Cellular/Molecular

Dopamine D_3 Receptors Regulate $GABA_A$ Receptor Function through a Phospho-Dependent Endocytosis Mechanism in Nucleus Accumbens

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The dopamine D_3 receptor, which is highly enriched in nucleus accumbens (NAc), has been suggested to play an important role in reinforcement and reward. To understand the potential cellular mechanism underlying D_3 receptor functions, we examined the effect of D_3 receptor activation on $GABA_A$ receptor ($GABA_AR$)-mediated current and inhibitory synaptic transmission in medium spiny neurons of NAc. Application of PD128907 [(4a*R*,10b*R*)-3,4a,4,10b-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol hydrochloride], a specific D_3 receptor agonist, caused a significant reduction of $GABA_AR$ current in acutely dissociated NAc neurons and miniature IPSC amplitude in NAc slices. This effect was blocked by dialysis with a dynamin inhibitory peptide, which prevents the clathrin/activator protein 2 (AP2)-mediated $GABA_A$ receptor endocytosis. In addition, the D_3 effect on $GABA_AR$ current was prevented by agents that manipulate protein kinase A (PKA) activity. Infusion of a peptide derived from $GABA_AR$ β subunits, which contains an atypical binding motif for the clathrin AP2 adaptor complex and the major PKA phosphorylation sites and binds with high affinity to AP2 only when dephosphorylated, diminished the D_3 regulation of IPSC amplitude. The phosphorylated equivalent of the peptide was without effect. Moreover, PD128907 increased $GABA_AR$ internalization and reduced the surface expression of $GABA_A$ receptor β subunits in NAc slices, which was prevented by dynamin inhibitory peptide or cAMP treatment. Together, our results suggest that D_3 receptor activation suppresses the efficacy of inhibitory synaptic transmission in NAc by increasing the phospho-dependent endocytosis of $GABA_A$ receptors.

Key words: **nucleus accumbens; dopamine D_3 receptor; $GABA_A$ receptor; trafficking; dynamin; protein kinase A**

Introduction

Since the discovery of dopamine D_3 receptors (Sokoloff et al., 1990), considerable research effort has been directed to the elucidation of their role in brain function. Based on gene sequence and pharmacological profile, the D_3 receptor is classified as a member of the D_2-like dopamine receptor family (Civelli et al., 1993; Gingrich and Caron, 1993). Unlike the D_2 receptor, which is widely expressed in several brain areas, the D_3 receptor is primarily restricted to parts of the limbic system (Sokoloff et al., 1990; Murray et al., 1994; Diaz et al., 2000), such as nucleus accumbens (NAc), a central relay structure implicated in motivated behaviors (Nicola et al., 2000; Nestler, 2004; Kalivas et al., 2005). This unique distribution of the D_3 receptor has suggested its potential role in reinforcement and reward (Levant, 1997; Richtand et al., 2001). Indeed, D_3-preferring agonists have been reported to decrease self-administration of cocaine (Caine and Koob, 1993; Pilla et al., 1999). Adaptive increases in D_3 receptors are found in brain reward circuits of cocaine overdose victims (Staley and Mash, 1996). Mutant mice lacking D_3 receptors show increased locomotor activity and hyperactivity in an exploratory test (Accili et al., 1996) and exhibit increased sensitivity to psychostimulants (Xu et al., 1997). Moreover, an association between D_3 receptor polymorphism and schizophrenia susceptibility has been identified (Crocq et al., 1992). Schizophrenic patients show a selective loss of D_3 receptor mRNA expression (Schmauss et al., 1993). Chronic treatment with antipsychotic drugs has been found to produce increases in D_3 receptor mRNA (Buckland et al., 1993). These results indicate that the D_3 receptor may be a useful target in the treatment of neuropsychiatric disorders, such as drug abuse and schizophrenia (Levant, 1997; Richtand et al., 2001).

Despite the findings on the involvement of D_3 receptors in motivation and motor behavior, the cellular mechanism underlying the action of D_3 receptors in NAc is essentially unknown. NAc is mainly composed of GABAergic medium spiny projection neurons (Chang and Kitai, 1985; Smith and Bolam, 1990), which form extensive recurrent axon collaterals to provide GABAergic innervation to adjacent spiny neurons (Pennartz et al., 1994). In addition, there are dense GABAergic afferents to NAc (Brog et al., 1993; Pennartz et al., 1994). Thus, the $GABA_A$ receptor

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($GABA_AR$), which mediates the inhibitory synaptic transmission network, plays a key role in regulating NAc neuronal activity and functions. It has been found that GABA transmission in NAc is altered after withdrawal from repeated cocaine (Xi et al., 2003), and dopamine depresses inhibitory synaptic transmission in NAc via a presynaptic D_1-like receptor (Nicola and Malenka, 1997). In this study, we examined the role of D_3 receptors in regulating GABA signaling of NAc medium spiny neurons. Results gained from this study may provide a molecular and cellular mechanism underlying D_3 receptor functions in NAc.

Materials and Methods

Slice preparation. All experiments were performed with the approval of State University of New York at Buffalo Animal Care Committee. Sprague Dawley rats (3–5 weeks old) were anesthetized by inhaling 2-bromo-2-chloro-1,1,1-trifluoroethane (1 ml/100 g) before decapitation (Feng et al., 2001; Chen et al., 2004). Brains were quickly removed and sliced with a Leica (Nussloch, Germany) VP1000S Vibratome. Slices were then incubated in artificial CSF (ACSF) bubbled with 95% O_2 and 5% CO_2.

Patch-clamp recording in dissociated neurons and slices. Dissociation procedure was similar as described previously (Yan and Surmeier, 1997). After incubation in a $NaHCO_3$-buffered saline, NAc slices were dissected and placed in oxygenated HEPES-buffered HBSS (Sigma, St. Louis, MO) containing protease (0.8–1.2 mg/ml; Calbiochem, La Jolla, CA) for 30 min. After enzyme digestion, tissue was rinsed three times in the low-Ca^{2+}, HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux Petri dish, which was then placed on the stage of a Nikon (Tokyo, Japan) inverted microscope.

Whole-cell current recording was performed using standard voltage-clamp techniques (Yan and Surmeier, 1997; Cai et al., 2002). The internal solution consisted of the following (in mM): 180 *N*-methyl-D-glucamine (NMG), 40 HEPES, 4 $MgCl_2$, 0.5 BAPTA, 12 phosphocreatine, 2 Na_2ATP, 0.2 Na_2GTP, and 0.1 leupeptin, pH 7.2–7.3 (265–270 mOsm/L). The external solution consisted of the following (in mM): 135 NaCl, 20 CsCl, 1 $MgCl_2$, 5 $BaCl_2$, 10 HEPES, 10 glucose, and 0.001 TTX, pH 7.35 (300–305 mOsm/L). Recordings were made with Axon Instruments (Palo Alto, CA) 200B patch-clamp amplifier that was controlled and monitored with a computer running pClamp (version 8) with a DigiData 1320 series interface (Axon Instruments). Membrane potential was held at 0 or −40 mV during recording. GABA (50 μM) was applied for 1 s every 30 s to minimize the desensitization-induced current amplitude decrease. Drugs were applied with a gravity-fed "sewer pipe" system. The array of application capillaries (~150 μm inner diameter) was positioned a few hundred micrometers from the cell under study. Solution changes were conducted with the SF-77B fast-step solution stimulus delivery device (Warner Instruments, Hamden, CT).

IPSC recording in slices was performed as described previously (Zhong et al., 2003). Patch electrode (5–8 MΩ) was filled with the following solution (in mM): 100 CsCl, 10 HEPES, 1 $MgCl_2$, 1 EGTA, 30 NMG, 5 MgATP, 0.5 Na_2GTP, 12 phosphocreatine, and 0.1 leupeptin, pH 7.2–7.3 (270–280 mOsm/L). NAc slice (300 μm) was placed in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus Optical, Melville, NY) and submerged in continuously flowing ACSF [in mM: 130 NaCl, 26 $NaHCO_3$, 3 KCl, 5 $MgCl_2$, 1.25 NaH_2PO_4, 1 $CaCl_2$, and 10 glucose, pH 7.4 (300 mOsm/L)]. CNQX (20 μM) and APV (40 μM) were added to block AMPA and NMDA receptors. TTX (1 μM) was also added when miniature IPSC (mIPSC) was recorded. Cells were visualized with a 40× water-immersion lens and illuminated with near infrared (IR) light, and the image was detected with an IR-sensitive CCD camera. A Multiclamp 700A amplifier (Axon Instruments) was used in the recording. Tight seals (2–10 GΩ) from visualized neurons were obtained by applying negative pressure. The membrane was disrupted with additional suction, and the whole-cell configuration was obtained. The access resistances ranged from 13 to 18 MΩ. Cell membrane potential was held at −70 mV.

To measure cell excitability, the whole-cell current-clamp technique (Zhong et al., 2003) was used to record spikes evoked by a 500-ms-duration depolarizing current pulse. The amplitude of injected current was adjusted so that six to seven spikes were elicited in the control ACSF solution. Patch electrodes were filled with the following internal solution (in mM): 60 K_2SO_4, 60 NMG, 40 HEPES, 4 $MgCl_2$, 0.5 BAPTA, 12 phosphocreatine, 2 Na_2ATP, 0.2 Na_3GTP, and 0.1 leupeptin, pH 7.2–7.3 (265–270 mOsm/L). Resting membrane potentials before and during PD128907 [*R*(+)-*trans*-3,4,4a,10b-tetrahydro-4-propyl-2*H*,5*H*-(1)benzopyrano(4,3-b)-1,4-oxazin-9-ol] application were 68.9 ± 1.8 and 69.5 ± 1.4 mV (n = 8), respectively.

Dopamine receptor ligands (4a*R*,10b*R*)-3,4a,4,10b-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol hydrochloride (PD128907), sulpiride, SCH23390 [*R* (+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride], and 4′-acetyl-*N*-[4-[4-(2-methoxyphenyl)-1-piperazinyl]butyl]-[1,1′-biphenyl]-4-carboxamide (GR103691) (Tocris Cookson, Ballwin, MO), as well as second-messenger reagents 8-(4-chlorophenylthio)-cAMP (cpt-cAMP), protein kinase inhibitor 6–22 (PKI_{6-22}), okadaic acid (OA), U73122 (1-[6[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), bisindolylmaleimide (Bis), wortmannin, cyclosporin A (CsA), and lavendustin A (Calbiochem) were made up as concentrated stocks in water or DMSO and stored at −20°C. Stocks were thawed and diluted immediately before use. The amino acid sequence for the dynamin inhibitory peptide (p4) is QVPSR-PNRAP. The peptide pepβ3 was synthesized corresponding to residues 401–412 (KTHLRRRSSQLK) of the rat $GABA_AR$ β3 subunit. An identical peptide (pepβ-phos) chemically phosphorylated at S408/S409 was synthesized as described previously (Kittler et al., 2005). The peptide pepβ3-[S→A] was synthesized based on the same amino acid sequence as pepβ3, except that S408/S409 were both changed to Ala (KTHLR-RRAAQLK). Neurons were dialyzed with various peptides for >20 min to get stabilized current before the effect of D_3 agonists was tested.

Data analyses of recordings in dissociated neurons were performed with AxoGraph (Axon Instruments) and Kaleidagraph (Albeck Software, Reading, PA). For analysis of statistical significance, Mann–Whitney *U* tests were used to compare the current amplitudes in the presence or absence of agonists. Mini Analysis program (Synaptosoft, Leonia, NJ) was used to analyze synaptic activity. Individual synaptic events with fast onset and exponential decay kinetics were captured with threshold detectors in Mini Analysis software. All quantitative measurements were taken 4–6 min after drug application. Miniature or spontaneous IPSCs (sIPSCs) of 3 min under each different treatment were used for obtaining cumulative distribution plots of the amplitudes and interevent intervals. Statistical comparisons of the synaptic currents were made using the Kolmogorov–Smirnov (K–S) test. ANOVA tests were performed to compare the differential degrees of current modulation between groups subjected to different treatment. Numerical values were expressed as mean ± SEM.

Immunocytochemical measurement of internalized receptors. Rat NAc cultures were prepared by modification of previously described methods (X. Wang et al., 2003). At 8–14 d *in vitro*, immunocytochemical experiments were performed to measure internalized $GABA_A$ receptors using procedures as we described previously (X. Wang et al., 2003). Briefly, surface $GABA_ARs$ were labeled in live cultured NAc neurons by 20 min incubation with an antibody directed against an extracellular region of $GABA_A$ receptor β2/3 subunits (1:50; Chemicon, Temecula, CA). After washing out the antibody, cells were treated with the D_3 agonist at 37°C for 10 min. After the treatment, cells were chilled on ice and surface stripped with an acidic solution (0.5 M NaCl, 0.2 N acetic acid) for 5 min. Cells were then fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and stained with a fluorescein-conjugated secondary antibody (1:200; Sigma) for 60 min at room temperature. After washing in PBS for three times, the coverslips were mounted on slides. Labeled cells were imaged using a 100× objective with a cooled CCD camera mounted on a Nikon microscope. All specimens were imaged under identical conditions and analyzed using identical parameters. Images were first thresholded to subtract the average background fluorescence, and then the level of internalized $GABA_AR$ immunoreactivity on the same length

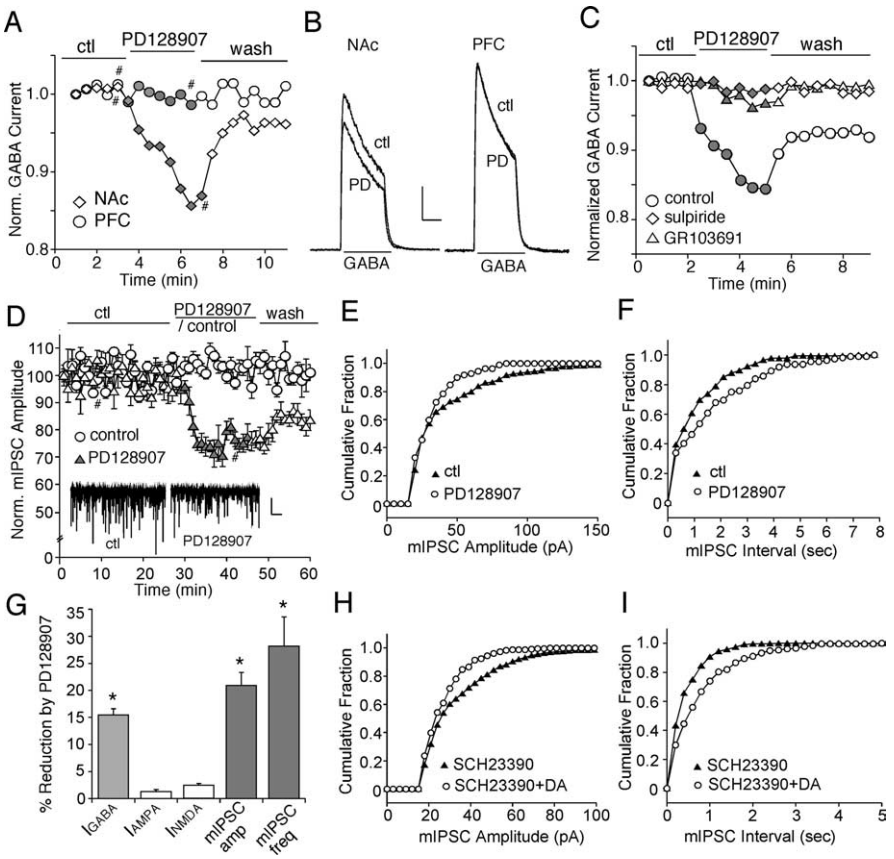


Figure 1. Activation of dopamine D_3 receptors reduced $GABA_A$ receptor-mediated current in nucleus accumbens. ***A***, Plot of normalized peak $GABA_AR$ current as a function of time and drug application in an isolated NAc medium spiny neuron and a dissociated PFC pyramidal neuron. Reduced current in response to the D_3 agonist PD128907 (10 μM) was shown in the NAc neuron but was absent in the PFC neuron. ***B***, Traces of GABA (50 μM)-evoked current taken from the records used to construct ***A*** (at time points denoted by #). Calibration: 0.5 nA, 0.5 s. ***C***, Plot of normalized peak $GABA_AR$ current in isolated NAc neurons with PD128907 applied in the presence or absence of the D_3 receptor antagonist sulpiride (20 μM) or GR103691 (5 μM). ***D***, Plot of normalized mIPSC amplitude in NAc neurons treated with or without PD128907. Each point represents the average peak (mean ± SEM) of synaptic currents within 1 min. Inset, Representative traces of mIPSCs (3 min each, at time points denoted by #). Calibration: 20 pA, 50 ms. ***E***, ***F***, Cumulative plots from the PD128907-treated neuron indicating that the distribution of mIPSC amplitude (***E***) and frequency (shown with interevent interval; ***F***) was decreased by the D_3 agonist. ***G***, Bar plot summary showing the different effect of PD128907 on I_{GABA}, I_{AMPA}, I_{NMDA}, mIPSC amplitude, and mIPSC frequency. ***H***, ***I***, Cumulative plots from a representative NAc medium spiny neuron indicating that the distribution of mIPSC amplitude (***H***) and frequency (shown with interevent interval; ***I***) was decreased by dopamine (DA; 100 μM) in the presence of the D_1-class antagonist SCH23390 (10 μM).

of dendrites and the same area of somas in treated versus untreated cells was compared. Quantitative analyses were conducted blindly.

Biochemical measurement of surface-expressed receptors. The procedure was similar to that described previously (X. Wang et al., 2003). After treatment, NAc slices were incubated with ACSF containing 1 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 20 min on ice. The slices were then rinsed three times in TBS to quench the biotin reaction, followed by homogenization in 500 μl of modified radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM $NaPO_4$, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml leupeptin). The homogenates were centrifuged at 14,000 × *g* for 15 min at 4°C. A total of 15 μg of protein was removed to measure total $GABA_AR$ β3 subunit. For surface protein, 150 μg of protein was incubated with 100 μl of 50% Neutravidin agarose (Pierce) for 2 h at 4°C, and bound proteins were resuspended in SDS sample buffer and boiled. Quantitative Western blots were performed on both total and biotinylated (surface) proteins using an antibody against β3 subunit (1:1000) (Kittler et al., 2004). Quantitative analyses were obtained with NIH Image.

Results

Activation of D_3 receptors reduces $GABA_AR$ current in dissociated NAc neurons and mIPSC in NAc slices

To understand the potential impact of D_3 receptors on GABAergic signaling in NAc, we first examined the effect of PD128907, a potent and highly selective D_3 receptor agonist (Pugsley et al., 1995; Sautel et al., 1995), on $GABA_A$ receptor-mediated whole-cell current in acutely dissociated NAc medium spiny neurons. Application of GABA (50 μM) evoked a partially desensitizing outward current that was completely blocked by the $GABA_A$ receptor antagonist bicuculline (30 μM; $n = 6$). Application of PD128907 (10 μM) caused a significant reduction of $GABA_AR$ current amplitude in NAc neurons (15.4 ± 1.2%; $n = 152$; $p < 0.01$, Mann–Whitney *U* test). This effect did not appear in D_3 receptor-lacking pyramidal neurons of prefrontal cortex (PFC) (2.5 ± 0.6%; $n = 5$; $p > 0.05$, Mann–Whitney *U* test). The time courses and current traces from representative cells in NAc and PFC were shown in Figure 1, *A* and *B*. The PD128907-induced reduction of $GABA_AR$ current in NAc neurons had a fast-onset kinetics, taking 2–4 min to stabilize. This effect recovered partially after PD128907 was washed out, suggesting a possible sustained inhibition by the D_3 agonist. To verify that D_3 receptors were mediating the modulation seen with PD128907, we tested D_3 antagonists. As shown in Figure 1*C*, in the presence of the D_2-class antagonist sulpiride (20 μM), PD128907 had little effect on $GABA_AR$ current (4.1 ± 0.5%; $n = 11$; $p > 0.05$, Mann–Whitney *U* test). Similarly, in the presence of GR103691 (5 μM), a specific D_3 receptor antagonist (Audinot et al., 1998), PD128907 failed to modulate $GABA_AR$ current (1.9 ± 0.4%; $n = 13$; $p > 0.05$, Mann–Whitney *U* test).

We next examined the effect of PD128907 on $GABA_A$ receptor-mediated IPSCs in NAc slices. TTX (1 μM) was added to NAc slices, and mIPSC was recorded in medium spiny neurons. As shown in Figure 1 *D–F*, bath application of PD128907 (10 μM) caused a strong reduction of the amplitude and frequency of mIPSC, which were stable throughout the recording period when no agonist was applied. The reduction reached a plateau 5–8 min after application of PD128907 and recovered partially. In a sample of neurons we examined (Fig. 1*G*), PD128907 decreased mean amplitude of mIPSC by 20.8 ± 2.5% ($n = 18$; $p < 0.001$, K–S test) and mean frequency of mIPSC by 28.1 ± 5.5% ($n = 18$; $p < 0.001$, K–S test).

To test the specificity of D_3 modulation of $GABA_A$ receptors, we also measured the effect of PD128907 on AMPA and NMDA receptors in NAc neurons. As shown in Figure 1*G*, PD128907 had no significant effect on either AMPA (100 μM)-evoked current (1.2 ± 0.3%, $n = 7$; $p > 0.05$, Mann–Whitney *U* test) or NMDA

(100 μM)-elicited current (2.4 ± 0.2%, $n = 10$; $p > 0.05$, Mann–Whitney U test).

To test whether the action of the D_3 agonist was mimicked by dopamine, we also measured the effect of dopamine (100 μM) on mIPSC. In the presence of the D_1-class receptor antagonist SCH23390 (10 μM), dopamine significantly reduced mIPSC amplitude (21.4 ± 1.2%; $n = 5$; $p < 0.001$, K–S test) and frequency (31.7 ± 3.8%; $n = 5$; $p < 0.001$, K–S test), similar to the effect of PD128907. A representative example is shown in Figure 1, *H* and *I*.

The D_3 modulation of $GABA_A$ receptors is blocked by dynamin inhibitory peptide

We next examined the potential mechanism underlying the D_3 inhibition of GABAergic signaling. The efficacy of synaptic inhibition is critically dependent on the number of $GABA_A$ receptors expressed on the neuronal surface (Kittler and Moss, 2003). One possibility is that $GABA_A$ receptors undergo clathrin/dynamin-dependent endocytosis (Kittler et al., 2000) after D_3 receptor activation. To test this, we examined the effect of PD128907 on $GABA_A$R current in NAc neurons dialyzed with the dynamin inhibitory peptide, which competitively blocks binding of dynamin to amphiphysin, thus preventing endocytosis (Gout et al., 1993; Lissin et al., 1998). As shown in Figure 2, *A* and *B*, when $GABA_A$R endocytosis was inhibited by intracellular administration of the dynamin inhibitory peptide (p4; 50 μM), PD128907 (10 μM) failed to suppress $GABA_A$R current, whereas the effect of PD128907 was intact in the presence of a scrambled control peptide (50 μM). As summarized in Figure 2*C*, PD128907 had little effect on $GABA_A$R current in the presence of the p4 peptide (3.5 ± 1.0%; $n = 8$; $p > 0.05$, Mann–Whitney U test), which was significantly ($p < 0.01$, ANOVA) different from the effect of PD128907 in the presence of the control peptide (16.2 ± 1.3%, $n = 7$; $p < 0.01$, Mann–Whitney U test).

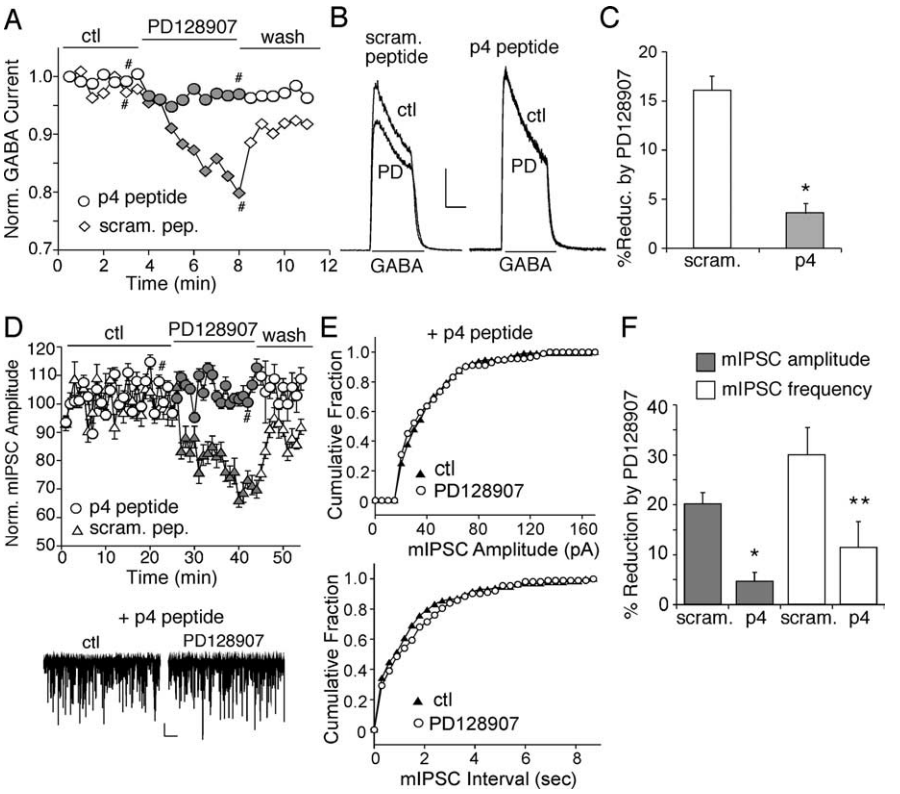


Figure 2. The dynamin inhibitory peptide blocked the D_3 effect on $GABA_A$ receptors. ***A***, Plot of normalized peak $GABA_A$R current in isolated NAc neurons dialyzed with the dynamin inhibitory peptide p4 (50 μM) or a scrambled control peptide (50 μM). ***B***, Representative traces taken from the records used to construct ***A*** (at time points denoted by #). Calibration: 1 nA, 0.5 s. ***C***, Bar plot summary showing the percentage reduction of $GABA_A$R current by PD128907 in the presence of different peptides in a sample of dissociated NAc neurons. *$p < 0.01$, ANOVA. ***D***, Plot of normalized mIPSC amplitude in NAc slices with the p4 peptide or a scrambled control peptide in pipette solution. Inset, Representative mIPSC traces (3 min each, at time points denoted by #). Calibration: 20 pA, 50 ms. ***E***, Cumulative plots from the p4 peptide-injected neuron indicating that PD128907 had little effect on the distribution of mIPSC amplitude (top) or frequency (bottom). ***F***, Bar plot summary showing the effect of PD128907 on mIPSC amplitude and frequency in the presence of different peptides. *$p < 0.01$; **$p < 0.05$, ANOVA.

Our then examined the involvement of $GABA_A$ receptor endocytosis in D_3 modulation of mIPSC in NAc slices. As shown in Figure 2, *D* and *E*, PD128907 was without effect on mIPSC amplitude in the cell dialyzed with the p4 peptide, whereas inclusion of a scrambled control peptide failed to block the PD128907 reduction of mIPSC amplitude. As summarized in Figure 2*F*, PD128907 caused a reduction of mIPSC amplitude by 21.0 ± 2.3% ($n = 8$; $p < 0.001$, K–S test) in the control peptide-injected group, which was significantly ($p < 0.01$, ANOVA) different from the effect of PD128907 in the p4 peptide-injected group (4.6 ± 1.9%; $n = 8$; $p > 0.05$, K–S test). The PD128907-induced reduction of mIPSC frequency was also significantly ($p < 0.05$, ANOVA) attenuated by the p4 peptide (control, 29.9 ± 5.6%, $n = 15$; p4, 11.3 ± 5.2%, $n = 8$).

The D_3 effect on $GABA_A$ receptors is counteracted by insulin

To further confirm that D_3 receptor activation regulates $GABA_A$R current via increased endocytosis of $GABA_A$ receptors, we tested whether insulin, which increases $GABA_A$R surface membrane expression (Wan et al., 1997), could counteract the effect of PD128907. As shown in Figure 3, *A* and *B*, bath application of insulin (0.1 μg/ml) markedly attenuated the effect of PD128907 on $GABA_A$R current in the dissociated NAc neuron. After insulin was washed out, PD128907 restored the capability to decease $GABA_A$R current. In a sample of neurons we tested (Fig. 3*C*), PD128907 had little effect on $GABA_A$R current in the presence of insulin (4.8 ± 0.6%; $n = 20$; $p > 0.05$, Mann–Whitney U test), which was significantly ($p < 0.01$, ANOVA) different from the effect of PD128907 in the absence of insulin (16.5 ± 1.2%; $n = 9$; $p < 0.01$, Mann–Whitney U test).

We next examined the impact of insulin on PD128907 regulation of mIPSC in NAc slices. As shown in Figure 3, *D* and *E*, in the absence of insulin, the mIPSC amplitude was stable over time, and application of PD128907 caused a significant reduction of mIPSC amplitude. Insulin treatment caused an enhancement of mIPSC amplitude (17.1 ± 3.6%; $p < 0.01$, K–S test), and subsequent application of PD128907 had no effect on mIPSC amplitude. As summarized in Figure 3*F*, PD128907 decreased mIPSC amplitude by 20.8 ± 2.5% ($n = 16$; $p < 0.001$, K–S test) in the control group, which was significantly ($p < 0.01$, ANOVA) different from the effect of PD128907 in insulin-treated group (5.9 ± 1.9%; $n = 7$; $p > 0.05$, K–S test). Insulin also significantly ($p < 0.01$, ANOVA) diminished the PD128907-induced reduc-

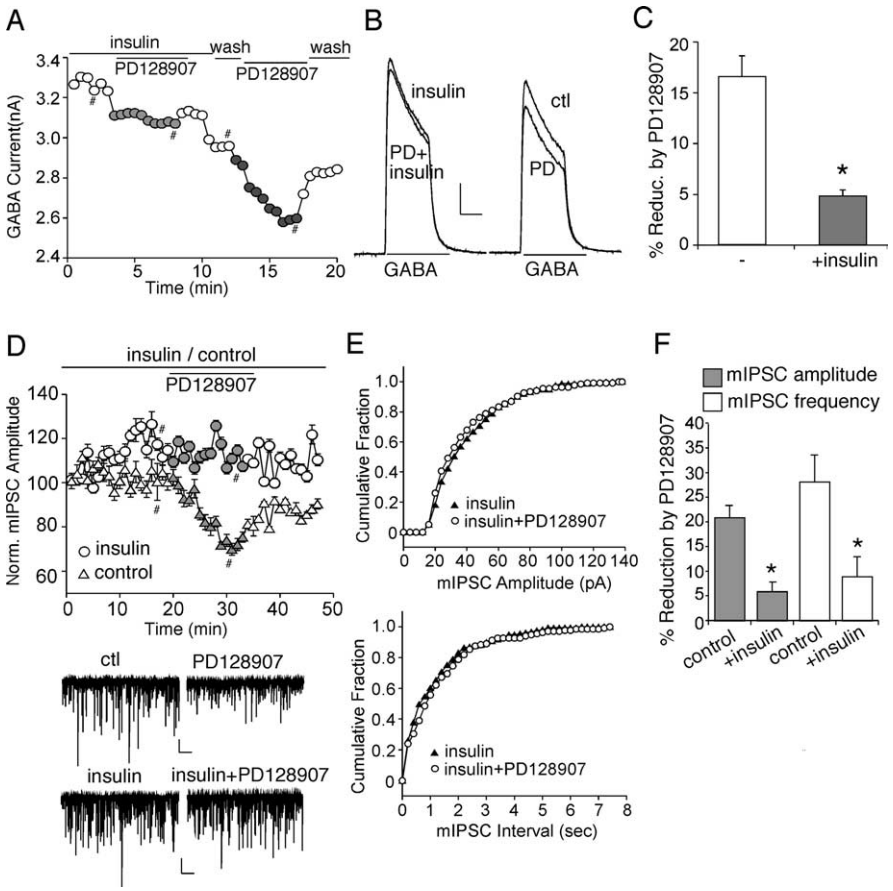


Figure 3. Insulin diminished the D_3 modulation of $GABA_A$ receptor current. ***A***, Plot of peak $GABA_AR$ current in an isolated NAc neuron showing the effect of PD128907 in the presence and absence of insulin (0.1 μg/ml). ***B***, Representative traces taken from the records used to construct ***A*** (at time points denoted by #). Calibration: 1 nA, 0.5 s. ***C***, Bar plot summary showing the percentage reduction of $GABA_AR$ current by PD128907 in the absence or presence of insulin. $*p < 0.01$, ANOVA. ***D***, Plot of normalized mIPSC amplitude in NAc slices treated with or without insulin (0.1 μg/ml). Inset, Representative mIPSC traces (3 min each, at time points denoted by #). Calibration: 20 pA, 50 ms. ***E***, Cumulative plots from the insulin-treated neuron indicating that PD128907 had little effect on the distribution of mIPSC amplitude (top) or frequency (bottom). ***F***, Bar plot summary showing the effect of PD128907 on mIPSC amplitude and frequency in the absence or presence of insulin. $*p < 0.01$, ANOVA.

tion of mIPSC frequency (control, 28.1 ± 5.5%, $n = 16$; insulin treated, 8.9 ± 4.1%, $n = 7$).

The D_3 regulation of $GABA_A$ receptors is protein kinase A dependent

The following set of experiments were designed to uncover the molecular mechanism that might be involved in the D_3 regulation of $GABA_AR$-mediated current in NAc neurons. The coupling of D_3 receptors to signal transduction systems in transfected cell lines have varied considerably, and evidence of cellular signaling mechanisms for the D_3 receptor in brain is lacking (Levant, 1997). Because D_2 receptors are linked to the inhibition of adenylate cyclase and cAMP formation, we speculate that the D_3 reduction of $GABA_AR$ current might be through the inhibition of protein kinase A (PKA). Previous studies have shown that PKA phosphorylation of $GABA_A$ receptor subunits exerts a powerful impact on $GABA_A$ channels (Porter et al., 1990; Moss et al., 1992).

To test the involvement of PKA, we examined whether the effect of D_3 receptors on $GABA_A$ receptor current can be prevented by agents that manipulate PKA activity. As shown in Figure 4, *A* and *B*, PD128907 had little effect on $GABA_AR$ current in the presence of the PKA activator cpt-cAMP (50 μM). Dialysis with the PKA inhibitory peptide PKI_{6-22} (20 μM) also prevented D_3 receptors from regulating $GABA_A$ receptor current (Fig. 4*C*). As summarized in Figure 4*D*, the effect of PD128907 was significantly ($p < 0.01$, ANOVA) attenuated by PKA-manipulating agents (cpt-cAMP, 39.8 ± 5.8% of control modulation, $n = 13$; PKI_{6-22}, 40.4 ± 4.6% of control modulation, $n = 17$).

The potential involvement of several other signaling molecules in the D_3 regulation of $GABA_AR$ current in NAc neurons was also investigated. As shown in Figure 4*D*, dialysis with the protein phosphatase 1/2A inhibitor OA (1 μM) did not block the effect of PD128907 (91.9 ± 4.2% of control modulation; $n = 23$). Moreover, the D_3 effect was not significantly altered by the phospholipase C (PLC) antagonist U73122 (1 μM; 96.6 ± 6.2% of control modulation; $n = 6$), the protein kinase C (PKC) antagonist Bis (1 μM; 91.2 ± 2.5% of control modulation; $n = 8$), the phosphoinositide 3-kinase (PI_3K) inhibitor wortmannin (3 μM; 97.5 ± 3.4% of control modulation; $n = 4$), the calcineurin inhibitor CsA (50 μM; 77.6 ± 2.6% of control modulation; $n = 5$), or the tyrosine kinase inhibitor lavendustin A (0.2 μM; 100.1 ± 4.7% of control modulation; $n = 7$). Together, these results suggest that D_3 receptor activation inhibits $GABA_AR$ current in NAc neurons through a specific PKA-dependent mechanism.

The D_3 modulation of $GABA_A$ receptors involves PKA phosphorylation-dependent endocytosis of $GABA_AR$ β subunit

It has been shown that $GABA_A$ receptors undergo endocytosis via the clathrin/activator protein 2 (AP2)-mediated mechanism (Kittler et al., 2000). Recently, an atypical binding motif that is conserved within the intracellular domains of all $GABA_A$ receptor β subunit isoforms for the AP2 complex has been identified (Kittler et al., 2005). This motif includes the major PKA phosphorylation sites, and PKA phosphorylation of these sites drastically reduces the affinity of the AP2 complex for $GABA_A$ receptor β subunit (Kittler et al., 2005). To test whether D_3/PKA modulates GABAergic transmission via a mechanism involving the phospho-dependent AP2 binding to $GABA_A$ receptors, we took advantage of the β3 peptides (pepβ3) that represents the AP2-binding region (residues 401–412; KTHLRRRSSQLK). The phosphorylated version of pepβ3 peptide (pepβ3-phos) that differs from pepβ3 only in PKA phosphorylation sites (S408 and S409) was used as a control. It has been found that pepβ3, but not pepβ3-phos, binds with high affinity to AP2 (Kittler et al., 2005).

As shown in Figure 5, *A* and *B*, dialysis with pepβ3 (200 μg/ml), which prevents AP2 binding to $GABA_ARs$ and thus blocks $GABA_A$ receptor endocytosis to the clathrin-coated pits, significantly attenuated the effect of PD128907 on sIPSC amplitude. In contrast, pepβ3-phos (200 μg/ml), which does not bind to AP2 with high affinity, failed to alter the PD128907-induced suppres-

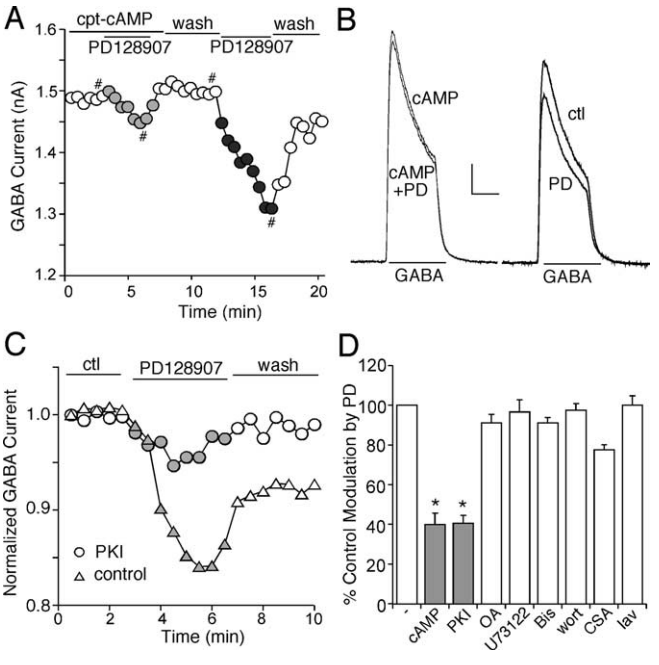


Figure 4. PKA was specifically involved in the D_3 regulation of $GABA_A$R current. ***A***, Plot of peak $GABA_A$R current in an NAc neuron showing the effect of PD128907 in the presence or absence of the PKA activator cpt-cAMP (50 μM). ***B***, Representative traces taken from the records used to construct ***A*** (at time points denoted by #). Calibration: 0.2 nA, 0.5 s. ***C***, Plot of normalized peak $GABA_A$R current in isolated NAc neurons dialyzed with or without the PKA inhibitor PKI_{6-22} (20 μM). ***D***, Bar plot summary showing the percentage control modulation of $GABA_A$R current by PD128907 in the absence or presence of various agents, including cpt-cAMP, PKI_{6-22}, okadaic acid (1 μM, phosphatase 1/2A inhibitor), U73122 (1 μM, PLC inhibitor), Bis (1 μM, PKC inhibitor), wortmannin (wort; 1 μM, PI_3K inhibitor), cyclosporin A (CSA; 50 μM, phosphatase 2B inhibitor), and lavendustin A (lav; 0.2 μM, tyrosine kinase inhibitor). *$p < 0.01$, ANOVA.

sion of sIPSC amplitude (Fig. 5*C*,*D*). To further confirm the phosphorylation dependence of the D_3 regulation of $GABA_A$ receptor endocytosis, we also examined the effect of another peptide, pepβ3-[S→A], which had the same amino acid sequence as pepb3, except that S408/S409 were both changed to Ala. This peptide is resistant to phosphorylation and thus always binds to AP2 with high affinity. As shown in Figure 5*E*, dialysis with pepβ3-[→] diminished the capability of PD128907 to reduce sIPSC amplitude. Figure 5*F* summarized the effect of PD128907 on sIPSC amplitude in the presence of different peptides. PD128907 had significantly ($p < 0.05$, ANOVA) smaller effect in cells loaded with pepβ3 (11.3 ± 4.7%; $n = 6$) compared with the effect of PD128907 in the presence of pepβ3-phos (27.6 ± 4.4%; $n = 8$). Moreover, perfusion with pepβ3-[S→A] significantly ($p < 0.01$, ANOVA) blocked the PD128907-induced reduction of sIPSC amplitude (6.6 ± 2.2%; $n = 6$). These results suggest that activation of D_3 receptors, by inhibiting PKA phosphorylation of $GABA_A$ receptors, increases $GABA_A$R binding to AP2 and thus $GABA_A$R endocytosis, leading to reduced GABAergic transmission.

D_3 receptor activation increases $GABA_A$R internalization and reduces surface $GABA_A$R expression through a mechanism dependent on dynamin and PKA

To provide a direct visualization of $GABA_A$ receptor endocytosis, we performed immunocytochemical experiments to detect $GABA_A$R internalization in cultured NAc neurons. Surface $GABA_A$Rs were first stained with an antibody to the extracellular region of β2/3 subunit (Ewert et al., 1990), and then, after the treatment with the D_3 agonist, surface-bound antibodies were stripped away so that only internalized $GABA_A$Rs were visualized. As shown in Figure 6*A*, application of PD128907 (10 μM, 10 min) triggered a strong internalization of $GABA_A$Rs. Quantification of fluorescently labeled, internalized $GABA_A$Rs in a sample of cells indicates that PD128907 increased $GABA_A$R internalization by 2.2 ± 0.2-fold ($n = 15$; $p < 0.001$, ANOVA).

To further test the impact of D_3 receptor activation on the expression of $GABA_A$Rs on the cell membrane, we performed surface biotinylation to measure levels of surface $GABA_A$R β3 subunit in NAc slices. Surface proteins were first labeled with sulfo-NHS-LC-biotin, and then biotinylated surface proteins were separated from nonlabeled intracellular proteins by reaction with Neutravidin beads. Surface and total proteins were subjected to electrophoresis and probed with an antibody against the β3 subunit. As shown in Figure 6*B*, treatment of NAc slices with PD128907 (10 μM, 10 min) reduced the level of surface $GABA_A$R β3 subunit, with no change in the total β3 subunit. This effect of PD128907 on the surface expression of β3 was blocked by pretreatment with the myristoylated (cell permeable) dynamin inhibitory peptide p4 (10 μM, 10 min) or cpt-cAMP (50 μM, 10 min). Quantitative analysis in a sample of experiments indicated that PD128907 decreased the level of surface β3 to 57.9 ± 14.4% of control ($n = 3$; $p < 0.01$, ANOVA). The dynamin inhibitory peptide slightly increased the level of surface β3 (109.1 ± 13.6% of control; $n = 3$) and prevented PD128907 from reducing the surface level of β3 (110.2 ± 9.0% of control; $n = 3$). PD128907 also had little effect on surface β3 in the presence of cpt-cAMP (104.0 ± 15.3% of control; $n = 3$). Together, these results suggest that D_3 receptor activation regulates the trafficking of $GABA_A$ receptors in a dynamin- and PKA-dependent manner.

To test the physiological consequence of the D_3-induced decrease in inhibitory transmission, we measured the effect of D_3 receptor activation on firing activity of NAc medium spiny neurons by quantifying the number of spikes evoked by depolarizing current pulses. As shown in Figure 7, *A* and *B*, bath application of PD128907 (10 μM) caused a significant increase in the firing rate, and this effect was abolished in the presence of the $GABA_A$ receptor antagonist bicuculline (10 μM). No changes on membrane potential, input resistance, action potential threshold, or kinetics were observed in response to PD128907 application. Bicuculline itself increased the firing rate by 31.0 ± 4.8% ($n = 6$). In a sample of NAc neurons we tested, the PD128907-induced increase in firing rate (28.1 ± 6.1%; $n = 8$) was significantly diminished in the presence of bicuculline (6.5 ± 3.1%; $n = 5$), suggesting that D_3 receptor activation is able to regulate neuronal excitability via modifying $GABA_A$R-mediated synaptic transmission.

Discussion

Although accumulative evidence indicates that the D_3 receptor in NAc is implicated in motivational behavior (Levant, 1997), its potential role in regulating synaptic activities in this limbic circuitry is not well established. Our present study provides evidence showing that activation of D_3 receptors depresses the $GABA_A$R-mediated current and inhibitory synaptic transmission but has little effect on NMDA receptor- or AMPA receptor-mediated current, suggesting that $GABA_A$Rs are specifically targeted by D_3 receptors in NAc.

$GABA_A$ receptors constitute the major inhibitory synaptic transmission network in the CNS (Moss and Smart, 2001). It has been shown that $GABA_A$ receptors in cultured hippocampal neurons undergo constitutive dynamin-dependent endocytosis by an association with adaptin AP2 complex (Kittler et al., 2000).

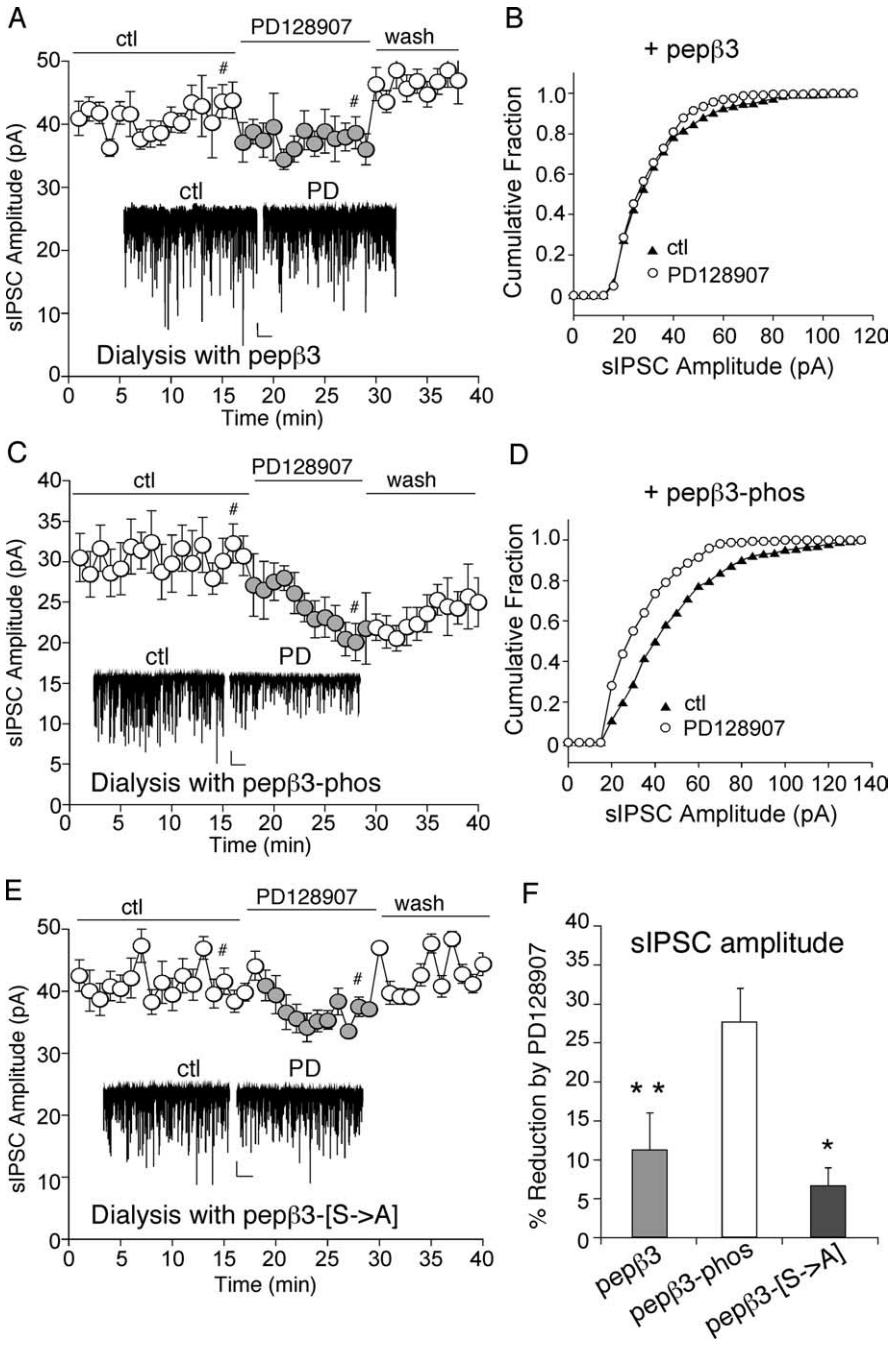


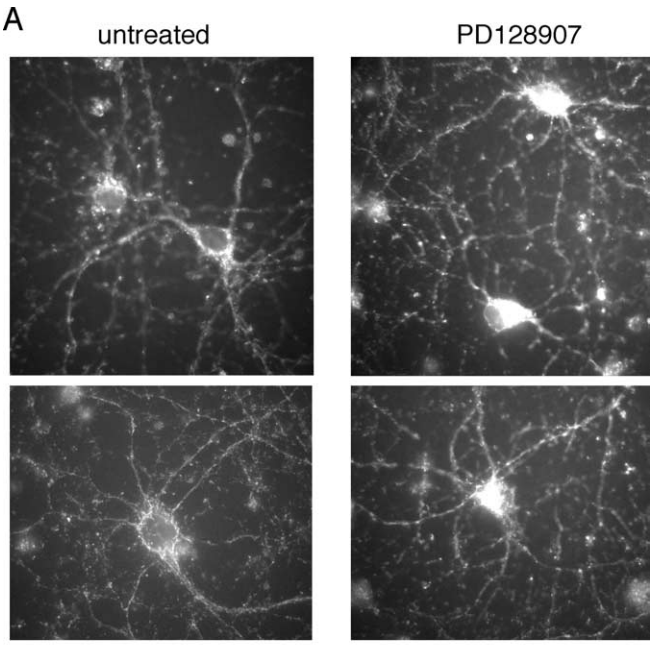
Figure 5. PKA phosphorylation-dependent endocytosis of $GABA_AR$ β subunit was involved in the D_3 modulation of postsynaptic $GABA_A$ receptors. ***A***, ***C***, ***E***, Plot of normalized sIPSC amplitude in NAc neurons dialyzed with the β3 subunit peptide (pepβ3, 200 μg/ml; ***A***) that represents the AP2-binding region (residues 401–412) or the phosphorylated version of pepβ3 peptide (pepβ3-phos, 200 μg/ml; ***C***) that differs from pepβ3 only in PKA phosphorylation sites (S408 and S409) or the phosphorylation-resistant β3 subunit peptide (pepβ3-[S⟶A], 200 μg/ml; ***E***) that the PKA phosphorylation sites have been mutated. Inset, Representative sIPSC traces (3 min each, at time points denoted by #). Calibration: 20 pA, 50 ms. ***B***, ***D***, Cumulative plots of sIPSC amplitude before (ctl) and after PD128907 application in the neurons injected with pepβ3 (***B***) or pepβ3-phos (***D***). ***F***, Bar plot summary showing the percentage reduction of sIPSC amplitude by PD128907 with pepβ3, pepβ3-phos, or pepβ3-[S⟶A] in pipette solutions. $^*p < 0.01$; $^{**}p < 0.05$, ANOVA.

Dynamin is proposed to be a universal membrane tubulation and fission molecule (Hinshaw, 2000; Praefcke and McMahon, 2004). It is involved in the endocytosis of not only $GABA_A$ receptors but also NMDA receptors and AMPA receptors (Carroll et al., 1999; Kittler et al., 2000; Lin et al., 2000). In this study, we found that inclusion of the dynamin inhibitory peptide that blocks the interaction between amphiphysin and dynamin abolished the PD128907-induced depression of $GABA_AR$ current in NAc neurons, supporting the notion that D_3 receptors regulate $GABA_AR$ function by affecting its endocytosis. This is further proved by biochemical experiments showing that D_3 receptor activation reduces the surface expression of $GABA_AR$ β subunit, and this effect is blocked by the cell-permeable dynamin inhibitory peptide.

The finding that insulin diminishes the D_3 effect on $GABA_AR$ current, in another way, suggests that a receptor endocytosis pathway is involved in the D_3 suppression of $GABA_AR$ function. Administration of insulin has been shown to increase the number of $GABA_A$ receptors on the plasma membrane surface (Wan et al., 1997) through Akt-mediated phosphorylation of $GABA_A$ receptors (Q. Wang et al., 2003). Recently, insulin has been reported to exert neuroprotection by counteracting the decrease in cell-surface $GABA_A$ receptors after oxygen-glucose deprivation in cultured cortical neurons (Mielke and Wang, 2005). Our present data suggest that insulin counteracts the D_3 receptor-induced internalization of $GABA_A$ receptors by recruiting membrane surface insertion of $GABA_A$ receptors.

The coupling of D_3 receptors to signal transduction systems in neurons is not very clear (Levant, 1997). In transfected cell lines, the D_3 receptor efficiently inhibits adenylyl cyclase and induces mitogenesis through a mechanism involving tyrosine phosphorylation (Griffon et al., 1997). In the present study, we found that the D_3 regulation of $GABA_A$ receptors in NAc neurons is specifically dependent on PKA, whereas many other signaling molecules, such as phosphatases, PLC, PKC, PI_3K, and tyrosine kinases, are not involved in the process.

Several lines of evidence have shown that PKA is involved in receptor trafficking. For example, activation of PKA inhibits the internalization of metabotropic glutamate receptors (Mundell et al., 2004). Inhibition of basal PKA activity induces internalization of epidermal growth factor receptor, which is abrogated by interfering with clathrin function (Salazar and Gonzalez, 2002). In neuronal cultures, the NMDA receptor-mediated AMPA receptor endocytic sorting is regulated by PKA (Ehlers, 2000). Recently, it has been found that $GABA_AR$ β subunits have an AP2-binding motif, which includes the major PKA phosphorylation sites, and PKA phosphorylation of these sites considerably reduces the affinity of the AP2 complex for $GABA_A$ receptor β subunits (Kittler et al., 2005). In this study, we found that the D_3 modulation of $GABA_AR$ function is attenuated by the nonphosphorylated β3 peptide that represents the AP2-binding region, although it is not af-



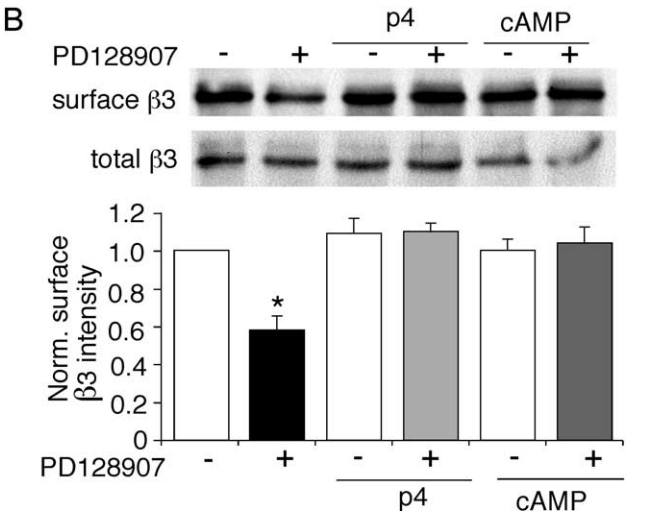


Figure 6. Activation of NAc D_3 receptors increased $GABA_AR$ internalization and reduced the $GABA_AR$ surface expression in a dynamin- and PKA-dependent manner. ***A***, Representative immunocytochemical images showing the internalized $GABA_ARs$ in cultured NAc neurons. The untreated cells showed a little $GABA_AR$ internalization, whereas the cells treated with PD128907 (10 μM, 10 min) showed significantly more staining for $GABA_ARs$ internalized from the plasma membrane. ***B***, Representative immunoblots and quantitation showing the surface $GABA_AR$ β3 subunit in NAc slices under different treatment conditions. PD128907 (10 μM, 10 min) decreased the level of surface β3 subunit, and this effect was abolished by pretreatment with the membrane-permeable dynamin inhibitory peptide (p4, 10 μM, 10 min) or cpt-cAMP (50 μM, 10 min). *$p < 0.01$, ANOVA.

fected by the phosphorylated β3 peptide. The phosphorylation-resistant β3 peptide that has mutated phosphorylation sites also blocked the D_3 effect on $GABA_AR$ function. Because the nonphosphorylated β3 peptide, which has high affinity for AP2, blocks the binding of AP2 complex to $GABA_A$ receptors and therefore prevents the endocytosis of $GABA_A$ receptors in clathrin-coated vesicles, it suggests that the D_3 regulation of GABAergic signaling is through a mechanism involving the phospho-dependent $GABA_AR$ internalization.

Based on the experimental data, we come up with the following model. In normal conditions, $GABA_A$ receptors undergo a balanced endocytosis and exocytosis. Activation of D_3 receptors inhibits PKA activity. As a result, PKA sites on $GABA_A$ receptor β subunits are dephosphorylated, which facilitates the binding of $GABA_AR$ to endocytotic machineries, such as AP2 complex, thus leading to an increased endocytosis of $GABA_A$ receptors and a depressed $GABA_AR$ function. Because dopamine receptors and PKA in NAc have been proposed to play important roles in drug abuse (Nestler, 2004), our present study on their regulation of $GABA_A$ receptors provides a potential cellular mechanism underlying the involvement of these molecules in motivated behaviors.

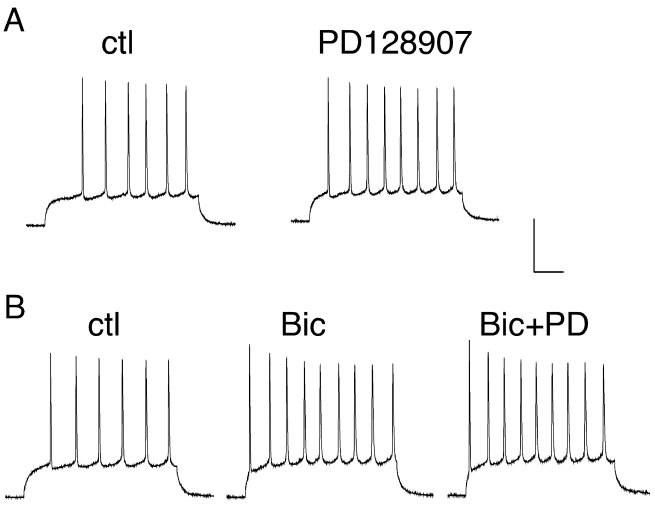


Figure 7. D_3 receptor activation increases NAc neuron excitability through a mechanism involving $GABA_A$ receptors. ***A***, ***B***, Representative traces of action potential firing illustrating the effect of PD128907 (10 μM) applied in the absence (***A***) or presence (***B***) of bicuculline (10 μM). Calibration: 40 mV, 100 ms.

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