a proportion of the cell population examined (Fig. 4*A*, bottom). Quantification of the cell surface localization of the GluR2 subunit, as shown in Figure 4*B*, with a cell-based ELISA revealed a significant but small decrease in HA–GluR2 subunit on the cell surface by D_2 receptor activation (control, 100%; quinpirole, 84.2 ± 2; $n = 12$; $p < 0.01$). Furthermore, the internalization of the GluR2 subunit during D_2 receptor activation could be blocked by the overexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene, which we also show to inhibit the D_2 receptor-mediated attenuation of AMPA-mediated excitotoxicity. Together, this suggests that the rescue from AMPA-mediated excitotoxicity may be a result of decreased GluR2 subunits at the cell surface during D_2 receptor activation. However, although we observed a ~45% decrease in AMPA-mediated cell death in cotransfected HEK-293T cells, there was only a ~16% decrease in AMPA receptor plasma membrane expression after D_2 activation. These results led us to examine whether another pathway is also involved in the D_2 receptor inhibition of AMPA-mediated cell death.

Quinpirole treatment uncouples NSF–GluR2 interaction and promotes p85–GluR2 interaction that leads to the activation of PI-3K

Phosphoinositide-3 kinases have been implicated in regulating many fundamental cellular responses, including proliferation, transformation, protection from apoptosis, superoxide production, cell migration, and adhesion. The ability of PI-3K to promote cell survival through the activation of protein kinase B/Akt provides a potential mechanism for the protective effects of quinpirole treatment (Franke et al., 1997; Hemmings, 1997; Marte and Downward, 1997; Cantley, 2002). Although several groups have reported that D_2 receptor activation may lead to PI-3K activation (Kihara et al., 2002; Nair et al., 2003), there have also been reports of AMPA receptors coupling to PI-3K (Man et al., 2003). Furthermore, our group has reported that dopamine D_1 receptors protect against NMDA-mediated excitotoxicity through enhancing the NR1–CaM–p85 subunit coupling that leads to the activation of PI-3K pathway (Lee et al., 2002a). Therefore, the quinpirole-mediated rescue we observed may in part be attributable to PI-3K activation. As the first step toward investigating our hypothesis, we tested the agonist effects on the

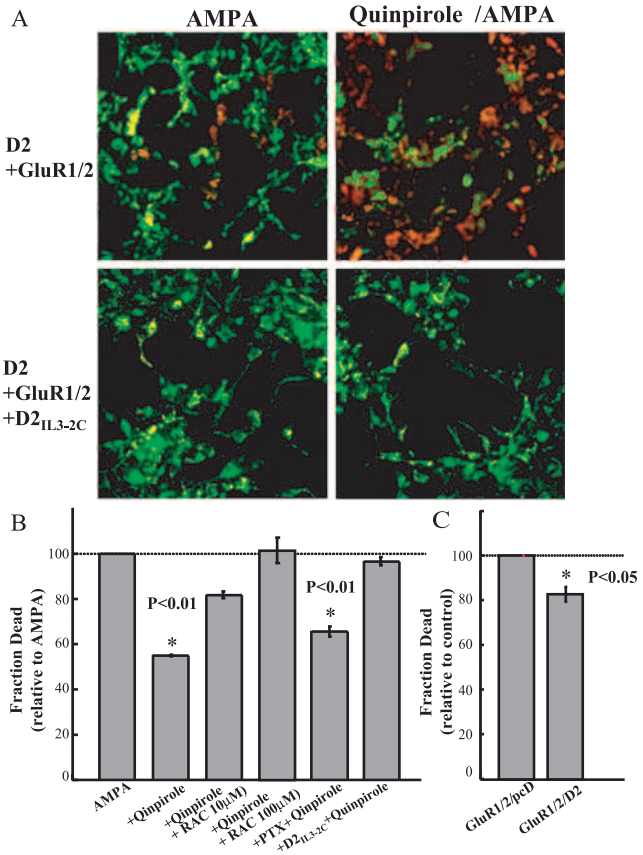

Figure 3. AMPA-induced cell death in HEK-293T cells coexpressing D_2Long and AMPA (GluR1/2) receptors is attenuated by D_2 receptor activation. ***A***, AMPA receptor stimulation (30 μM AMPA/25 μM cyclothiazide, 24 h at room temperature) induced apoptosis, as detected with an ApoAlert Mitosensor kit. Pretreatment of cells with 10 μM quinpirole for 20 min reduced the amount of apoptotic cells. The protective effect of quinpirole is attenuated during coexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene (bottom). ***B***, Quantification of AMPA receptor-mediated toxicity through quantitative measurements of PI fluorescence after indicated treatments. The numbers reported were normalized to the fraction of dead cells in samples treated with 30 μM AMPA/25 μM cyclothiazide only (AMPA). Pretreatment with 10 μM quinpirole significantly reduced cell toxicity, an effect that was partially blocked by a 20 min pretreatment with 10 μM raclopride (RAC) but not by PTX. Coexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene almost completely blocked the rescue seen with 10 μM quinpirole. Data were analyzed by ANOVA, followed by *post hoc* Student–Newman–Keuls test. An asterisk indicates a significant difference from the AMPA group ($p < 0.01$; $n = 6$). ***C***, Coexpression of D_2 receptors, along with GluR1/2 subunits, in HEK-293T cells exhibits a small but significant inhibition of AMPA-mediated cell death even in the absence of D_2 receptor agonist. Data were analyzed by Student's *t* test. The asterisk indicates a significant difference from GluR1/2/pcD group ($p < 0.05$; $n = 4$).

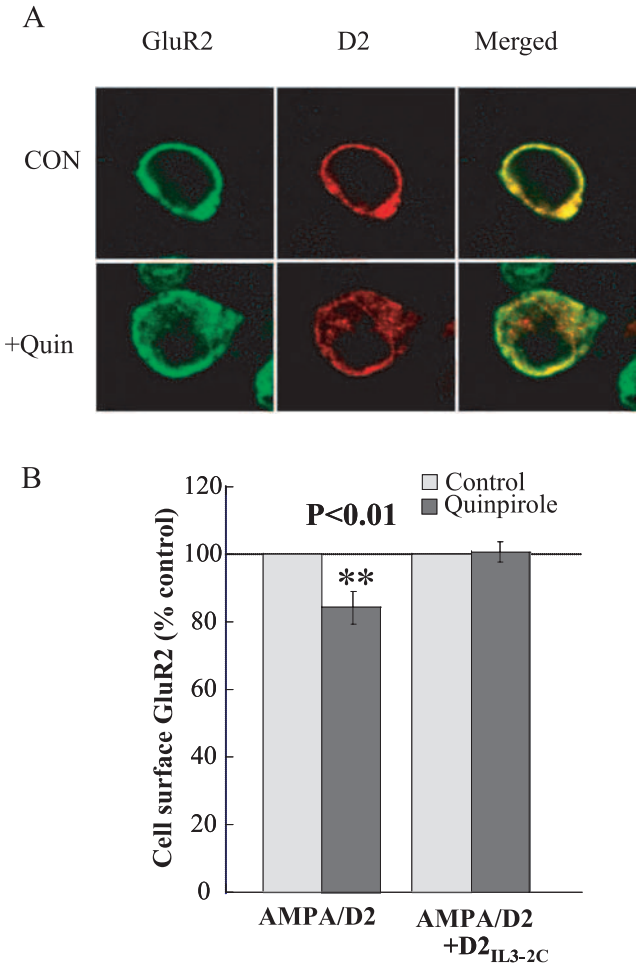

Figure 4. D_2 receptor activation results in a decrease in GluR2 AMPA receptor subunits localized at the cell surface. ***A***, Confocal microscopy of HEK-293T cells cotransfected with D_2Long receptor and AMPA receptor GluR1 and HA–GluR2 subunits. Both nontreated (CON) and 10 μM quinpirole-treated (+QUIN) cells were fixed and subsequently probed for D_2 receptor labeling that was detected with a Cy3 (red)-conjugated secondary antibody. The HA–GluR2 labeling was detected with an FITC (green)-conjugated secondary antibody. ***B***, Quantification of GluR2 expression at the plasma membrane using a cell-based ELISA (see Materials and Methods). In HEK-293T cells coexpressing D_2 receptor and GluR1/GluR2 subunit, cells that were treated with 10 μM quinpirole show a small but significant decrease in cell surface HA–GluR2 localization. This decrease in cell surface localization was blocked by coexpression of $D_{2(IL3\text{-}2C)}$ mini-gene. Data were analyzed by Student's *t* test. Double asterisks indicate a significant difference from the nontreated group ($p < 0.01$; $n = 6$).

D_2–NSF–GluR2 protein complex formation. As seen from the coimmunoprecipitation experiments in Figure 5*A* (top), quinpirole treatment of hippocampal lysates significantly increased the interaction between the D_2 receptor and NSF but decreased the GluR2–NSF coupling (Fig. 5*A*, middle). Previously, it has been suggested that full-length NSF is required for the NSF–GluR2 coupling (Nishimune et al., 1998), and, therefore, it is not surprising that there is a decrease in the GluR2–NSF interaction. In addition, this reduction in GluR2–NSF coupling not only frees the GluR2 subunit from NSF but allows the GluR2 subunit to potentially bind to other proteins. In fact, quinpirole treatment not only disrupted the D_2–GluR2 interaction and disengages NSF from GluR2 but promotes the interaction between the p85 subunit of PI-3K and GluR2 (Fig. 5*A*, bottom). Quinpirole stimulation did not significantly alter the initial levels of solubilized protein or the levels of directly immunoprecipitated proteins (data not shown). Next, to examine whether this increase in the p85–

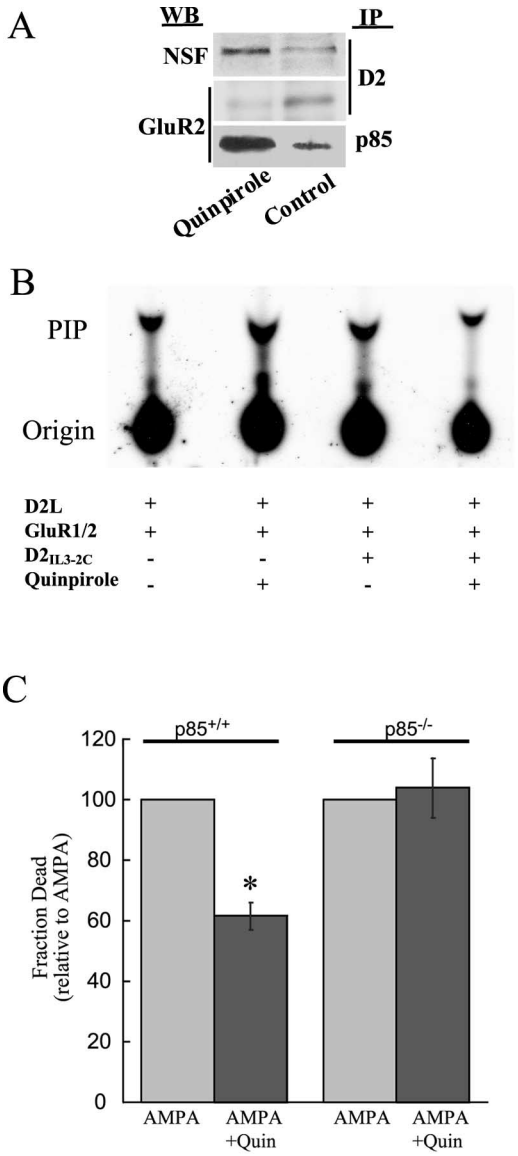

Figure 5. The attenuation of AMPA receptor-mediated toxicity is mediated by an increased GluR2–p85 interaction and correlative increase in PI-3K activity. ***A***, Western blot (WB) analysis of coimmunoprecipitation samples from hippocampal lysates that were pretreated with 10 μM quinpirole reveals an increase in the D_2–NSF interaction with a concomitant decrease in NSF–GluR2 interaction. Quinpirole pretreatment also enhances the p85–GluR2 interaction. IP, Immunoprecipitation. ***B***, PI-3K activities in anti-p85 immunoprecipitates from Cos-7 cells coexpressing D_2Long and GluR1/2 subunits 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. Quinpirole pretreatment (10 μM) significantly enhanced the activity of PI-3K, as indicated by the increase in ^{32}P-incorporated lipid products, an effect that was blocked by coexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene. ***C***, To confirm that D_2 receptors inhibit AMPA-mediated cell death via a PI-3-dependent pathway, we tested the D_2-protective effect on AMPA-mediated cell death in fibroblast lines derived from wild-type and PI-3K-deficient p85$\alpha^{-/-}$ embryos cotransfected with D_2 and GluR1/2 subunits. Activation of D_2 receptors exhibited no protective effects on AMPA-induced cell death in p85$\alpha^{-/-}$ cells. In contrast, D_2 receptor activation effectively protected cells from AMPA toxicity in wild-type cells. +QUIN, Quinpirole treated. Data were analyzed by Student's *t* test. The asterisk indicates a significant difference from the AMPA group ($p < 0.01$; $n = 3$).

GluR2 interaction during D_2 activation leads to an increase in PI-3K activity, we used the PI-3K assay using lysates from Cos-7 cells coexpressing the D_2Long receptor and GluR1 and GluR2 subunits and, when indicated, with the $D_{2(IL3\text{-}2C)}$ mini-gene. It was unnecessary to transfect recombinant NSF because Cos-7

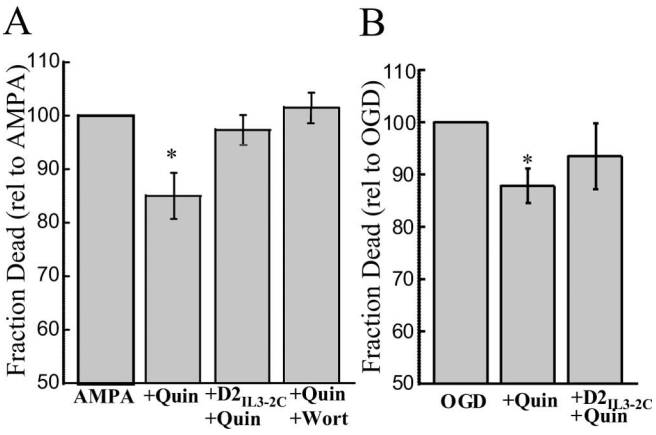

Figure 6. AMPA-induced and OGD-induced neurotoxicity of dissociated primary cultures of hippocampal neurons. ***A***, Pretreatment with 10 μM quinpirole (Quin) reduced the apoptosis induced by 30 μM AMPA (coincubation of 10 μM MK-801 and 2 μM nimodipine). Quantification of AMPA-induced toxicity of rat hippocampal neurons through PI fluorescence measurements show a reduction in toxicity after quinpirole pretreatment, an effect that was removed during coexpression of $D_{2(IL3\text{-}2C)}$ or with the cotreatment of 100 nM wortmannin (Wort). Data were analyzed by ANOVA, followed by *post hoc* Student–Newman–Keuls test. The asterisk indicates a significant difference from the AMPA group ($p < 0.01$; $n = 4$). ***B***, PI fluorescence quantitative measurements of rat hippocampal neuron toxicity after cell death was induced by OGD. Quinpirole pretreatment was able to significantly reduce the toxicity induced by OGD. Data were analyzed by ANOVA, followed by *post hoc* Student–Newman–Keuls test. The asterisk indicates a significant difference from OGD group ($p < 0.01$; $n = 7$).

cells exhibit significant endogenous NSF expression (supplemental Fig. C, bottom, available at www.jneurosci.org as supplemental material). As shown in Figure 5*B*, during quinpirole treatment, there is a significant increase in PI-3K activity, as indicated by the increase in ^{32}P-incorporated lipid products that has migrated on the TLC plates. However, this increase is significantly reduced during coexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene. To confirm that a PI-3 dependent pathway is involved in the D_2 receptor inhibition of AMPA-mediated cell death, we tested the D_2 receptor-protective effect on AMPA-mediated cell death in fibroblast lines derived from wild-type and PI-3K p85$\alpha^{-/-}$ embryos cotransfected with D_2 and GluR1/2 subunits (Fruman et al., 1999). As shown in Figure 5*C*, activation of D_2 receptors exhibited no protective effects on AMPA-induced cell death in p85$\alpha^{-/-}$ cells. In contrast, D_2 receptor activation effectively protected cells from AMPA toxicity in wild-type cells. Thus, these data confirmed that D_2 receptor stimulation protects cells from AMPA-mediated cell death through the activation of the PI-3K pathway by promoting a GluR2–p85 interaction.

D_2 receptor activation modulates AMPA receptor-mediated excitotoxicity and ischemic stress in hippocampal neuronal cultures

Primary dissociated cultures of rat hippocampal neurons were used to confirm that the modulation of AMPA receptor-mediated cell death by activation of D_2 receptors occurs in a more physiologically relevant system. Quinpirole pretreatment of hippocampal neurons, in the presence of NMDA receptor and Ca^{2+} channel antagonists [10 μM (+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801) and 2 μM nimodipine, respectively] resulted in a small but significant decrease in AMPA-induced toxicity (~15%) during pretreatment with quinpirole (Fig. 6*A*). Moreover, we also show that the cell-permeant TAT–$D_{2(IL3\text{-}2C)}$ peptide (Aarts et al., 2002) was able to effectively block the D_2 receptor-dependent rescue from AMPA-mediated toxicity (Fig. 6*A*). We also show that wortmannin is capable of inhibiting the quinpirole-mediated rescue from AMPA-induced toxicity, which provides additional evidence that the quinpirole-induced rescue from AMPA-toxicity is indeed mediated by the activation of PI-3K.

AMPA-mediated toxicity is often considered a contributing, if not an underlying, causative factor in ischemia. Therefore, to verify the implication of the D_2–NSF–GluR2 interaction in ischemia, we used the OGD protocol as an *in vitro* model of ischemia to assess the effectiveness of quinpirole to rescue cells from another neurotoxic stress. As shown in Figure 6*B*, quinpirole pretreatment was again able to rescue a small but significant number of cells from OGD-induced toxicity (~12%), and this rescue effect was blocked by the cell-permeant TAT–$D_{2(IL3\text{-}2C)}$ peptide. Together, the data suggest that the modulation of the D_2–NSF–GluR2 interaction may play a key role in the D_2 receptor-mediated rescue from ischemia-induced cell death.

Discussion

In this report, we provide evidence in both cotransfected cells and dissociated primary cultures of hippocampal neurons that activation of D_2 receptors inhibits AMPA receptor-mediated excitotoxicity. Activation of D_2 receptors in cotransfected cells with quinpirole results in a ~45% decrease in AMPA-mediated toxicity. In addition, this rescue appears to be mediated by the shuttling of NSF from GluR2 to the D_2 receptor, which increases GluR2 subunit accessibility by other proteins. The carboxyl tail of the GluR2 subunit is responsible for the interaction with many distinct proteins (Dong et al., 1997; Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Xia et al., 1999), especially the $GluR2_{CT833-853}$ region, which directly couples to AP-2, NSF, and PI-3K p85 subunit (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Braithwaite et al., 2002; Lee et al., 2002b; Man et al., 2003). Interestingly, we show that quinpirole treatment enhances the GluR2–p85 interaction, which in turn is correlated to an increase in PI-3K activation, which initiates anti-apoptotic pathways. In addition, cotreatment of wortmannin, a PI-3K inhibitor, effectively blocks the rescue effect of quinpirole on AMPA-induced toxicity.

AMPA receptors are ligand-gated ion channels that are classically regulated by G-protein-coupled receptors through the activation of downstream second-messenger systems, which can involve the phosphorylation of the ligand-gated ion channel (Price et al., 1999; Snyder et al., 2000; Chao et al., 2002, 2003; Vanhoose and Winder, 2003; Gomes et al., 2004; Mangiavacchi and Wolf, 2004). Recently, our group has demonstrated that G-protein-coupled dopamine D_1/D_5 receptors can modulate ligand-gated ion channel NMDA/$GABA_A$ receptors through direct protein–protein interactions, respectively (Liu et al., 2000; Lee et al., 2002a; Pei et al., 2004). Furthermore, several groups have reported that direct protein–protein interactions form the basis of a molecular mechanism that participates in the modulation of AMPA receptor-mediated functions, albeit most of these interacting proteins are intracellular accessory proteins (Dong et al., 1997; Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Xia et al., 1999). In this study, we have shown through the *in vitro* binding assay that dopamine D_2 receptors do not directly interact with AMPA receptors. Nonetheless, D_2 receptors can still form a protein complex with AMPA receptors that is mediated by the GluR2 subunit and the third intracellular loop of D_2 receptors. Furthermore, NSF directly interacts with both the GluR2 subunit and dopamine D_2 receptors, as shown in Figure 2, implicating NSF as the possible identity of the accessory protein in the D_2–

AMPA interaction. NSF may serve not only as an anchoring protein for GluR2 but may also occlude sites on the GluR2 and prevent it from interacting with other proteins, namely p85. In this study, we provide evidence that D_2 receptor activation results in an increase in the D_2–NSF interaction and decrease in the D_2–GluR2 interaction, which provides circumstantial evidence that NSF may be shuttling between the two receptors depending on the activation state of the D_2 receptor. However, at this point in time, it is unclear what role NSF may have on D_2 receptor function given that coexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene does not affect cAMP accumulation (our unpublished observations). The uncoupling of NSF from GluR2 during quinpirole administration appears to increase the GluR2–p85 interaction. More importantly, this increase in the GluR2–p85 complex is associated with an increase in PI-3K activation that rescues cells from AMPA-mediated toxicity. Alternatively, NSF may be constitutively coupled to the GluR2 subunit to facilitate GluR2 subunit plasma membrane expression and, when uncoupled from the GluR2 subunit during D_2 receptor agonist stimulation, leads to the inability of NSF to maintain functional AMPA receptors at the cell surface and, subsequently, less calcium influx through AMPA receptors. However, this appears unlikely to be the major mechanism involved in the D_2 receptor-mediated rescue from AMPA toxicity because we only observed <20% decrease in GluR2 subunits localized at the cell surface during D_2 receptor stimulation in cells cotransfected with the D_2Long and AMPA receptor GluR1/2 subunits, which is unlikely to completely account for the ~45% rescue seen during quinpirole treatment. From our data, it is more likely that the activation of PI-3K that is facilitated by the GluR2–p85 interaction has a more significant contribution to the D_2 receptor-regulated rescue from AMPA toxicity (as illustrated in supplemental Fig. D, available at www.jneurosci.org as supplemental material), given that this effect is abolished during wortmannin treatment and abolished in fibroblasts derived from $p85^{-/-}$ mice.

The quinpirole-mediated rescue effect is D_2 receptor dependent given that the rescue is inhibited by the antagonist raclopride in a concentration-dependent manner. Although the mere coexpression of D_2 receptors appears to have a protective effect, which we speculate to be attributable to constitutive D_2 receptor activity in the absence of D_2 agonists, this effect is small compared with the rescue seen with quinpirole treatment. In addition, the observed D_2 modulation of AMPA-mediated function is regulated by an agonist-dependent protein–protein coupling/uncoupling among D_2–NSF–GluR2 complex. Thus, as illustrated by coimmunoprecipitation, affinity purification, and *in vitro* binding assays, D_2 receptors directly couple to NSF through the $D_{2(IL3\text{-}2C)}$ region of the third intracellular loop. Dopamine D_2 receptors activate downstream signaling pathways mainly by coupling to $G_{i/o}$-proteins. The inability of PTX, which can uncouple the D_2 receptor from $G_{i/o}$-proteins, to block the protective effect of D_2 activation suggests the involvement of an alternative mechanism to the classical G-protein-coupled pathway. Furthermore, the ability of NSF to coimmunoprecipitate with the D_2 receptor, specifically with the $D_{2(IL3\text{-}2C)}$ region of the receptor, together with the observation that overexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene significantly blocked D_2 receptor inhibition of AMPA receptor-mediated cell death without affecting D_2 receptor downstream cAMP signaling, suggests that the D_2–NSF protein–protein interaction or the uncoupling of the GluR2–NSF interaction may play a role in the observed D_2 modulation of AMPA-mediated cell death. However, the $D_{2(IL3\text{-}2C)}$, which couples directly to NSF, is also a potential site for the $G_{i/o}$-protein coupling (Malek et al., 1993; Van Leeuwen et al., 1995; Robinson and Caron, 1996; Lachowicz and Sibley, 1997; Filteau et al., 1999; Senogles et al., 2004). This raises the possibility that PTX would not be able to block the D_2 receptor-protective effect because the NSF–D_2 interaction could possibly occlude $G_{i/o}$-proteins from coupling to the D_2 receptor, which would inhibit the activation of G_i-dependent pathways. Therefore, we tested the ability of D_2 receptors to inhibit adenylyl cyclase in cells coexpressing D_2 receptors with $D_{2(IL3\text{-}2C)}$ mini-gene compared with cells expressing D_2 receptor alone. The coexpression of the $D_{2(IL3\text{-}2C)}$ mini-gene shows no effect on the ability of D_2 receptors to inhibit adenylyl cyclase (data not shown). These data suggests that $D_{2(IL3\text{-}2C)}$ is not the exclusive region by which $G_{i/o}$-proteins couple to D_2 receptors, and it is unlikely that the observed quinpirole-mediated rescue from AMPA-induced toxicity is through the activation of G-proteins.

Dopamine D_2 receptors express two molecular isoforms that arise from alternative splicing, the D_2Long and D_2Short receptor. The D_2Long receptors are identical with D_2Short receptors except for the insertion of 29 amino acids within the third cytoplasmic loop of D_2 receptors (Dal Toso et al., 1989; Giros et al., 1989; Monsma et al., 1989; Selbie et al., 1989). We used the D_2Long receptor mainly on the basis that D_2Long receptors are localized predominantly on postsynaptic membranes, whereas D_2Short receptors are localized predominantly on presynaptic terminals. In addition, as our results have shown, the critical region within the D_2 receptor involved in the interaction with NSF is located within $D_{2(IL3\text{-}2C)}$, a region n which there is no sequence variation between the D_2Long and D_2Short receptor. Therefore, it would appear that both D_2Long and D_2Short receptors would be able to functionally modify AMPA receptors. However, because of the predominant presynaptic localization of the receptor, the ability of the D_2Short receptor to modulate AMPA receptor-mediated excitotoxicity may be limited.

Ischemic stroke is a worldwide public health problem and one of the leading causes of death. Elevation of extracellular glutamate after cerebral ischemia is thought to play a major role in the pathophysiological processes leading to death of ischemic brain tissue. Thus, modulation of glutamate receptor-mediated neurotoxicity has been a focus of major research to discover a neuroprotective treatment for ischemia. Our study has shown that the coupling/uncoupling of the D_2–NSF/NSF–AMPA protein–protein interactions enable D_2 receptors to modulate AMPA receptor-mediated toxicity, highlighting the molecular mechanism that underlies how D_2 receptors and AMPA receptors communicate and provides a new avenue to identify specific targets for therapeutics to modulate glutamate receptor-governed diseases, such as stroke.

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