Interactions between Histamine H₃ and Dopamine D₂ Receptors and the Implications for Striatal Function

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Abstract

The striatum contains a high density of histamine H₃ receptors, but their role in striatal function is poorly understood. Previous studies have demonstrated antagonistic interactions between striatal H₃ and dopamine D₁ receptors at the biochemical level, while contradictory results have been reported about interactions between striatal H₃ and dopamine D₂ receptors. In the present study, by using reserpinized mice, we demonstrate the existence of behaviorally significant antagonistic postsynaptic interactions between H₃ and D₁ and also between H₃ and dopamine D₂ receptors. The selective H₃ receptor agonist imetit inhibited, while the H₃ receptor antagonist thioperamide potentiated locomotor activation induced by either the D₁ receptor agonist SKF 38393 or the D₂ receptor agonist quinpirole. High scores of locomotor activity were obtained with H₃ receptor blockade plus D₁ and D₂ receptor co-activation, i.e., when thioperamide was co-administered with both SKF 38393 and quinpirole. Radioligand binding experiments in striatal membrane preparations showed the existence of a strong and selective H₃-D₂ receptor interaction at the membrane level. In agonist/antagonist competition experiments stimulation of H₃ receptors with several H₃ receptor agonists significantly decreased the affinity of D₂ receptors for the agonist. This kind of intramembrane receptor-receptor interactions are a common biochemical property of receptor heteromers. In fact, by using Bioluminescence Resonance Energy Transfer techniques in co-transfected HEK-293 cells, H₃ (but not H₄) receptors were found to form heteromers with D₂ receptors. The present study demonstrates an important role of postsynaptic H₃ receptors in the modulation of dopaminergic transmission by means of a negative modulation of D₂ receptor function.

Keywords

Histamine H₃ receptor; dopamine D₂ receptor; receptor heteromer; striatum

1. Introduction

The histaminergic system consists of a group of about 64,000 neurons whose cellular bodies are localized in one single hypothalamic nucleus, the tuberomammillary nucleus and which

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project extensively to all major parts of the forebrain, brainstem, cerebellum and spinal cord (Schwartz et al., 1991; Brown et al., 2001; Haas and Panula, 2003). The central actions of histamine are mediated by G protein-coupled receptors, mostly of the H₁, H₂ and H₃ subtype. Another histamine receptor, the H₄ subtype, has recently been cloned, but it is predominantly expressed in bone marrow, eosinophils and mast cells, with little expression in the brain (Oda et al., 2000; de Esch et al., 2005). The histaminergic system is involved in arousal, which is related to its innervation to the cortex, thalamus and basal forebrain (Brown et al., 2001), but the role of histamine in other parts of the forebrain are not well understood.

H₃ receptors were first identified as autoreceptors, localized in histaminergic nerve terminals (Arrang et al., 1983), but they were later on found to act as heteroreceptors which modulate the release of various neurotransmitters (Leurs et al., 2005). The striatum contains one of the highest densities of H₃ receptors in the brain (Pollard et al., 1993; Anichtchik et al., 2001; Pilot et al., 2002). In the striatum, presynaptic H₃ receptors have also been identified in glutamatergic and dopaminergic terminals, where they seem to exert an inhibitory role of glutamate and dopamine release (Schlicker et al., 1993; Doreulee et al., 2001; Molina-Hernandez et al., 2000, 2001; Munzar et al., 2004). However, the major localization of striatal H₃ receptors is postsynaptic, in the GABAergic striatal efferent neurons, where they are co-localized with D₁ and D₂ receptors in the D₁ receptor-containing GABAergic dynorphinergic and the D₂ receptor-containing GABAergic enkephalinergic neurons, respectively (Ryu et al., 1994; Pillot et al., 2002). This would give the rationale for the existence of functional postsynaptic H₃-D₁ and H₃-D₂ receptor interactions. In fact, antagonistic interactions between striatal H₃ and D₁ receptors have been observed at the biochemical level, by which stimulation of H₃ receptor decreases cAMP accumulation and GABA release in striatal slices (Arias-Montano et al., 2001; Sanchez-Lemus and Arias-Montano, 2004). On the other hand, contradictory results about biochemical interactions between striatal H₃ and D₂ receptors have been reported (Hussain et al., 2002; Pillot et al., 2002).

In the present study, by analyzing the effects of H₃ receptor agonists and antagonists on the locomotor activation induced by the D₁ or D₂ receptor agonists in reserpinized mice, we demonstrate the existence of behaviorally significant antagonistic postsynaptic H₃-D₁ and H₃-D₂ receptor interactions. We then show the existence of significant antagonistic H₃-D₂ receptor interactions at the membrane level in radioligand binding experiments in striatal membrane preparations. Finally, we demonstrate that H₃ receptors can form heteromers with D₂ receptors in co-transfected mammalian cells.

2. Materials and Methods

2.1. Locomotor activity in reserpinized mice

Male Swiss Webster mice (Charles River Laboratories, Inc., Wilmington, MA), experimentally naive at the start of the study and initially weighing 20 to 25 g, were housed four per cage with food and water available ad libitum. Mice were housed in a temperature- and humidity-controlled room and were maintained on a 12-h light/dark cycle (lights on from 6:00 to 18:00 h). Animals were used only once for locomotor activity experiments, which were conducted during the light phase one week after their arrival. 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. Four locomotor activity chambers (Coulbourn Instruments, Allentown, PA) were used. Mouse open fields were 25.40 × 25.40 × 40.64 (high) cm. The beams were spaced 1.52 cm apart providing a 0.76 cm spatial resolution. Each chamber was equipped with one sensor ring used to accurately track the locomotion of each subject. Sessions programming and data recording made use of the TruScan Photobeam Activity System interface and the data acquisition TruScan 99 software package (Coulbourn Instruments) running on a PC compatible via the L18-16S/C Linc interface card (Coulbourn

Instruments). Reserpine, the D₁-like receptor agonist (+/-) – SKF 38393, the D₂-like receptor agonist (-)-quinpirole and the H₃ receptor antagonist thioperamide were purchased from Sigma (St. Louis, MO, USA). The H₃₋₄ receptor agonist imetit dihydrobromide was purchased from Tocris (Ellisville, MO, USA). Reserpine was dissolved in a drop of glacial acetic acid which was made up to volume with 5.5% glucose and was administered (5 mg/kg) subcutaneously (s.c.) 20 h prior to the start of the locomotor activity recording. This dose of reserpine has been previously shown to produce pronounced striatal dopamine depletion in mice (more than 90% and 95% depletion 3 and 24 h after reserpine administration, respectively) (Starr et al., 1987), while histamine levels in the mouse brain are very little affected by reserpine (see Muroi et al., 1991). All the other drugs were dissolved in sterile saline and administered intraperitoneally (i.p.). The volume of injection was 10 ml/kg for all drugs. Imetit and thioperamide were administered 20 and 15 min prior to the locomotor activity recording, respectively, and SKF 38393 and quinpirole were administered immediately before the locomotor activity recording. Locomotor activity was recorded immediately after the animals, either reserpinized or non-reserpinized, were introduced in the open field without any acclimatization period. All values (in cm of ambulation) registered per 10 min were transformed (square root; Ferré et al., 1991, 1994) and the average of the results obtained during the 10-min periods of the first hour of recording were used for calculations. Differences between differently treated groups of animals were analyzed by ANOVA followed by post-hoc Newman-Keuls test.

2.2. Membrane preparation and protein determination

Membrane suspensions from sheep brain striatum were processed as described previously (Casadó et al., 1990). Tissue was disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for three 5 s-periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4. Membranes were obtained by centrifugation at 105,000 g (40 min, 4°C), and the pellet was resuspended and recentrifuged under the same conditions. The pellet was stored at -80°C and was washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard.

2.3. Radioligand binding experiments

For competition experiments, membrane suspensions from sheep brain striatum (0.5 mg of protein/ml) were incubated for 2 h at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ with 1.4 nM of the D₂ receptor antagonist [³H]YM-09151-2 (NEN Perkin Elmer) and increasing concentrations of the D₂-like receptor agonist quinpirole (triplicates of 13 different competitor concentrations from 0.1 nM to 100 µM; Sigma) or the D₂ receptor antagonist YM-09151-2 (triplicates of 8 different competitor concentrations from 0.01 nM to 1 µM; Sigma) in the absence or the presence of 20 nM of the H₃ receptor agonist R-α-methylhistamine (RAMH, Sigma), imetit dihydrobromide (Tocris) or imipip dihydrobromide (Tocris) or 20 nM RAMH plus 1 µM of the H₃ receptor antagonist thioperamide. Nonspecific binding was determined in the presence of 50 µM raclopride (Sigma) and confirmed that the value was the same as calculated by extrapolation of the displacement curves. To test the H₃ receptor antagonist effect on D₂ receptor ligand binding, membrane suspension from sheep brain striatum (0.5 mg of protein/ml) were incubated for 2 h at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ with 1.4 nM of the D₂ receptor antagonist [³H]YM-09151-2 and 0, 0.1 or 1 µM of thioperamide. In all cases, free and membrane-bound ligand were separated by rapid filtration of 500 µl aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters (Brandel) embedded in 0.3% polyethylenimine, which were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer,

Boston, MA, USA) with an efficiency of 62% (Sarrió et al., 2000). Binding data from competition experiments were analyzed by nonlinear regression to the previously described equations (Casadó et al., 1992, Sarrió et al., 2000), using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK). Goodness of fit was tested according to reduced χ^2 value given by the nonlinear regression program and, in all cases, biphasic over monophasic competition curves were selected according to a $p < 0.01$ using an F test (Ciruela et al., 2006). The equilibrium dissociation constants for the high-affinity (K_{DH}) and low-affinity (K_{DL}) binding sites were determined from IC_{50} values using the Cheng-Prusoff relation (Cheng and Prusoff, 1973). Results are given as parameter values $\pm$ S.E.M. from four independent experiments. Differences in K_{DH} and K_{DL} in the presence and absence of H_3 agonists were tested for significance (two-tailed; $p < 0.05$) using Student's t test for unpaired samples.

2.4. Expression vectors

A plasmid encoding the cDNA of the human H_3 receptor was provided by Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (San Diego, California). The H_3 receptor without its stop codon was amplified using sense and antisense primers harboring a unique EcoRI site. The fragment was then subcloned to be in-frame with Enhanced Yellow variant of green Fluorescent Protein (EYFP) into the EcoRI site of pEYFP-N1 (Clontech, Heidelberg, Germany) to provide the plasmid H_3 -YFP, which expresses EYFP on the C-terminal ends of the receptor. The D_2 -*RLuc* construct was generated by subcloning the cDNA of the human D_2 receptor without its stop codon between the NheI and Xho I restriction sites of the *Renilla luciferase*-fusion protein expression vector (pRLuc-N1, PerkinElmer). Constructs were verified by nucleotide sequencing. A pcDEF3 plasmid encoding the human cDNA of the H_4 receptor fused to EYFP was used as negative control.

2.5. Cell culture and transfection

HEK-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM glutamine (all from Invitrogen, Grand Island, NY, USA), at 37 °C in an atmosphere of 5% CO_2 . Cells were seeded in 35-mm diameter wells of 6-well plates and transient transfection with the corresponding fusion protein cDNAs was performed the following day using the calcium phosphate precipitation method (Jordan et al., 1996). Cells were harvested 48 h after transfection and used for BRET experiments. The empty vector pcDNA3.1 was used to equilibrate the total amount of transfected DNA.

2.6. cAMP determination

HEK-293 cells were grown in 25 cm^2 flasks to 80% confluence and were transfected with 5 μ g of cDNA corresponding to D_2 , H_3 or H_4 receptors or the fusion proteins D_2 -*RLuc*, H_3 -YFP or H_4 -YFP. Cells were incubated in serum-free medium for 16 h before the experiment and then were preincubated with 50 μ M zardaverine (Tocris) as phosphodiesterase inhibitor for 10 min at 37°C and treated for 10 min with 10 μ M quinpirole, 100 nM RAMH, 10 μ M imetit and/or 10 μ M forskolin (Sigma). To stop the reaction cells were placed on ice and washed with ice-cold phosphate-buffered saline. Cells were incubated with 200 μ L of $HClO_4$ (4%) for 30 min and 1.5 M KOH was added to reach neutral pH and samples were centrifuged. The supernatant was frozen at -20°C. Accumulation of cAMP was measured with Cyclic AMP (3H) Assay System (Amersham Biosciences, Uppsala, Sweden) as described in the manual from the manufacturer. Non-paired Student's t test was used for statistics.

2.7. Bioluminescence Resonance Energy Transfer (BRET)

HEK-293 cells were transfected with 500 ng/well of the cDNA construct coding for D_2 -*RLuc*, acting as BRET donor, and increasing amounts (0.5–9 μ g/well) of the cDNA construct

coding for the BRET acceptor H₃-YFP or the negative control H₄-YFP. After 48 h of transfection cells were washed twice with Hanks' balanced salt solution HBSS (137 mM NaCl, 5 mM KCl, 0.34 mM Na₂HPO₄×12H₂O, 0.44 mM KH₂PO₄, 1.26 mM CaCl₂×2H₂O, 0.4 mM MgSO₄×7H₂O, 0.5 mM MgCl₂, 10 mM HEPES, pH 7.4) supplemented with 0.1% glucose (w/v), detached by gently pipetting and resuspended in the same buffer. Sample protein concentration was determined to control cell number, using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards. Cell suspension (20 µg of protein) was dispensed in duplicates into 96-well black microplates with a transparent bottom (Porvair, King's Lynn, UK) and the fluorescence was measured using a Mithras LB940 fluorescence-luminescence detector (Berthold, Bad Wildbad, Germany) with an excitation filter of 485 nm and an emission filter of 535 nm. For BRET measurement, 20 µg of cell suspension were distributed in duplicates into 96-well white opaque microplates (Porvair) and coelenterazine H (Molecular Probes Europe, Leiden, The Netherlands) was added at a final concentration of 5 µM, after 1 min the readings were collected using sequential integration of signals detected at 440 to 500 nm and 510 to 590 nm. The same samples were incubated for 10 min and the luminescence was measured. Cells expressing BRET donors alone were used to determine background. The BRET ratio is defined as [(emission at 510–590)/(emission at 440–500)] – Cf where Cf corresponds to (emission at 510–590)/(emission at 440–500) for the -Rluc construct expressed alone in the same experiment. Curves were fitted using a nonlinear regression equation, assuming a single binding site with GraphPad Prism software (San Diego, CA, USA).

3. Results

3.1. H₃-D₁ and H₃-D₂ receptor interactions in reserpinized mice

Significant differences in locomotor activity were observed among groups of reserpinized mice that received different doses of the D₁ receptor agonist SKF 38393 (0, 5 or 15 mg/kg, i.p.) with or without the previous administration of the H₃ receptor antagonist thioperamide (3 or 10 mg/kg, i.p.) (one-way ANOVA: $p < 0.0001$; $n = 7-15$ /group) (Fig. 1a). Post-hoc Newman-Keuls tests demonstrated that the highest dose of SKF 38393 (15 mg/kg, i.p.) produced a modest but significant locomotor activation when administered alone ($p < 0.05$). This effect was significantly potentiated by the previous administration of the H₃ receptor antagonist thioperamide (3 and 10 mg/kg, i.p.; $p < 0.01$ in both cases), which was not significantly effective when administered alone (Fig. 1a). Furthermore, thioperamide (3 and 10 mg/kg, i.p.) induced locomotor activation when administered previously to the low ineffective dose of SKF 38393 (5 mg/kg, i.p.; $p < 0.05$ in both cases) (Fig. 1a). Significant differences in locomotor activity were also observed among groups of reserpinized mice that received different doses of the D₂-like receptor agonist quinpirole (0, 0.15 or 0.5 mg/kg, i.p.) with or without the previous administration of thioperamide (3 or 10 mg/kg, i.p.) (one-way ANOVA: $p < 0.0001$; $n = 7-15$ /group) (Fig. 1b). Post-hoc Newman-Keuls tests demonstrated that the highest dose of quinpirole (0.5 mg/kg, i.p.) produced a modest but significant locomotor activation when administered alone ($p < 0.05$), which was significantly potentiated by the previous administration of the H₃ receptor antagonist thioperamide (3 and 10 mg/kg, i.p.; $p < 0.01$ in both cases) (Fig. 1b). These results indicate that in reserpinized mice the already demonstrated high constitutive activity of H₃ receptors (Morisset et al., 2000) or an endogenous histaminergic tone acting on H₃ receptors is able to counteract the effects of both D₁ and D₂ receptor stimulation.

Significant differences in locomotor activity were also observed among groups of reserpinized mice that received SKF 38393 (15 mg/kg, i.p.) with or without the previous administration of the H₃ receptor agonist imetit (10 mg/kg, i.p.) and with or without the previous administration of thioperamide (3 or 10 mg/kg, i.p.) (one-way ANOVA: $p < 0.01$; $n = 6-13$ /group) (Fig. 2).

Post-hoc Newman-Keuls tests demonstrated that imetit significantly decreased locomotor activation induced by SKF 38393 ($p < 0.01$), but not when thioperamide was also co-administered (Fig. 2). Similarly, significant differences in locomotor activity were observed among groups of reserpinized mice that received quinpirole (0.5 mg/kg, i.p.) with or without the previous administration of imetit (10 mg/kg, i.p.) and with or without the previous administration of thioperamide (3 or 10 mg/kg, i.p.) (one-way ANOVA: $p < 0.05$; $n = 8/\text{group}$) (Fig. 2). Post-hoc Newman-Keuls tests demonstrated that imetit significantly decreased locomotor activation induced by quinpirole ($p < 0.05$), but not when thioperamide was also co-administered (Fig. 2). In fact, when co-administered with imetit, thioperamide did not produce any potentiation of the motor effects induced by quinpirole or SKF 38393, strongly supporting an involvement of H_3 receptors in the locomotor effects of thioperamide. The results indicate that stimulation of H_3 receptors with exogenous agonists antagonize the locomotor activation induced by D_1 or D_2 receptor stimulation. Neither thioperamide (3 and 10 mg/kg, i.p.) or imetit (10 mg/kg, i.p.) significantly modified the exploratory locomotor activity in non-habituated, non-reserpinized mice (data not shown).

Finally, we analyzed the effects of H_3 receptor blockade on the locomotor activation induced by the simultaneous activation of D_1 and D_2 receptors. Significant differences in locomotor activity were observed among groups of reserpinized mice that received different combinations of doses of SKF 38393 (5 or 15 mg/kg, i.p.) and quinpirole (0.15 or 0.5 mg/kg, i.p.) with or without the previous administration of thioperamide (3 mg/kg, i.p.) (one-way ANOVA: $p < 0.0001$; $n = 6\text{--}15/\text{group}$) (Fig. 3). As previously reported (Rubinstein et al., 1988; Starr et al., 1987; Ferré et al., 1991, 1994), co-administration of SKF 38393 and quinpirole in reserpinized mice produced a synergistic motor activating effect. Thus, post-hoc Newman-Keuls tests demonstrated that co-administration of low ineffective doses of SKF 38393 (5 mg/kg, i.p.) and quinpirole (0.15 mg/kg, i.p.) produced a significant locomotor activation ($p < 0.05$), and co-administration of the effective doses of SKF 38393 (15 mg/kg, i.p.) and quinpirole (0.5 mg/kg, i.p.) produced a greater than additive activating effect ($p < 0.01$) (Fig. 3). Importantly, the additional administration of thioperamide (3 mg/kg, i.p.) strongly potentiated the activating effects of both combinations of D_1 and D_2 receptor agonists (Fig. 3) ($p < 0.05$ and $p < 0.01$ for the combinations with low and high doses of dopamine agonists, respectively) (Fig. 3). In all the behavioral experiments, very similar effects were obtained with both doses of thioperamide used (3 and 10 mg/kg, i.p.). This dose range was similar to other studies in mice (see for instance, Brabant et al., 2006). Our results indicate that there is a ceiling effect at 3 mg/kg thioperamide, since pilot studies showed no significant effects with 1 mg/kg thioperamide (data not shown), indicating the existence of a small dose-dependent window for the H_3 receptor-dependent effects of thioperamide.

3.2. Striatal H_3 - D_2 intramembrane receptor interaction

To investigate the possible existence of a striatal H_3 - D_2 intramembrane receptor interaction, competition experiments with the D_2 receptor antagonist [^3H] YM-09151-2 (1.4 nM) as radioligand and increasing concentrations of the D_2 receptor agonist quinpirole as displacer were carried out in the presence or the absence of a high concentration (95% receptor saturation) of the selective H_3 receptor agonist RAMH (20 nM). As shown in Fig. 4a, both competition curves are biphasic (significantly better fitting than monophasic; F test: $p < 0.01$) and in the presence of the H_3 receptor agonist a pronounced shift to the right in the competition curve was observed. From four independent experiments (different membrane preparations) in the absence of H_3 receptor agonist the K_{DH} and K_{DL} values for quinpirole binding to D_2 receptor were 0.9 ± 0.2 nM and 1046.0 ± 172.2 nM, respectively, whereas in the presence of RAMH the K_{DH} and K_{DL} values were 52.5 ± 8.2 nM and 4030.0 ± 75.5 nM, respectively (Student's unpaired t test: $p < 0.01$ in both cases). Additional experiments using the same concentration than RAMH (20 nM; more than 95% receptor saturation) with the H_3 receptor agonists imetit and immepip

produced a similar effect than RAMH (one experiment with each agonist performed in triplicate). K_{DH} values in the presence of imetit and immepip were 13.0 nM and 14.3 nM, respectively, and K_{DL} values were 4600 nM and 5500 nM, respectively (Fig. 4a). These results indicate that H_3 receptor activation induces a pronounced loss of affinity of both affinity states of the D_2 receptor for agonists.

The H_3 receptor agonist-mediated loss of affinity was specific for the D_2 agonist since no change in the maximum binding or the K_D value for the YM-09151-2 binding to D_2 receptor was observed in competition experiments using 1.5 nM [3H] YM-09151-2 and increasing concentrations of YM-09151-2 in the absence or the presence of 20 nM RAMH, imetit or immepip (Table 1), also indicating that H_3 receptor agonists are not D_2 receptor ligands. Accordingly, high concentrations of H_3 receptor agonists did not change 1.5 nM [3H] YM-09151-2 binding and only very high thioperamide concentrations significantly diminished the binding (Fig. 4b). Nevertheless, thioperamide was able to completely counteract the effect of RAMH on D_2 receptor agonist binding (Fig. 4c).

3.3. H_3 - D_2 receptor heteromerization

Since intramembrane receptor interactions are a functional property of some G protein-coupled receptor heteromers (Agnati et al., 2003; Ferré et al., 2007), we used the BRET approach to demonstrate the ability of H_3 receptors to heteromerize with D_2 receptors. Fusion of *Rluc* to D_2 receptor or fusion of YFP to H_3 or H_4 receptors did not prevent receptor functionality, as determined by cAMP production in the presence of forskolin (Fig. 5A). The effect was significant and the degree of inhibition (around 30%) was similar to that observed with receptors without fusion proteins (Fig. 5A). BRET measurements were performed in transiently co-transfected HEK cells using a constant amount of D_2 -*RLuc* and increasing amounts of H_3 -YFP. A positive and saturable BRET signal for the transfer of energy between D_2 -*RLuc* and H_3 -YFP was also obtained (Fig. 5B). From the saturation curve a $BRET_{max}$ of 0.052 ± 0.004 and a $BRET_{50}$ of 7.6 ± 1.6 was calculated. The pair D_2 -*RLuc* and H_4 -YFP was used as a negative control, since the human H_4 receptor is closely related to the human H_3 receptor (31% sequence identity at the protein level, which increases to 54% in the transmembrane region) (de Esch et al., 2005). As shown in Fig. 5B this pair gave a linear non-specific BRET signal, indicating selectivity in the interaction between D_2 -*RLuc* and H_3 -YFP. HEK cells transfected with 0.5 μ g cDNA corresponding to D_2 -*RLuc* expressed 1.5 pmol/mg protein of the receptor determined by radioligand binding. Analogously, cells transfected with increasing amounts of cDNA corresponding to the H_3 -YFP expressed 0.02 to 0.5 pmols/mg of the receptor protein. According to these values, in $BRET_{50}$ conditions, the receptors expression was near the range of receptors expressed in sheep brain striatum (0.8 pmols/mg protein for D_2 receptor and 0.1 pmols/mg protein for H_3 receptor; values determined from saturation experiments using 0.02–10 nM [3H]YM 09151-2 and 0.02–10 nM [3H]RAMH, respectively).

4. Discussion

The evaluation of locomotor activity induced by dopamine receptor agonists in reserpinized mice is a very useful in vivo model to study the function of striatal postsynaptic D_1 and D_2 receptors, which are predominantly localized in GABAergic dynorphinergic and GABAergic enkephalinergic neurons, respectively, without the influence of endogenous dopamine (Rubinstein et al., 1988; Starr et al., 1987; Ferré et al., 1991, 1994b). By using the reserpinized mouse model, in the present study, we demonstrate the existence of H_3 receptor-mediated negative modulation of D_1 and D_2 receptor function. In reserpinized mice, the selective H_3 receptor agonist imetit inhibited, while the H_3 receptor antagonist thioperamide potentiated the locomotor activation induced by either the D_1 receptor agonist SKF 38393 or the D_2 receptor agonist quinpirole.

GABAergic enkephalinergic and GABAergic dynorphinergic neurons give rise to two striatal efferent systems which connect the striatum with the output structures of the basal ganglia: the substantia nigra pars reticulata and the internal segment of the globus pallidus (entopeduncular nucleus in rodents) (Gerfen, 2004). These are called “direct” and “indirect” pathways. The direct pathway is made of GABAergic dynorphinergic neurons, which directly connect the striatum with the output structures. The indirect pathway consists of GABAergic enkephalinergic neurons, which connect the striatum with the external segment of the globus pallidus (globus pallidus in rodents), GABAergic neurons which connect the globus pallidus with the subthalamic nucleus and glutamatergic neurons which connect the subthalamic nucleus with the output structures (Gerfen, 2004). Stimulation of the direct pathway results in motor activation and stimulation of the indirect pathway produces motor inhibition. Dopamine and dopamine agonists induce motor activation by activating the direct pathway (acting on stimulatory D₁ receptors localized in GABAergic dynorphinergic neurons) and by depressing the indirect pathway (acting on inhibitory D₂ receptors localized in GABAergic enkephalinergic neurons) (Gerfen, 2004). The striatum contains a high density of postsynaptic H₃ receptors, which are co-localized with both D₁ and D₂ receptors in the GABAergic dynorphinergic and enkephalinergic neurons, respectively (Ryu et al., 1994; Pillot et al., 2002). Previous studies have underscored the role of presynaptic H₃ receptors (see introduction). The present study adds a new dimension to the role of histamine in basal ganglia physiology, by underscoring the role of postsynaptic H₃ receptors in the modulation of D₁ and D₂ receptor function.

In the present study, we demonstrate the existence of an antagonistic intramembrane interaction between H₃ and D₂ receptors in striatal tissue, by which stimulation of H₃ receptors significantly decreases the ability of an agonist, but not an antagonist, to bind to the D₂ receptor. The antagonistic H₃-D₂ intramembrane interaction is particularly pronounced when compared with similar interactions involving striatal D₂ receptors (Agnati et al., 2003). Thus, H₃ receptor agonists produced a clear shift to the right in the competitive-inhibition curve of the D₂ receptor antagonist [³H]YM-09151-2 *versus* the D₂-like receptor agonist quinpirole, which was reversed by thioperamide. The analysis of the competition curves in the presence and absence of the H₃ receptor agonist RAMH indicated that this effect was due to 50-fold and 4-fold H₃ receptor-mediated decreases in the affinities of the high and low affinity states of the D₂ receptor for the agonist binding, respectively. Therefore, this negative cross-talk could be the main mechanism responsible for the postsynaptic H₃-D₂ receptor negative interaction observed in our reserpinized mouse model and could also explain the biochemical results reported by Hussain et al. (2002), who found that an H₃ receptor antagonist counteracts the increase in the striatal expression of the immediate-early gene *c-fos* induced by the D₂ receptor antagonist haloperidol. In fact, it would be difficult to understand the H₃-D₂ functional cross-talk at the level of the intracellular signaling cascades interaction, because both D₂ and H₃ receptors couple to Gi/o proteins and their stimulation inhibits adenylyl cyclase (Lovenberg et al., 1999; Drutel et al., 2001; Neve et al., 2004; Leurs et al., 2005). Therefore, the pronounced antagonistic intramembrane H₃-D₂ receptor interaction most probably plays a key modulatory role of the function of the GABAergic enkephalinergic neuron.

Intramembrane receptor-receptor interaction implies a crosstalk that does not involve any signaling pathway, but an allosteric modification of one receptor secondary to stimulation of an adjacent receptor (Agnati et al., 2003; Franco et al., 2003; Ferré et al., 2007). An intramembrane receptor-receptor interaction constitutes a common biochemical characteristic of receptor heteromers (Ferré et al., 2007). Therefore, the existence of an antagonistic intramembrane H₃-D₂ receptor interaction in brain striatum strongly suggests the existence of a direct protein-protein interaction between both receptors. In fact, in the present study, we demonstrate the existence of H₃-D₂ receptor heteromerization in transfected cells by using

BRET techniques. Thus, according to the recent UPHAR guidelines (Pin et al., 2007), the present study strongly suggests the existence of GPCR heteromers in the striatum.

Previous studies on neurotransmitter (GABA) release in striatal slices (Arias-Montano et al., 2001) and the present studies in reserpinized mice indicate the existence of antagonistic H₃-D₁ receptor interactions. Different from H₃ receptors, D₁ receptor couples to Gs/olf proteins and its main signaling pathway is the stimulation of the adenylyl cyclase-PKA cascade (Neve et al., 2004). The opposite effects of H₃ and D₁ receptors on adenylyl-cyclase predict the existence of antagonistic interactions of both receptors in the GABAergic dynorphinergic neuron. In fact, Sanchez-Lemus and Arias-Montano (2004) have recently demonstrated that H₃ receptor activation efficiently inhibits D₁ receptor-mediated adenylyl cyclase activation in rat striatal slices. The same authors could not find evidence for the existence of an H₃-D₁ intramembrane receptor interaction (Sanchez-Lemus and Arias-Montano, 2004). Therefore, an antagonistic H₃-D₁ receptor interaction at the second messenger level, with its consequent modulation of the function of the GABAergic dynorphinergic, might be mostly responsible for the antagonistic H₃-D₁ receptor interaction observed at the behavioral level.

In Parkinson's disease a preferential degeneration of the nigrostriatal dopa-minergic system produces striatal dopamine depletion with the consequent impairment of the functioning of basal ganglia circuits, which is associated with hypokinesia. Reserpinized mice are used as a model to evaluate the possible antiparkinsonian activity of a drug. In the present study the H₃ receptor antagonist thioperamide did not produce locomotor activation in reserpinized mice, but it potentiated the locomotor activation induced by the D₁ receptor agonist SKF 38393 or by the D₂ receptor quinpirole. Furthermore, very high scores of locomotor activity were obtained with H₃ receptor blockade plus D₁ and D₂ receptor co-activation, i.e., when thioperamide was co-administered with both SKF 38393 and quinpirole. Although these results suggest that H₃ receptor antagonists could be used as an adjuvant to dopamine receptor agonists in Parkinson's disease, it must however be pointed out that thioperamide was not found to potentiate L-DOPA-mediated effects in another model of Parkinson's disease, the unilateral 6-OH-dopamine lesioned rat (Huotari et al., 2000). Those negative results could be related to the neuroadaptations that develop upon chronic dopamine denervation. For instance, the upregulation of dopamine or H₃ receptors observed after striatal dopamine denervation (Ryu et al., 1994, 1996; Anichtchik et al., 2000) could be accompanied by a reduction in H₃-D₁ or H₃-D₂ receptor interactions. Nevertheless, a possible antiparkinsonian role of drugs acting on striatal H₃ receptors cannot be discarded and other disorders involving the cortico-striatal-thalamo-cortical circuits (Huntington's disease, Tourette syndrome, obsessive compulsive disorder, schizophrenia, drug abuse) could benefit from a therapeutic approach based on the H₃ receptor-mediated negative modulation of D₁ and D₂ receptor function.

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Figure 1.

Effects of the H_3 receptor ligands on locomotor activity induced by dopamine receptor agonists in reserpinized mice. Results represent means + S.E.M. of the average of the transformed values (square root of cm of ambulation) obtained during the 10-min periods of the first hour of recording. **a)** Effect of saline or the H_3 receptor antagonist thioperamide (Thiop; 3 and 10 mg/kg, i.p.) on the locomotor activation induced by saline or the D_1 receptor agonist SKF 38393 (SKF; 5 and 15 mg/kg, i.p.; $n = 8-16$ /group). **b)** Effect of saline or the H_3 receptor antagonist thioperamide (Thiop; 3 and 10 mg/kg, i.p.) on the locomotor activation induced by saline or the D_2 receptor agonist quinpirole (Quin; 0.15 and 0.5 mg/kg, i.p.; $n = 9-15$ /group). * and **: significantly different compared to the control, saline-saline, group (ANOVA and post-hoc Newman-Keuls test: $p < 0.05$ and $p < 0.01$, respectively); ##: significantly different compared to the group treated with highest doses of SKF 38393 (15 mg/kg, i.p.) or quinpirole (0.5 mg/kg, i.p.) without thioperamide pretreatment (ANOVA and post-hoc Newman-Keuls test: $p < 0.01$).

Figure 2.

Effects of the H_3 receptor agonist imetit (10 mg/kg, i.p.) on the locomotor activation induced by the D_1 receptor agonist SKF 38393 (SKF; 15 mg/kg, i.p.) or the D_2 receptor agonist quinpirole (Quin; 0.5 mg/kg, i.p.) with or without co-administration of the H_3 receptor antagonist thioperamide (Thiop; 3 and 10 mg/kg, i.p.) in reserpinized mice ($n = 8-12$ /group). Results represent means + S.E.M. of the average of the transformed values (square root of cm of ambulation) obtained during the 10-min periods of the first hour of recording. * and **: significantly different compared to the respective saline group (treated with SKF 38393 or quinpirole alone; ANOVA and post-hoc Newman-Keuls test: $p < 0.05$ and $p < 0.01$, respectively).

Figure 3.

Effects of the H_3 receptor antagonist thioperamide (Thiop; 3 mg/kg, i.p.) on the locomotor activation induced co-administration of the D_1 receptor agonist SKF 38393 (SKF; 5 or 15 mg/kg, i.p.) and the D_2 receptor agonist quinpirole (Quin; 0.15 or 0.5 mg/kg, i.p.) in reserpinized mice ($n = 7-11$ /group). Results represent means + S.E.M. of the average of the transformed values (square root of cm of ambulation) obtained during the 10-min periods of the first hour of recording. The dashed line represents the mean of the control, saline-saline, group. * and **: significantly different compared to the control, saline-saline, group (represented by the dashed line) (ANOVA and post-hoc Newman-Keuls test: $p < 0.05$ and $p < 0.01$, respectively); # and ##: significantly different compared to the group treated with the same doses of the

dopamine receptor agonists without thioperamide pretreatment (ANOVA and post-hoc Newman-Keuls test: $p < 0.05$ and $p < 0.01$, respectively).

Figure 4.

H₃ receptor ligand-mediated modulation of D₂ receptor agonist binding in sheep striatal membranes. (A and C) Competition experiments of the D₂ receptor antagonist [³H] YM-09151-2 (1.4 nM) versus increasing concentrations of the D₂ receptor agonist quinpirole. In A, experiments were performed in the absence (■) or in the presence of the H₃ receptor agonists RAMH (●), imnepip (○) or imetit (▼) (20 nM in all cases). In C, experiments were performed in the presence of 20 nM RAMH (●) or 20 nM RAMH plus 1 μM thioperamide (Δ). Data are means ± S.D. from representative experiments performed in triplicate. (B) Displacement of 1.4 nM [³H] YM-09151-2 binding by 0.1 μM (black bars) or 1 μM (white bars) of the H₃ receptor agonists RAMH, imnepip or imetit or the H₃ receptor antagonist thioperamide. Values are expressed as specific binding percentage respect to the specific binding in absence of H₃ receptor ligands. Data are means ± S.E.M. Significant differences were calculated by Student's *t* test for unpaired samples (**: *p*<0.01).

Figure 5.

A. Production of cAMP by D₂-RLuc, H₃-YFP and H₄-YFP constructs and by the same receptors without fusion proteins in HEK-293 cells. HEK-293 cells transfected with 5 µg of cDNA corresponding to D₂-RLuc, H₃-YFP or H₄-YFP constructs were treated with 10 µM forskolin plus the D₂ receptor agonist quinpirole (10 µM), the H₃ receptor agonist RAMH (0.1 µM) or the H₃-H₄ receptor agonist imetit (10 µM). In the same conditions, cells transfected with the same amount of cDNA corresponding to the receptors not fused to bioluminescent or fluorescent proteins induced a 25–30% reduction of forskolin-induced cAMP. Results are expressed as percentage of the effect of forskolin alone (mean + SEM; n = 4); * and **: significantly different compared to forskolin alone (non-paired Student's *t* test: p<0.05 and p<0.01, respectively). **B.** D₂-H₃ receptor heteromerization in HEK-293 cells. BRET saturation curve obtained from experiments with HEK-293 cells co-expressing D₂-RLuc and H₃-YFP (■) constructs or D₂-RLuc and H₄-YFP (○) constructs as a negative control. Co-transfections were

performed with increasing amounts of plasmid for H₃-YFP or H₄-YFP whereas the D₂-RLuc constructs were maintained constant. Both fluorescence and luminiscence of each sample were measured before every experiment to confirm equal expression of D₂-RLuc while monitoring the increase of the acceptor. The relative amounts of BRET acceptor are expressed as the ratio between the fluorescence of the acceptor and the *luciferase* activity of the donor. YFP₀ corresponds to the fluorescence value of cells expressing D₂-RLuc alone. BRET data are expressed as means $\pm$ S.D. of three to thirteen different experiments grouped as a function of the amount of BRET acceptor.

Table 1

Competition experiments of the D₂ receptor antagonist [³H] YM-09151-2 with increasing concentrations of YM-09151-2 in the absence or the presence of H₃ receptor agonists.

Ligand	Parameters	
	K _D (nM)	B _{max} (pmol/mg protein)
YM-09151-2	5.4 ± 0.6	0.811 ± 0.092
YM-09151-2 + RAMH	4.4 ± 0.6	0.890 ± 0.101
YM-09151-2 + Imetit	4.2 ± 0.4	0.864 ± 0.097
YM-09151-2 + Immepip	4.4 ± 0.4	0.865 ± 0.097

Parameter values obtained by fitting data according to the one site model. Data are means ± SD values (of 3 experiments with triplicates). K_D is the equilibrium dissociation constant and B_{max} is the maximum binding.