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Heteromeric Nicotinic Acetylcholine–Dopamine Autoreceptor Complexes Modulate Striatal Dopamine Release

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In the striatum, dopamine and acetylcholine (ACh) modulate dopamine release by acting, respectively, on dopamine D_2 autoreceptors and nicotinic ACh (nACh) heteroreceptors localized on dopaminergic nerve terminals. The possibility that functional interactions exist between striatal D_2 autoreceptors and nACh receptors was studied with *in vivo* microdialysis in freely moving rats. Local perfusion of nicotine in the ventral striatum (shell of the nucleus accumbens) produced a marked increase in the extracellular levels of dopamine, which was completely counteracted by co-perfusion with either the non-α_7 nACh receptor antagonist dihydro-β-erythroidine or the D_{2-3} receptor agonist quinpirole. Local perfusion of the D_{2-3} receptor antagonist raclopride produced an increase in the extracellular levels of dopamine, which was partially, but significantly, counteracted by coperfusion with dihydro-β-erythroidine. These findings demonstrate a potent crosstalk between G protein-coupled receptors and ligand-gated ion channels in dopaminergic nerve terminals, with the D_2 autoreceptor modulating the efficacy of non-α_7 nACh receptor-mediated modulation of dopamine release. We further demonstrate physical interactions between β_2 subunits of non-α_7 nicotinic acetylcholine receptors and D_2 autoreceptors in co-immunoprecipitation experiments with membrane preparations from co-transfected mammalian cells and rat striatum. These results reveal that striatal non-α_7 nicotinic acetylcholine receptors form part of heteromeric dopamine autoreceptor complexes that modulate dopamine release.

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INTRODUCTION

In the central nervous system (CNS), ligand-gated ion channels can be found presynaptically, in nerve terminals, where they control neurotransmitter release (Engelman and MacDermott, 2004). Among them, nicotinic acetylcholine (nACh) receptors constitute a particular example, as nACh receptor-mediated modulation (stimulation) of neurotransmitter release is more pronounced than the relatively low numbers of neuronal nACh receptors might predict, suggesting that this is a main function of nACh receptors in the brain (Wonnacott, 1997). Thus, nACh receptors play mostly a modulatory role in the CNS, in contrast to neuromuscular junctions and autonomic ganglia, where nACh receptors mediate postsynaptic, fast, excitatory neurotransmission (Wonnacott, 1997; Vizi and Lendvai, 1999).

Neuronal nACh receptors are heteromeric pentamers made of a heterogeneous family of eight different subunits (α_{3-7}, β_{2-4}). Neuronal nACh receptors can be subdivided into α-bungarotoxin-sensitive or homomeric α_7 nACh receptors and α-bungarotoxin-insensitive or heteromeric non-α_7 nACh receptors. In the striatum, most nACh receptors are heteromeric, containing α and β subunits. Almost all of these heteromeric receptors contain the β_2 subunit, whereas α_4 and α_6 are the most represented α subunits (Zoli *et al*, 2002). Dopaminergic terminals seem to be the predominant localization of striatal nACh receptors (Wonnacott *et al*, 2000; Jones *et al*, 2001; Zoli *et al*, 2002) and results obtained from numerous experiments performed both *in vitro* (striatal synaptosomes or slices) and *in vivo* (microdialysis) have demonstrated that stimulation of

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these presynaptic nACh receptors results in dopamine (DA) release (Mifsud *et al*, 1989; Nakamura *et al*, 1992; Toth *et al*, 1992; Nisell *et al*, 1994; Sacaan *et al*, 1995; Clarke and Reuben, 1996; Marshall *et al*, 1997; Wonnacott *et al*, 2000; Kulak *et al*, 2001; Zhou *et al*, 2001; Ferrari *et al*, 2002; Grady *et al*, 2002; Champtiaux *et al*, 2003).

G-protein-coupled receptors (GPCRs) can also be found in presynaptic nerve terminals. Dopaminergic nerve terminals possess DA D_2 autoreceptors, which, when stimulated, inhibit dopaminergic neurotransmission. Multiple mechanisms have been suggested to be involved in the D_2 autoreceptor-mediated modulation of DA release. These include the reduction of membrane excitability by increasing a K^+ conductance (Cass and Zahniser, 1991; Congar *et al*, 2002), the decrease of DA synthesis and packaging (Onali *et al*, 1988; Pothos *et al*, 1998) and the upregulation of the DA transporter (Gulley and Zahniser, 2003). The present study demonstrates a new and important functional mechanism that allows D_2 autoreceptors to decrease dopaminergic neurotransmission, based on the existence of functional and physical interactions between D_2 autoreceptors and non-α_7 nACh receptors in the striatum.

MATERIALS AND METHODS

Subjects and Drugs

Male Sprague–Dawley rats, weighing 300-350 g, were used in all experiments. Animals were maintained in accordance with guidelines of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse, NIH. (−)-Nicotine hydrogen tartrate salt ([−]-1-methyl-2-[3-pyridyl] pyrrolidine), quinpirole hydrochloride, and raclopride tartrate were purchased from Sigma Chemical Co. (St Louis, MO); dihydro-β-erythroidine hydrobromide (DHβE) was purchased from RBI (Research Biochemicals International, Natick, MA). Cocaine HCl was obtained from the National Institute on Drug Abuse (NIDA Pharmacy, Baltimore, MD).

In Vivo Microdialysis

Concentric microdialysis probes were prepared as described previously (Pontieri *et al*, 1995). Animals were anesthetized with a solution of 4.44% chloral hydrate and 0.97% Na pentobarbital (NIDA Pharmacy, Baltimore, MD) and probes were implanted in the shell of the NAc (coordinates with respect to bregma: anterior, +2.2; lateral, −1.0; ventral, 7.7 from the dura). Experiments were performed on freely moving rats 24 h after probe implantation. All drugs were freshly dissolved in a Ringer solution (147 mM NaCl, 4 mM KCl, and 2.2 mM CaCl2) and pH was corrected when necessary. Ringer solution, either pure (during drug preperfusion and wash-out periods) or containing different concentrations of DA and nACh receptor agonists or antagonists alone or in combination (drug perfusion period), was pumped through the dialysis probe at a constant rate of 1 μl/min and samples were collected at 20-min intervals. Each animal was used to study the effect of one treatment by local administration (perfusion by reverse dialysis). At the end of the experiment, rats were killed with an overdose of Equithesin and methylene blue was perfused through the probe. The brain was removed and placed in a 10% formaldehyde solution, and coronal sections were cut to verify probe location. Dialysate DA content was measured by reverse high-performance liquid chromatography coupled to an electrochemical detector, as described in detail previously (Pontieri *et al*, 1995). The statistical analysis used was the 'summary measures' method (Matthews *et al*, 1990), using the mean of the three values previous to drug administration (basal value) and the mean of the three values during drug perfusion (perfusion value) per animal as the summary measures. Repeated measures analysis variance (ANOVA) (one-way and two-way) with Bonferroni *post hoc* comparisons were used to analyze differences within (basal *vs* perfusion) and between treatments (GraphPad-Prism version 4 software, San Diego, CA). The *p*-values shown in the figures refer to differences between basal *vs* perfusion values for each treatment.

Plasmid Constructs

The *myc* epitope (EQKLISEEDL) was introduced between Thr_{32} and Arg_{33}, after the signal peptide (Met_1-Ser_{27}), of the human α_4 subunit of the nACh receptor (kindly provided by JM Lindstrom, Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, PA) using a PCR mutagenesis approach (Ferré *et al*, 2002) and cloned into the *Hin*dIII/*Eco*RI sites of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). The cDNA encoding the human β_2 subunit of the nACh receptor (also provided by JM Lindstrom) was cloned into the *Eco*RI site of pcDNA3.1. The human D_{2S} receptor containing a hemaglutinin (HA) epitope (YPYDVPDYALV) between Asp_2 and Pro_3 (kindly provided by SL Milgram, Department of Cell and Molecular Physiology and the Curriculum in Neurobiology, The University of North Carolina at Chapel Hill, Chapel Hill, NC) was cloned into *Hin*dIII/*Xba*I sites of pcDNA3.1. The sequences of the cDNAs and their orientation in the vectors were confirmed by DNA sequencing.

Antibodies

The primary antibodies were: goat anti-α_4 nACh receptor polyclonal antibody (A-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-β_2 nACh receptor polyclonal antibody (H-92; Santa Cruz Biotechnology), rat anti-β_2 nACh receptor monoclonal antibody (Clone mAb270; Sigma), rabbit anti-D_2 receptor polyclonal antibody (D_2-246-316; previously; Bjelke *et al*, 1996), mouse anti-*c-myc* monoclonal antibody (Clone 9E10; Sigma), mouse anti-HA monoclonal antibody (Clone 12CA5; Roche Applied Sciences, Nutley, NJ). The secondary antibodies were: horseradish-peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL), HRP-conjugated rabbit anti-goat IgG (Pierce), and HRP-conjugated anti-rabbit IgG TrueBlot™ (eBioscience, San Diego, CA).

Cell Culture, Transfection and Membrane Preparation

HEK-293 cells were grown in DMEM (Sigma) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 10% (v/v) foetal bovine serum at 37°C, and in an atmosphere of 5% CO2. Cells were passaged

when 80–90% confluent. For the transient expression of proteins, HEK-293 cells growing in 25 cm^2 flasks were transiently transfected with 3 μg of DNA by calcium phosphate precipitation (Jordan *et al*, 1996). Membrane suspensions from rat striatum or from transfected HEK cells were obtained as described previously (Casadó *et al*, 1990; Burgueño *et al*, 2004).

Immunoprecipitation and Western Blot

Rats were killed with an overdose of Equithesin and the brain was rapidly removed and striata dissected out. Membranes from transiently transfected HEK cells or rat striatum were solubilized in ice-cold lysis buffer (PBS, pH 7.4, containing 1% (v/v) Nonidet P-40) for 30 min on ice. Solubilized preparations were then centrifuged at 13 000*g* for 30 min. Supernatant (1 mg/ml) was processed for immunoprecipitation as described previously (Burgueño *et al*, 2003; Ferré *et al*, 2002), each step conducted with constant rotation at 0–4°C, and incubated overnight with the indicated antibody. Forty microliters of a suspension of protein G crosslinked to agarose beads were added and the mixture was incubated overnight. Beads were washed twice with ice-cold lysis buffer, twice with ice-cold lysis buffer containing 0.1% (v/v) Nonidet P-40, once with ice-cold Tris-buffered saline, pH 7.4, and aspirated to dryness with a 28-gauge needle. Subsequently, 30 μl of sodium dodecyl sulfate (SDS)-PAGE sample buffer (8 M Urea, 2% SDS, 100 mM DTT, 375 mM Tris, pH 6.8) was added to each sample. Immune complexes were dissociated by heating to 37°C for 2 h and resolved by SDS-polyacrylamide gel electrophoresis in 10% gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a semidry transfer system and immunoblotted with the indicated primary antibody and then the appropriate HRP-conjugated goat secondary antibody. Immunoreactive bands were developed using a chemiluminescent detection kit.

RESULTS

Nicotine-Induced DA Release in the NAc Depends on Non-α_7 nACh Receptors

Basal extracellular levels of dialysate DA from the shell of the NAc were 3.7 ± 0.2 nM ($n = 65$). Local perfusion of 1 and 10 mM of nicotine in the NAc markedly increased extracellular levels of DA (ANOVA: $p < 0.001$ in both cases; maximal increases of about 600% and 300% from basal values, respectively). The concentration of 1 mM of nicotine produced a significantly more potent effect than 10 mM of nicotine (two-way ANOVA: $p < 0.05$) (Figure 1). The most effective concentration of nicotine (1 mM) was chosen for the next experiments. Perfusion with 0.1 mM, but not 0.01 mM, of the broad-spectrum non-α_7 nACh receptor antagonist DHβE (Chavez-Noriega *et al*, 1997) produced a small but significant decrease in the extracellular concentration of DA (ANOVA: $p < 0.05$; a maximal decrease of about 30% from basal values) (Figure 2). The simultaneous perfusion of DHβE (0.1 mM) completely counteracted the DA release induced by 1 mM of nicotine (Figure 2).

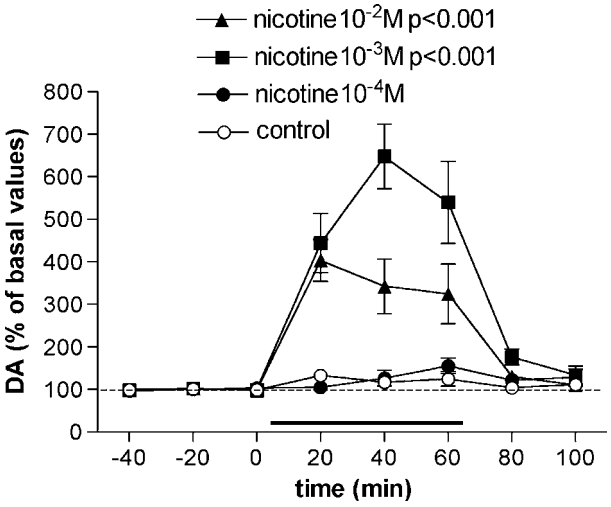

Figure 1 Extracellular concentrations of DA in the shell of the NAc after local perfusion of nicotine (0 (control), 0.1, 1, and 10 mM). The horizontal line shows the period of perfusion. The results represent means ± SEM of the percentage of basal values of the extracellular concentrations of DA ($n = 4$–6 per group). Basal values were the means of three values before drug perfusion. Nicotine 1 and 10 mM produced a significant increase in the extracellular concentration of DA. Nicotine 1 mM was significantly more effective than nicotine 10 mM (two-way ANOVA: $p < 0.05$). The *p*-values refer to the significant differences between basal *vs* perfusion values for each treatment.

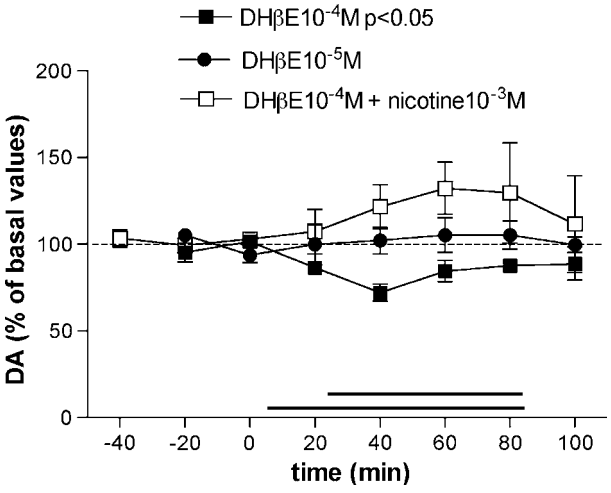

Figure 2 Extracellular concentrations of DA in the shell of the NAc after local perfusion of the non-α_7 nACh receptor antagonist DHβE with or without coperfusion with nicotine (1 mM). The horizontal lines show the periods of perfusion; the upper line corresponds to nicotine and the lower line corresponds to DHβE. The results represent means ± SEM of the percentage of basal values of the extracellular concentrations of DA ($n = 5$–6 per group). Basal values were the means of three values before drug perfusion. DHβE 0.1 mM produced a significant decrease in the extracellular concentration of DA and counteracted the increase in DA levels induced by nicotine 1 mM. The *p*-values refer to the significant differences between basal *vs* perfusion values for each treatment.

DA D_2 Receptor Stimulation Counteracts Nicotine-Induced DA Release

Local perfusion of 0.001 and 0.01 mM of the D_{2-3} receptor agonist quinpirole produced a dose-dependent decrease in

extracellular levels of DA in the NAc (ANOVA: $p<0.05$ and $p<0.001$, respectively; maximal decreases of about 20 and 50% from basal values, respectively). The concentration of 0.01 mM of quinpirole produced a significantly more potent effect than 0.001 mM of quinpirole (two-way ANOVA: $p<0.05$) (Figure 3). When coperfused with nicotine (1 mM), the lowest effective concentration of quinpirole (0.001 mM) completely counteracted nicotine-induced DA release (Figure 4). In contrast, quinpirole (1 μM) did not significantly modify the increase in extracellular levels of DA induced by the local perfusion of cocaine (0.01 mM). Thus, cocaine produced maximal increases of about 100% from basal values in the presence and absence of quinpirole (ANOVA: $p<0.01$ in both cases) (Figure 4).

Non-α_7 nACh Receptor Blockade Counteracts DA Release Induced by DA D_2 Receptor Blockade

Local perfusion with the D_{2-3} receptor antagonist raclopride produced a dose-dependent increase in extracellular levels of DA in the NAc, which was significant at concentrations of 0.001 and 0.01 mM (ANOVA: $p<0.01$ and $p<0.001$, respectively; maximal increases of about 100 and 200% from basal values, respectively) (Figure 5). When coperfused with the non-α_7 nACh receptor antagonist DHβE (0.1 mM), the effect of raclopride (0.01 mM) was significantly decreased (two-way ANOVA: $p<0.01$; to a maximal increase of DA of about 80% from basal values) (Figure 5).

Co-immunoprecipitation of Non-α_7 nAChRs and D_2 Autoreceptors from Co-Transfected HEK Cells and Striatal Tissue

The possible existence of heteromeric receptor complexes between α_4 or β_2 subunits of the non-α_7 nACh receptors and the D_2 autoreceptor, which corresponds to the short isoform of the D_2 receptor or D_{2S} (Khan *et al*, 1998; Usiello *et al*, 2000; Rougé-Pont *et al*, 2002), was first studied in transiently co-transfected HEK cells. In extracts of cells

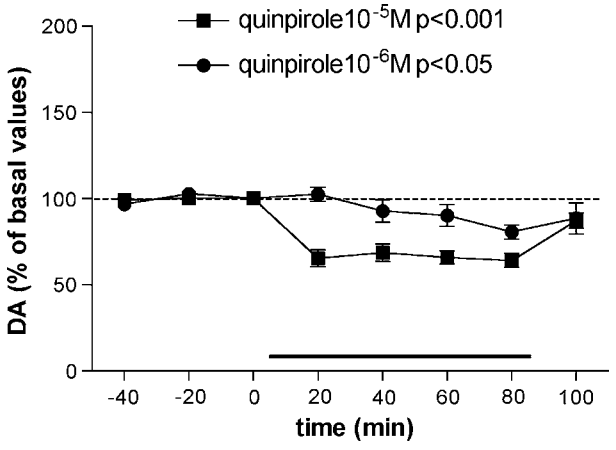

Figure 3 Extracellular concentrations of DA in the shell of the NAc after local perfusion of the D_{2-3} receptor agonist quinpirole. (0.001 and 0.01 mM). The horizontal line shows the period of perfusion. The results represent means ± SEM of the percentage of basal values of the extracellular concentrations of DA ($n=4$–5 per group). Basal values were the means of three values before drug perfusion. Quinpirole 0.001 and 0.01 mM produced a significant decrease in the extracellular concentration of DA. Quinpirole 0.01 mM was significantly more effective than quinpirole 0.001 mM (two-way ANOVA: $p<0.05$). The *p*-values refer to the significant differences between basal *vs* perfusion values for each treatment.

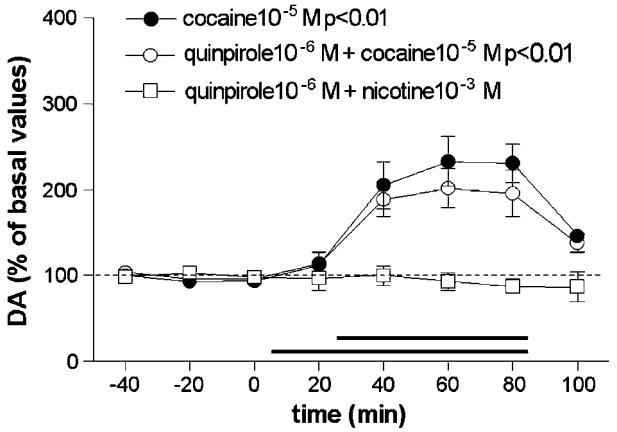

Figure 4 Extracellular concentrations of DA in the shell of the NAc after local perfusion of nicotine (0.1 mM) or cocaine (0.01 mM), with or without coperfusion with the D_{2-3} receptor agonist quinpirole (0.001 mM). The horizontal lines show the periods of perfusion; the upper line corresponds to cocaine or nicotine and the lower line corresponds to quinpirole. The results represent means ± SEM of the percentage of basal values of the extracellular concentrations of DA ($n=5$–6 per group). Basal values were the means of three values before drug perfusion. Quinpirole (0.001 mM) completely counteracted the increase in DA levels induced by nicotine 1 mM. Cocaine produced a significant increase in the extracellular concentration of DA, which was not significantly modified by quinpirole. The *p*-values refer to the significant differences between basal *vs* perfusion values for each treatment.

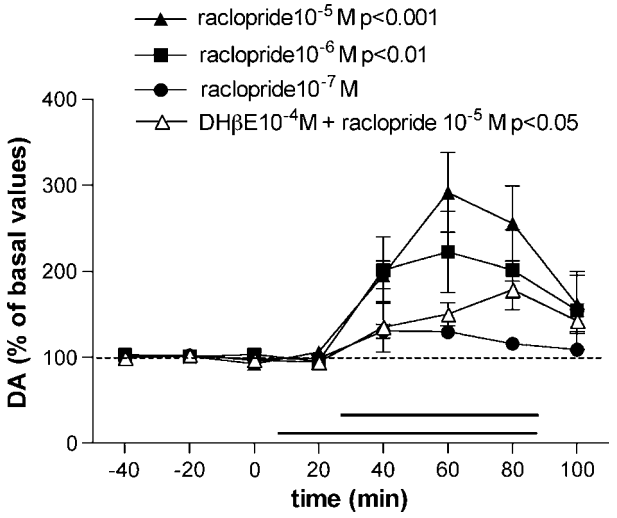

Figure 5 Extracellular concentrations of DA in the shell of the NAc after local perfusion of the D_{2-3} receptor antagonist raclopride (0.0001, 0.001, and 0.01 mM) with or without coperfusion with of the non-α_7 nACh receptor antagonist DHβE (0.1 mM). The horizontal lines show the period of perfusion; the upper line corresponds to raclopride and the lower line corresponds to DHβE. The results represent means ± SEM of the percentage of basal values of the extracellular concentrations of DA ($n=5$–6 per group). Basal values were the means of three values before drug perfusion. Raclopride 0.001 and 0.01 mM produced a significant increase in the extracellular concentration of DA. DHβE significantly decreased the increase in the extracellular concentration of DA induced by raclopride (0.01 mM) (two-way ANOVA: $p<0.01$). The *p*-values refer to the significant differences between basal *vs* perfusion values for each treatment.

transfected with α_4-*myc* subunit, mouse anti-*c-myc* antibody immunoprecipitate revealed a band of ~70 kDa, which corresponds to the α_4 subunit of the human nACh receptor (Figure 6a). In extracts of cells transfected with β_2 subunit, rat anti- β_2 nACh receptor antibody immunoprecipitate revealed a band of ~55 kDa, which corresponds to the β_2 subunit of the human nACh receptor (Figure 6a). In extracts of cells transfected with D_{2S}-HA receptor, mouse anti-HA antibody immunoprecipitate revealed a broad band of ~55–75 kDa, which corresponds to the human DA D_{2S} receptor (Figure 6a). This broad immunoblot detection of D_{2S} receptor most probably represents the different glycosylated states that this receptor shows when expressed in a heterologous system, as described previously (Fishburn *et al*, 1995). In extracts from cells co-transfected with α_4-*myc* and β_2 subunits, anti-*c-myc* antibody co-immunoprecipitated the β_2 subunit of the nACh receptor and anti- β_2 antibody co-immunoprecipitated the α_4 subunit of the nACh receptor (Figure 6a). Thus, as expected, in co-transfected HEK cells, α_4 and β_2 subunits form heteromeric complexes. When D_{2S}-HA receptor was co-transfected with both α_4-*myc* and β_2 subunits, anti-*c-myc* and anti- β_2 antibodies were able to co-immunoprecipitate the D_{2S} receptor and, conversely, mouse anti-HA antibody co-immunoprecipitated α_4 and β_2 subunits of the nACh receptor (Figure 6a). Importantly, the α_4 subunit of the nACh receptor did not co-immunoprecipitate with D_{2S} receptor in the absence of the β_2 nACh receptor subunit (Figure 6a), indicating that the D_{2S} receptor is able to establish heteromeric complexes with the nACh receptor by selectively interacting with the β_2 subunit.

We then analyzed the possible existence of heteromeric complexes in native brain preparations. To this end, we carried out similar co-immunoprecipitation experiments in soluble extracts from rat striatal membranes. As displayed in Figure 6b, the rabbit anti-β_2 nACh receptor antibody immunoprecipitated a band of ~55 kDa corresponding to the β_2 nACh receptor (Figure 6b, lane 3, IB:β_2) (similar results were found using the rat anti-β_2 nACh receptor antibody, data not shown) and the rabbit anti-D_2 receptor antibody immunoprecipitated a broad band of ~75-90 kDa corresponding to the D_2 receptor (Figure 6b, lane 2, IB:D_2), as expected. Again, several glycosylated states of the D_2 receptor tissue have been described in native brain (Clagett-Dame and McKelvy, 1989; Bjelke *et al*, 1996), which matches our current results. The divergence in D_2 receptor size between native tissue and transient expression might reflect the different glycosylation machinery in both systems. Importantly, the anti-D_2 receptor antibody was able to co-immunoprecipitate the β_2 subunit of the nACh receptor (Figure 6b, lane 2, IB:β_2) and, conversely, the rabbit anti-β_2 nACh receptor antibody co-immunoprecipitated the D_2 receptor (Figure 6b, lane 3, IB:D_2) (similar results were found using the rat anti-β_2 nAChR antibody, data not shown). Although the D_2 receptor antibody used in these experiments does not differentiate between the short and long isoforms of D_2 receptor (D_{2S} and D_{2L}, respectively), in the striatum the β_2 subunits only colocalize with the D_{2S} receptor in the dopaminergic cell terminals (see Discussion). Therefore, these results indicate that heteromeric receptor complexes of D_2 autoreceptors and nACh receptors containing β_2 subunits are present in the striatum in native

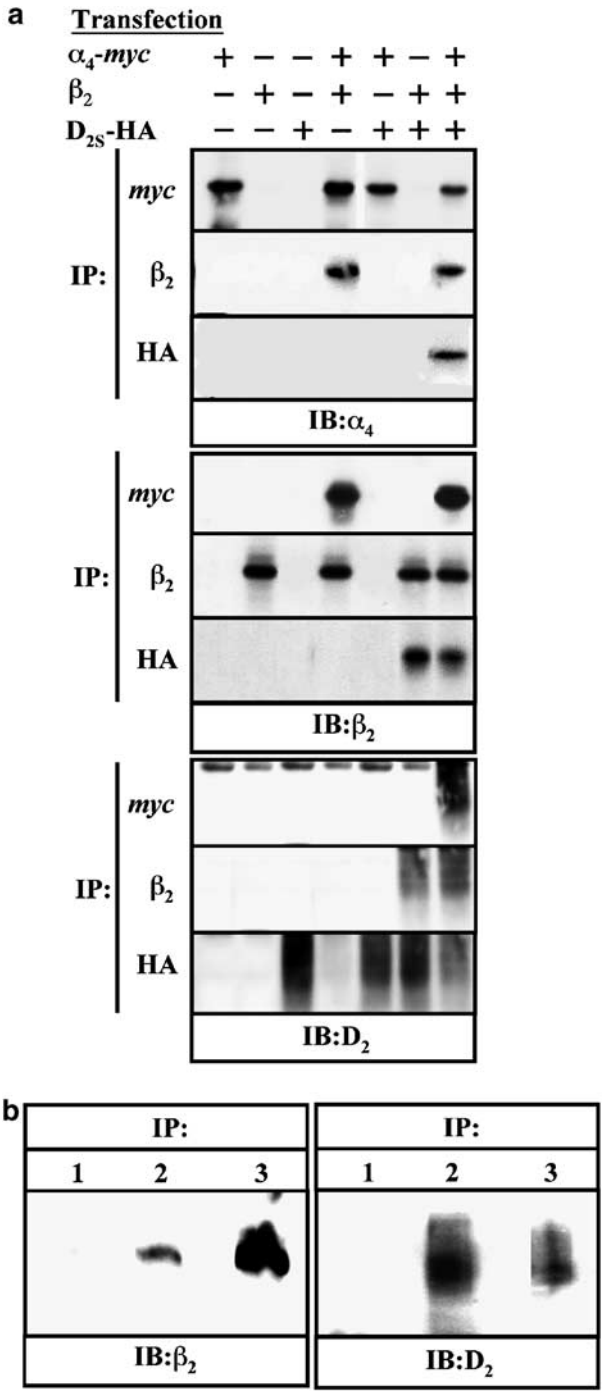

Figure 6 Co-immunoprecipitation of nACh and D_2 receptors. (a) HEK-293 cells transiently transfected with the indicated human cDNAs were solubilized and processed for immunoprecipitation (IP) with mouse anti-*myc* (1 μg), rat anti-β_2 nAch receptor (1 μg), and mouse anti-HA (1 μg) antibodies. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted (IB) using goat anti-α_4 nACh receptor antibody (1/500), rabbit anti-β_2 nACh recepor antibody (1/500), and rabbit anti-D_2 receptor antibody (1/2000). The immunoreactive bands were visualized by chemiluminescence. (b) Rat striatal membranes were solubilized and processed for IP with rabbit irrelevant IgG (lane 1), rabbit anti-D_2 receptor polyclonal antibody (lane 2), and rabbit anti-β_2 nACh receptor polyclonal antibody (lane 3). Immunoprecipitates were analyzed by SDS-PAGE and IB using rabbit anti-D_2 receptor antibody (1/2000) and rabbit anti-β_2 nACh receptor antibody (1/500). A HRP-conjugated anti-rabbit IgG TrueBlot™ was used as a secondary antibody in order to avoid IgG crossreactivity. The immunoreactive bands were visualized by chemiluminescence.

tissue, corroborating the previous results in transiently transfected cells.

DISCUSSION

In agreement with previous findings (Mifsud *et al*, 1989; Nakamura *et al*, 1992; Toth *et al*, 1992; Nisell *et al*, 1994; Marshall *et al*, 1997; Ferrari *et al*, 2002), we found that local perfusion of nicotine in the striatum (NAc) significantly increases the extracellular concentration of DA. Two main mechanisms that may be involved in this effect of nicotine have been suggested: (1), a direct stimulation of DA release by the activation of non-α_7 nACh receptors localized in dopaminergic terminals and (2), stimulation of DA release secondary to glutamate release (and activation of ionotropic glutamate receptors in the dopaminergic terminals) by the activation of α_7 nACh receptors localized in glutamatergic terminals (Toth *et al*, 1992; Kaiser and Wonnacott, 2000; Wonnacott *et al*, 2000; Zhou *et al*, 2001; Champtiaux *et al*, 2003; Rassoulpour *et al*, 2005). Our finding that the non-α_7 nACh receptor antagonist DHβE completely counteracted nicotine-induced DA release demonstrates that non-α_7 nACh receptors play a fundamental role in the local DA-releasing effects of nicotine in the NAc. In fact, most striatal nACh receptors are heteromeric non-α_7 nACh receptors (Zoli *et al*, 2002).

As in a recent study by Ferrari *et al*, (2002) we found that the extracellular concentration of DA in the NAc remained elevated during the whole period of nicotine perfusion (60 min) and went back to basal levels when nicotine perfusion was stopped. This effect was completely dependent on functional nACh receptors, as it was blocked with the nACh antagonist DHβE. These results are difficult to reconcile with the results obtained using *in vitro* models (fast voltametry in striatal slices), which suggest that exposure to DA induces a fast and potent desensitization of striatal non-α_7 nACh that modulate DA release and that nicotine behaves as a non-α_7 nACh receptor antagonist (Zhou *et al*, 2001; Rice and Cragg, 2004). This suggests that the *in vitro* models (striatal slices) do not adequately model the *in vivo* situation. Nevertheless, in our study, desensitization could play some role in the weaker effects of nicotine at higher concentrations.

In previous *in vivo* microdialysis experiments, local perfusion with the non-selective nACh receptor antagonist mecamylamine was reported to be ineffective or even to induce an increase in striatal extracellular levels of DA (Nakamura *et al*, 1992; Nisell *et al*, 1994; Marshall *et al*, 1997; Fu *et al*, 2000). However, mecamylamine is non-selective, acting on both α_7 and non-α_7 nACh receptors, and also has been shown to block NMDA receptors (Snell and Johnson, 1989). To our knowledge, this is the first report of the effects of local perfusion in the NAc of a selective non-α_7 nACh receptor antagonist, DHβE, which produced a small but significant decrease in the extracellular concentration of DA. This indicates that endogenous ACh tonically modulates DA release by acting on non-α_7 nACh receptors, as previously suggested by Zhou *et al*, (2001).

The effects of local and systemic administration of D_2 receptor agonists and antagonists on striatal DA release have been repeatedly shown in the literature (for specific effects in the NAc see, for instance, See *et al*, 1991; Ferré and Artigas, 1995) and they have been demonstrated to depend on D_{2S} autoreceptors (Khan *et al*, 1998; Usiello *et al*, 2000; Rougé-Pont *et al*, 2002). In agreement, we found that local perfusion of the D_{2-3} receptor agonist quinpirole significantly decreased, whereas the D_{2-3} receptor antagonist raclopride increased, the extracellular concentration of DA in the NAc. A major finding of the present study was that a concentration of quinpirole (1 μM) that slightly inhibited DA release (20% below basal values) completely counteracted the very large increase in the extracellular levels of DA (about 600% above basal values) induced by nicotine in the NAc. On the other hand, the same concentration of quinpirole (1 μM) did not significantly modify cocaine-induced DA release. These findings demonstrate a potent crosstalk between GPCRs and ligand-gated ion channels in dopaminergic nerve terminals, with the D_2 autoreceptor modulating the efficacy of non-α_7 nACh receptor-mediated modulation of DA release. Furthermore, the significant counteractive effect of DHβE on raclopride-induced increases in the extracellular concentrations of DA strongly suggests that inhibition of non-α_7 nACh striatal receptor function is a main mechanism by which D_2 autoreceptors control DA release.

An increasing number of receptor interactions are being demonstrated to depend on their physical association, forming functional heteromeric receptor complexes and often heterodimers (Bouvier, 2001; Devi, 2001; Agnati *et al*, 2003; Lee *et al*, 2003). This includes the possibility of heteromeric receptor complexes of GPCRs and ligand-gated ion channels, such as the DA D_1-NMDA and the DA D_5-$GABA_A$ receptor interactions (reviewed in Agnati *et al*, 2003). The present experiments with co-transfected HEK cells demonstrate that the D_{2S} receptor is able to establish heteromeric complexes with the nACh receptor by selectively interacting with the β_2 subunit when coexpressed in the same cells. Furthermore, an antibody against the β_2 subunit of the nAch receptor was able to co-immunoprecipitate D_2 receptors from membrane preparations of the rat striatum and, conversely, a D_2 receptor antibody was able to co-immunoprecipitate the β_2 subunit of the nAch receptor. Since in the striatum the β_2 subunits of the nAch receptor are mostly localized in the dopaminergic cell terminals (Wonnacott *et al*, 2000; Jones *et al*, 2001; Zoli *et al*, 2002), where they are colocalized with D_{2S} autoreceptors, the present results show that non-α_7 nAChRs containing β_2 subunits form part of a heteromeric D_{2S} autoreceptor complex, which exerts strong control over striatal DA release.

The term autoreceptor was introduced to define those receptors localized in nerve terminals that respond to the neurotransmitter released by the same neuron (Langer, 1974). Later, the term autoreceptor also included receptors localized in the somatodendritic region that respond to somatodendritic neurotransmitter release (Aghajanian and Bunney, 1977). Functionally, autoreceptors act as a feedback mechanism inhibiting neurotransmitter release. The term heteroreceptor is used for presynaptic receptors capable of regulating (stimulating or inhibiting) the release of neurotransmitter other than their own, which is one of the main functions of nACh receptors in the central nervous system (Wonnacott, 1997). The term heteromeric auto-

receptor complex expands the concepts of autoreceptor and heteroreceptor, to include them in a functional macromolecular complex. The present study shows that striatal dopaminergic neurotransmission is under the control of heteromeric autoreceptor complexes containing D_2 autoreceptors and non-α_7 ACh heteroreceptors. It still remains to be determined if the functional interaction between these two receptors depends on their physical interaction and if they establish a direct physical interaction ('true heteromerization'), as co-immunoprecipitation does not discard the existence of intermediate linking proteins. Nevertheless, the experiments in co-transfected HEK cells favor the hypothesis of a selective direct interaction between β_2 subunits of the nACh receptors and D_2 autoreceptors, as the α_4 subunit of the nACh receptor could co-immunoprecipitate with D_{2S} receptor only in the presence of the β_2 nACh receptor subunit. Otherwise, the heteromeric nACh-DA autoreceptor complexes should also contain some proteins that would be constitutively expressed in HEK cells and that would physically and specifically link the β_2 nACh receptor subunit to the D_{2S} receptor. It must also be pointed out that D_2 autoreceptors and non-α_7 nACh receptors are not only co-localized in the terminals of dopaminergic cells but also in their soma and dendrites (Aghajanian and Bunney, 1977), suggesting that heteromeric receptor complexes are also present in the ventral tegmental area. The present study provides new evidence against the generalized simplistic notion of the neurotransmitter receptor as a single functional entity and, to our knowledge, it is the first example of presynaptic heteromeric receptor complexes that include ligand-gated ion channels and GPCRs that modulate neurotransmitter release.

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