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Interplay Among Catecholamine Systems: Dopamine Binds to a_2-adrenergic Receptors in Birds and Mammals

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Abstract

Dopaminergic and adrenergic receptors are G protein-coupled receptors considered to be different based on their pharmacology and signaling pathways. Some receptor subtypes that are members of one family are actually closer in phylogenetic terms to some subtypes belonging to the other family suggesting that the pharmacological specificity among these receptors from different families is not perfect. Indeed, evidence is accumulating that one amine can cross-talk with receptors belonging to the other system. However, most of these observations were collected *in vitro* using artificial cell models transfected with cloned receptors, so that the occurrence of this phenomenon *in vivo* as well as its distribution in the central nervous system is not known. In this study, the pharmacological basis of possible *in vivo* interactions between dopamine and α_2-adrenergic receptors was investigated in quail, zebra finches and rats. Binding competitions showed that dopamine displaces the binding of the selective α_2-adrenergic ligand, [^{3}H]RX821002, in the brain of the three species with an affinity about 10 to 28 fold lower than that of norepinephrine. Dopamine also displaces with an affinity 3 fold lower than norepinephrine the binding of [^{3}H]RX821002 to human α_{h2A}-adrenergic receptors expressed in Sf9 cells. The anatomical distribution of this interaction was assessed in brain slices of quail and rat based on autoradiographic methods. Both norepinephrine and dopamine significantly displace [^{3}H]RX821002 binding in all brain nuclei considered. Together these data provide evidence for interaction between the dopaminergic and noradrenergic systems in the vertebrate brain albeit with species variations.

Keywords

autoradiography; binding competition; bird; [^{3}H]RX821002; catecholamines

Introduction

For each neurotransmitter, there is molecular and cellular diversity of the cognate receptors and their subtypes (Vernier et al., 1995). The catecholamines, dopamine (DA) and norepinephrine (NE), are no exception, both act on G protein-coupled receptors (GPCR) that are divided into different classes and sub-classes based on their pharmacology and signaling pathways. Dopaminergic receptors are divided into D1-type and D2-type receptors, referred to as D1-like and D2-like receptors. In mammals, two genes coding for receptor subtypes have been found to meet the pharmacological criteria for the D1 receptor class (D_{1A}/D_1 and D_{1B}/D_5), but genes coding for other subtypes of this sort have been found to exist in non-

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mammalian vertebrates (D_{1c} and D_{1d}). Similarly, genes for three subtypes of the D2 receptor type have been isolated in jawed vertebrates (D_2, D_3 and D_4; Callier et al., 2003; Civelli et al., 1993; Jaber et al., 1996; Missale et al., 1998). Adrenergic receptors (AR) are divided into three main classes (α_1-AR, α_2-AR and β-AR) each of which is represented by three subtypes in humans, other mammals and some other vertebrates (α_{1A}, α_{1B} and α_{1C}; α_{2A}, α_{2B} and α_{2C}; β_1, β_2, and β_3 ; Bylund et al., 1994; Docherty, 1998; Harrison et al., 1991a; Ruffolo et al., 1993). A fourth subtype of α_2-AR has been found in zebrafish (Ruuskanen et al., 2004). This pattern of variability of catecholaminergic receptors is thought to have arisen from successive gene duplications of a common ancestral gene over the course of evolutionary time (Callier et al., 2003; Harrison et al., 1991b; Kapsimali et al., 2003; Ruuskanen et al., 2004). Upon duplication, redundant copies that were not eliminated provided opportunities for novel signaling patterns and acquired new functions and patterns of distribution. Interestingly, however, morphological and functional features indicate that α_2-adrenergic receptors appear closer to D2 receptors in phylogenetic terms than to the other catecholaminergic receptors, while β-adrenergic receptors are closer to D1 receptors (Donnely et al., 1994; Vernier et al., 1995).

This relative similarity based on morphological and genetic criteria between receptors that are pharmacologically thought to be distinct raises the question of the extent of the binding specificity of adrenergic versus dopaminergic receptors. Is it possible that one catecholamine interacts also with the receptors of the other and vice-versa? Both molecules are indeed chemically close differing only by the presence of a β-hydroxyl group borne by norepinephrine. This extra hydroxyl group thus presumably confers the selectivity for NE. However, this selectivity is apparently not perfect. Indeed, evidence is accumulating that one amine can crosstalk with the pharmacologically defined receptors or transporters belonging to the other system. For instance, it has been shown that norepinephrine transporter can re-uptake dopamine (Carboni et al., 2001; Carboni et al., 1990; Moron et al., 2002; Valentini et al., 2004) and that DA may exert some of its effects via binding to pharmacologically defined noradrenergic receptors and vice-versa (Aguayo and Grossie, 1994; Cornil et al., 2002; Lanau et al., 1997; Newman-Tancredi et al., 1997; Nyronen et al., 2001; Rey et al., 2001; Ruuskanen et al., 2005; Zhang et al., 1999; Zhang et al., 2004).

Most of these observations were collected *in vitro* using artificial cell models expressing cloned receptors, so that it is not known whether such phenomenon occurs in vivo and what its distribution in the central nervous system is. Although they are considered as different systems for anatomical and functional reasons, there are reciprocal projections between the major neuronal groups of the dopaminergic and noradrenergic systems and several regions receive inputs from both (e.g. locus coeruleus, prefrontal cortex, bed nucleus of the stria terminalis, the shell of the nucleus accumbens, preoptic/hypothalamic area; (Smeets and Gonzalez, 2000). These regions thus provide a potential ground for cross-talk between one amine and the other's receptors. In a recent study, we explored the existence of such an interaction in whole brain homogenates and in one particular specialized neural system, namely the nuclei of the song system of a songbird, the zebra finch (Cornil et al., 2008). We found that DA has an affinity for α_2-AR about 10 to 28 fold lower than that of NE and that both NE and DA substantially displace the binding of [^{3}H]RX821002, a specific radioligand for α_2-AR, in most song nuclei.

In the present study, utilizing the same approach, we sought to determine how general this conclusion was to avian and mammalian taxa and the extent to which such interactions might occur in other neural systems. As in the zebra finch study, the first part of this study was designed to investigate the neuropharmacological basis for such an interaction in brain homogenates. Then, binding competitions were performed on brain slices using autoradiographic methods in order to determine where in the brain this interaction occurs.

Experiments were conducted on brain tissue derived from three different species (Rats, zebra finches and Japanese quail) and on Spodoptera frugiperda (Sf9) cell membranes expressing the human α_{2A}-adrenergic receptor. We selected quail and rats because there is evidence that dopamine functionally interacts α_2-AR in these species. We also studied an avian species distantly related to quail, the zebra finch, as well as a human α_2-adrenergic receptor subtype expressed in a cell line to provide a basis for comparison with the rat data. This subtype expressed in an insect cell also served as a control that our observations relied on an interaction with α_2-AR and not with another GPCR. We chose the selective α_2-adrenergic antagonist, [^{3}H]RX821002, as a marker of α_2-AR since it has been described as a radioligand of choice to detect the total population of α_2-AR in several tissues and species (Diez-Alarcia et al., 2006; Halme et al., 1995; O'Rourke et al., 1994; Ruuskanen et al., 2005). Indeed, this ligand does not select between different α_2-receptor subtypes and lacks binding at I_1 and I_2 imidazoline receptors, however it displays a relatively high affinity for $5HT_{1A}$ receptors (Clarke and Harris, 2002) making it necessary to check that our observations do not result from binding to these receptors. With this in mind, we measured the ability of dopamine and norepinephrine as well as various catecholaminergic and non-catecholaminergic ligands to displace its binding. Overall, this study provides the first demonstration of a direct interaction of dopamine with α_2-AR in birds and mammals and suggests that this interaction is widespread in the brain. The zebra finch binding data have been reported previously as part of a separate study that focused on the specialized song control system but they are presented here to provide additional comparative data to other species presented in this paper (Cornil et al., 2008).

Methods

Experimental animals

Sexually mature male (n=19) and female (n=13) Japanese quail (*Coturnix japonica*), male (n = 5) and female (n=5) zebra finches (*Taeniopygia guttata*) and male (n = 10) and female (n=9) long evans rats (*Rattus norvegicus*) were used for this study. The quail and zebra finches were purchased from a local breeder. Quail were housed in individual cages in a 14L:10D photoperiod. Zebra finches were housed in same sex groups in a photoperiod of 14L:10D. Long Evans rats were purchased from Charles river (Raleigh, NC, USA) and were housed individually in a 12L:12D light regimen. All animals were provided with food and water *ad libitum*. All procedures were approved by the Johns Hopkins Animal Care and Use Committee.

Drugs

[^{3}H]RX821002 (45.0 – 67.0 Ci/mmol; Amersham Biosciences, Piscataway, NJ, USA) was the ligand selected to label α_2-adrenergic receptors. Phentolamine hydrochloride (α-adrenergic antagonist; obtained from Sigma-Aldrich, Inc.) was used to define the non-specific binding. For specificity experiments, R-(−)-Apomorphine HCl Hemihydrate (non-selective dopaminergic agonist), clonidine hydrochloride (α_2-adrenergic agonist with affinity for I1 imidazoline receptors), clozapine (atypical antipsychotic, antagonist at D4, 5-HT2, and muscarinic receptors), dopamine, epinephrine, haloperidol (antipsychotic, D2 antagonist), idazoxan hydrochloride (α_2-adrenergic antagonist; I2 imidazoline receptor agonist; I1 imidazoline receptor antagonist), L-745,870 hydrochloride (D4 receptor antagonist), (±)-norepinephrine (+)-bitartrate salt, oxymetazoline hydrochloride (partial α_{2A}-adrenergic agonist; agonist at 5-HT1A, 5-HT1B and 5-HT1D serotonin receptors; mixed agonist-antagonist at 5-HT1C serotonin receptors), prazosin hydrochloride (α_1-adrenergic antagonist with some affinity for α_{2b} and α_{2C}-adrenergic receptors), (±)propranolol hydrochloride (β-adrenergic antagonist; 5-HT1/5-HT2 serotonin receptor antagonist; cardiac depressant), (−)quinpirole hydrochloride (D2 agonist with some selectivity for D3 sites),

S(−)raclopride (+)-tartrate salt (D2 antagonist), RX821002 hydrochloride (α_2-adrenergic antagonist lacking affinity for I1 and I2 imidazolin receptors but with a relatively high affinity for 5HT1A receptors), 5-hydroxytryptamine (serotonin) hydrochloride (5-HT), R-(+)-SCH-23390 hydrochloride (D1 antagonist), (±)-SKF-38393 hydrochloride (D1-receptor agonist), spiperone (D2 antagonist with a higher affinity for D4), (S)-(−)-Sulpiride (antipsychotic, D2 antagonist) and yohimbine hydrochloride (α_2-adrenergic antagonist) were purchased from Sigma-Aldrich.

Tissue preparation

Brains were collected from all subjects via rapid decapitation. For the binding competition and specificity experiments, brains were rapidly dissected out of the skull, the white matter was removed and the remaining gray matter from each brain was then minced and molded into a cylindrical plastic mold (≈ 5 mm high with a radius of ≈ 5 mm). Different molds were made for female and male tissue. Four brains per homogenate were used for quail (except one mold that contained three brains) and rat, while five brains per homogenate were used for zebra finch. One homogenate was used per condition (species and sex) for zebra finches and rats. More homogenates were needed for quail because this was the species first employed for the preliminary studies establishing the assay method. Therefore, 3 homogenates were used for males and 2 for females in this species. Repeated assays conducted to evaluate the binding properties of different ligands were performed with strictly one homogenate per drug. The female quail homogenate containing only three brains was used only for the 1st set of competitions (see below). Baseline and total bindings were checked and did not differ between homogenates. Molded brain homogenates were then immediately frozen on powdered dry ice and stored at −70°C until use. For autoradiography, the brains were rapidly dissected out of the skull and immediately frozen on powdered dry ice and stored at −70°C until all brains use. The material collected from these three species has been processed in a coordinated fashion in all the experiments described here below so that tissue from the three species was processed in parallel.

[^{3}H]RX821002 binding

The binding competition assays were performed on homogenized brain tissue mounted on glass slides as described previously (Ball et al., 1995; Ball et al., 1989; Nock et al., 1985). This method is commonly used to establish and validate conditions for the competition studies that would subsequently be performed in intact brain slices with the use of autoradiographic procedures to assess the anatomical distribution of the interaction sites (see for further discussion of this aspect see Cornil et al. 2008). Molded brain homogenates (see previous text for preparation) were cut into 16μm-thick sections using a cryostat and thaw mounted onto gel-coated microscope slides. One single section was collected per slide. The slides were dried and stored at −20°C until use. After drying at room temperature, slides were pre-incubated in buffer (50 nM Tris-HCl, pH 7.5 at 25°C with 1mM $MgCl_2$) for 30 min at room temperature. Slides were then incubated for one hour at room temperature in 5nM [^{3}H]RX821002 buffer with different concentrations of several unlabeled compounds. Five slides were incubated without any competitor to determine the total binding. Non-specific binding was determined in five other slides by addition of a saturating concentration of a non-selective α-adrenergic ligand, phentolamine (10μM). Five slides were also incubated for each concentration of the competitors. After one hour, slides were washed twice for 5 min in ice-cold buffer. Then each slice was immediately wiped off the slide with a filter paper (Whatman, Cat. No 1001042). The filter paper was then placed into a scintillation vial filled with scintillation cocktail (Scintiverse®, Fisher Scientific) and binding was estimated using a scintillation counter.

[^{3}H]RX821002 binding on membranes

Membranes of S*f*9 cells expressing cloned human α-adrenergic receptor subtype 2A were obtained from Perkin-Elmer ($h\alpha_{2A}$; Perkin-Elmer, Boston, MA, USA; product No. 6110113) and were diluted (1:100, final concentration: 0.014 mg protein/ml) in incubation buffer (75 mM Tris-Hcl pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA) as recommended by the manufacturer. They were incubated for 1h in 96-well plates containing 5nM [^{3}H]RX821002 buffer and several concentrations of various cold competitors. Each condition was tested in triplicates. Then, they were washed 9x on GF/C filter (using a Whatman Minifold-1 dot-blot system) with 300tl of ice-cold 50 mM Tris-HCl, pH 7.4. The filter dots were cut and placed into scintillation vials filled with scintillation cocktail (Scintiverse®, Fisher Scientific). Binding was estimated using a scintillation counter.

[^{3}H]RX821002 autoradiography

The main goal of the autoradiography experiments was to illustrate the binding displacement of [^{3}H]RX821002 and assess the distribution of this interaction in the two species we focused on, namely quail and rats. As a previous autoradiographic study showed a similar interaction in the song control system of zebra finches (Cornil et al., 2008), the anatomical localization of this interaction was thus not investigated in finches.

Quail (7 males and 7 females) and rat (6 males and 5 females) brains were cut into 16tm-thick sections using a cryostat and thaw mounted onto gel-coated microscope slides. Six series of slides were collected so that, on each slide, consecutive sections were 80 μm apart. Depending on their size, 5 to 10 sections were mounted per slide. Four series were used for the autoradiography while another series was Nissl stained in order to verify the location of brain regions. The slides were dried and stored at −20°C until use. After drying at room temperature, slides were pre-incubated in buffer (50 nM Tris-HCl, pH 7.5 at 25°C with 1mM $MgCl_2$) for 30 min at room temperature. Slides were then incubated for one hour at room temperature in 5nM [^{3}H]RX821002 buffer with no competitor (total binding, series 1), phentolamine (10 μM, non-specific, series 2), norepinephrine (0.1 μM, series 3) or dopamine (1 μM, series 4). One hour later, the slides were washed twice for five min in ice-cold buffer followed by a quick dip in ice-cold distilled water. Sections were fan dried, placed in X-ray cassettes and exposed to tritium-sensitive hyperfilm® (Amersham) for quail brains or BioMax® MR films (Kodak) for rat brains along with standards (ART-123; American Radiolabeled Chemicals Inc., St Louis, MO) containing concentrations of tritium ranging from 0.00 to 489.1 μCi/g. The films were developed after 9 weeks (for tritium-sensitive hyperfilm®) or 12 weeks (for BioMax® MR films). The density of α_2-adrenoceptor binding was analyzed in various brain regions identified by comparison with adjacent sections counterstained with the Nissl staining method. Images from the films were projected from a light box to a camera connected to a Macintosh computer. Image J 1.34s (Wayne Rasband, NIH, USA) was used to analyze receptor density within each region. Adjacent sections incubated in the different conditions tested or counterstained for anatomical purpose were stacked in Image J so that binding density could be measured in superposed areas. Mean density was calculated from the densities taken from the left and right side of each nucleus at the level of its maximal extension. The optical densities of the tritium standards that had been apposed to film along with the sections were converted into approximate fmol/mg protein of bound [^{3}H]RX821002 as described by (Casto and Ball, 1996). Specific binding was determined by subtracting binding in the presence of the α_2-antagonist phentolamine from binding observed in the absence of phentolamine. Brain structures were identified based on the atlas of the quail or chicken brain (Baylé et al., 1974; Kuenzel and Masson, 1988) and the atlases of the rat brain (Paxinos and Watson, 1996; Swanson, 2003). The photomicrographs shown in figures 9 and 11 have been adjusted for brightness and contrast.

Data analysis

Competition binding curves and subsequent calculations (IC_{50} and K_i) were analyzed using nonlinear regression analysis (Prism, Version 4.0a; GraphPad software, San Diego, CA, USA). Data were fit to a model assuming binding to one site and a Hill coefficient ≤ 1. The equation of this model is: Y=Ymax/(1+10^(X-LogIC_{50})) in which Y is the percentage of [^{3}H]RX821002 bound and X the concentration of competitor. IC_{50} values were converted to K_i value using the Cheng and Prusoff equation: $K_i = IC_{50}/(1+[L/K_D])$, where L is the concentration of the radioligand (5nM) and K_D is the equilibrium dissociation constant of the radioligand. Different values of K_D were used for the different species based on the K_D available in the literature: 0.83 for quail and zebra finch (Riters and Ball, 2002), 0.5 for rats (Happe et al., 2004) and 0.91 for humans (Halme et al., 1995).

Data from competition experiments were analyzed by two- or three-way ANOVA with the species, the sex and the incubation conditions as independent factors. When main effects were detected, analyses were followed by Fisher's PLSD post-hoc tests. All data are shown as means ± S.E.M. Effects were considered significant for $p < 0.05$. Analyses were carried out with the Macintosh version of Super Anova, version 1.11 (Abacus Concepts, Inc., Berkeley, CA) or with SPSS 13.0 for Mac OS X (SPSS Inc., Chicago, IL).

Results

Pharmacology of [^{3}H]RX821002 at quail, zebra finch and rat α_2-adrenergic receptors

Initial competition binding experiments performed to ensure and compare the specificity of [^{3}H]RX821002 for α_2-adrenergic sites in the three species studied revealed no sex or species differences ($p>0.2015$ and $p>0.1952$) as well as no interaction between the three factors (species × sex × concentration; $p>0.1761$) for either of these unlabeled ligands. Concentration effects were observed with adrenergic ligands and, in some cases, with dopaminergic ligands (See Fig. 1 for Post-hoc comparisons). The non-selective α-adrenergic antagonist, phentolamine ($F_{3,27} = 70.2524$, $p = 0.0001$, Fig. 1A), and the non-selective α-adrenergic agonist, epinephrine ($F_{2,27} = 66.4059$, $p = 0.0001$, Fig. 1B), shifted the binding of [^{3}H]RX821002 in a concentration-dependent fashion as indicated by the post-hoc analyses. Similarly, two α_2-adrenergic antagonists, RX821002 and yohimbine, also inhibited [^{3}H]RX821002 binding in a concentration-dependent manner ($F_{3,24} = 57.8987$, $p = 0.0001$, Fig. 1D, and $F_{3,24} = 79.4047$, $p = 0.0001$, Fig. 1E). In addition, micromolar concentrations of the α_1-adrenergic antagonist, prazosin, significantly di splaced, but by 50% only, the binding of [^{3}H]RX821002 ($F_{2,19} = 24.4437$, $p = 0.0001$, Fig. 1C). The incubation with high concentrations of some dopaminergic drugs also resulted in a significant reduction of the binding of the labeled α_2-adrenergic ligand. Indeed, the non-selective dopaminergic agonist, apomorphine, as well as SCH-23390, a D1 antagonist, almost completely inhibited [^{3}H]RX821002 binding at a concentration of 10μM, while no such displacement was seen at lower concentrations ($F_{2,18} = 56.0183$, $p = 0.0001$, Fig. 1F, and $F_{2,13} = 38.7855$, $p= 0.0001$, Fig. 1G, respectively). By contrast, the co-incubation with the unlabeled D2 antagonist, sulpiride, did not yield any significant binding reduction ($F_{2,13} = 2.7925$, $p = 0.0980$, Fig. 1H).

It seems thus possible that dopaminergic drugs interfere with [^{3}H]RX821002 binding. To test this possibility further, we examined the ability of a second set of adrenergic and dopaminergic ligands to displace [^{3}H]RX821002 binding. In a first step, all the drugs were tested in male and female quail in order to evaluate the existence of a potential sex difference (Fig. 2). While concentration effects were observed in a certain number of cases, no sex difference ($p>0.2468$) and no interaction between the sex and the concentration of the ligand ($p>0.4998$) was identified for either compounds. As illustrated in Fig. 2A–C, the

three α_2-adrenergic ligands tested (idazoxan, an α_2-adrenergic antagonist with some affinity for imidazoline receptors; oxymetazoline, a partial α_{2a}-adrenergic agonist interacting with some affinity for 5-HT receptors and clonidine, an α_2-adrenergic agonist with some affinity for imidazoline sites) significantly inhibited [^{3}H]RX821002 binding ($F_{3,10}$ = 36.1124, p = 0.0001; $F_{3,10}$ = 44.5668, p = 0.0001 and $F_{4,10}$ = 44.8859, p = 0.0001, respectively). The post-hoc analysis reveals that nanomolar concentrations of oxymetazoline and clonidine displaced the binding of the adrenergic ligand, while higher concentrations (10 μM) of idazoxan are required to compete with this binding. The β-adrenoceptor antagonist, propranolol, as well as serotonin (5-HT) did not modify [^{3}H]RX821002 binding ($F_{2,6}$ = 1.7365, p = 0.2541, Fig. 2D; $F_{2,6}$ = 1.6256, p = 0.2728, Fig. 2E). Interestingly, most dopaminergic drugs induced a significant binding displacement (SKF-38393, a D1-receptor antagonist [$F_{3,10}$ = 10.5356, p = 0.0019]; Haloperidol, a D2-receptor antagonist [$F_{3,12}$ = 8.7537, p = 0.0038]; Spiperone, a D2 antagonist with a higher affinity for D4 and some affinity for α_{1B} receptor and 5-HT_{2A} and 5-HT_1 receptors [$F_{3,10}$ = 7.3378, p = 0.0069]; quinpirole, a D2 agonist with some selectivity for D3 sites [$F_{2,6}$ = 11.5199, p = 0.0088]; L 745,870, a selective D4 receptor antagonist [$F_{4,12}$ = 16.1064, p = 0.0001]; clozapine, a selective antagonist for D4 receptors with antagonist properties at 5-HT_{2A}, 5-HT_{2C}, 5-HT_6 and 5-HT_7 receptors [$F_{4,12}$ = 16.6329, p = 0.0001]). The only exception is the non-selective D2 receptor antagonist, raclopride, which did not compete at all with the binding of [^{3}H]RX821002 ($F_{2,6}$ = 1.2051, p = 0.3631, Fig. 2J). As indicated by post-hoc analyses, these effects appear to result essentially from an effect of the highest concentration. Only the D4 selective drugs, clozapine and L 745,870, interfere with [^{3}H]RX821002 binding at all concentrations with exception the lowest.

The same set of drugs was also tested on zebra finch and rat tissue and compared with quail results. Given that no sex difference and no interaction between the sex and the concentrations tested was detected previously in quail, only male tissue was used in these experiments. As illustrated in figure 3, a two-way ANOVA with concentrations and species as independent factors revealed no significant species effect (p > 0.0001) and no interaction for most drugs (p>0.3534). A significant species effect was however detected for clozapine and haloperidol ($F_{2,15}$= 7.5652, p = 0.0053, Fig. 3K, and $F_{2,9}$= 4.5256, p = 0.0322, Fig. 3G, respectively). The post-hoc analyses indicate that these effects result from a higher effect of these ligands in the two avian species as compared to the rat. As observed previously, idazoxan, oxymetazoline and clonidine reduced the binding of [^{3}H]RX821002 in a concentration dependent fashion (p = 0.0001), while propranolol and 5-HT did not affect its binding ($F_{3;9}$ = 3.6926, p = 0.0675, Fig. 3D; $F_{3;8}$ = 3.6054, p = 0.0708, Fig. 3F). Again, the highest concentrations of all dopaminergic ligands (p<0.0053), with the exception of raclopride ($F_{2;9}$ = 2.0268, p = 0.1876), significantly interfered with RX821002 binding.

Pharmacology of [^{3}H]RX821002 at human α_{2A}-adrenergic receptors

The binding competition experiments described here above show that high concentrations of most dopaminergic drugs tested interact with the binding site of RX821002. Two hypotheses may explain a displacement of the adrenergic ligand by dopaminergic drugs: either these drugs bind to α_2-adrenergic receptors and thus interfere with RX821002 binding at this site or RX821002 interacts with dopaminergic sites. To answer this question, binding competitions were performed with these drugs on membranes of S*f*9 cells (insect cells devoid of endogenous G protein-coupled receptors (GPCR) commonly found in vertebrates cells; (Knight et al., 2003) expressing the human α_{2a}-receptor subtype. The results illustrated in figure 4 show that most dopaminergic drugs tested significantly inhibited the binding of [^{3}H]RX821002 (Apomorphine, $F_{6,7}$ = 54.8775, p = 0.0001; SCH-23390, $F_{6,7}$ = 27.5063, p = 0.0002; clozapine, $F_{6,7}$ = 62.9375, p = 0.0001; SKF-38393, $F_{3,5}$ = 21.2729, p = 0.0028; L 745,870, $F_{3,5}$ = 48.8819, p = 0.0004; Haloperidol, $F_{3,5}$ = 18.5177, p = 0.0039). While

apomorphine, SCH-23390 and clozapine showed a clear concentration-dependent action (Fig. 4A–C), the highest concentration of SKF-38393, L 745,870 and haloperidol (Fig. 4D–F) had to be used to reduce the binding of the adrenergic ligand by 30% only. In addition, quinpirole did not produce any significant effect ($F_{3,7} = 0.2863$, $p = 0.8341$, Fig. 4G). These data thus indicate that apomorphine, SCH-23390 and clozapine display a substantial affinity for α_{2a}-adrenergic receptors, while other drugs only weakly interact with this receptor subtype.

Affinities of norepinephrine and dopamine at quail, zebra finch and rat α_2-adrenergic receptors

In order to compare affinities of these agonists for α_2-adrenergic receptors in the three species studied, we generated inhibition curves of [^{3}H]RX821002 binding by norepinephrine and dopamine in brain tissue from male and female quail, zebra finch and rat. These curves were created based on the results of two to six experiments (see Table 1, for details) and analyzed by non-linear regression analysis. The results show that co-incubation of [^{3}H]RX821002 with increasing concentrations of norepinephrine (NE) and dopamine (DA) concentration-dependently displaced the binding of the radioligand (Fig. 5). The affinity (K_i) of norepinephrine and dopamine for α_2-adrenergic receptors was calculated from IC_{50} values obtained from the inhibition curves of individual experiments using the Cheng-Prusoff equation (Table 1). Norepinephrine has an affinity 10 to 28-fold higher than dopamine for α_2-adrenergic sites. The comparison of these K_i values between species and sexes (3-way ANOVA) reveals a significant difference between the incubation with norepinephrine or dopamine ($F_{1,30} = 18.3675$, $p = 0.0002$), a significant species difference ($F_{2,30} = 5.4928$, $p = 0.0093$) as well as an interaction between the species and the incubation conditions ($F_{2,30} = 4.2338$, $p = 0.0240$), but no sex difference ($F_{1,30} = 0.0497$, $p = 0.8250$). The post-hoc analyses (Fig. 6) indicate that these results are mainly explained by a significantly higher K_i of dopamine in zebra finch than all other K_i ($p<0.0017$) and by a difference between the K_i of dopamine in quail and the K_i of norepinephrine in rat ($p=0.0375$). Together these data demonstrate that in this preparation dopamine does interact with the binding of the α_2-adrenergic radioligand and that a species difference may exist in the affinities of dopamine and norepinephrine for α_2-adrenergic receptors.

Affinities of norepinephrine and dopamine at human α_{2A}-adrenergic subtype

The inhibition curves of [^{3}H]RX821002 binding by norepinephrine and dopamine were also generated to compare affinities of the endogenous transmitters for α_{2A}-receptors subtypes expressed in membranes of S*f*9 cells. Inhibition curves were generated in three experiments and all data were analyzed by non-linear regression analysis. [^{3}H]RX821002 binding was concentration-dependently inhibited by increasing concentrations of both norepinephrine and dopamine (Fig. 7). The inhibition curves are very close to each other. Although norepinephrine has a higher affinity than dopamine for the adrenergic subtype ($F_{1,66} = 59.36$, $p<0.0001$), this difference in affinity is only 3 folds (Table 1). Therefore, dopamine also binds the human α_{2A}-subtype.

[^{3}H]RX821002 autoradiography – Binding distribution

Figures 8 and 9 illustrate the distribution of [^{3}H]RX821002 binding in the brain of male quail. The densest binding (>20 fmol/mg protein) was detected in the preoptic nucleus, the supraoptic nucleus and the posterior part of the medial bed nucleus of the stria terminalis whose boundaries are outlined by the binding density. Intermediate density (between 10 and 20 fmol/mg tissue) was observed in the anterior part of the medial bed nucleus of the stria terminalis, the lateral and medial septum, the lateral preoptic area, the paraventricular nucleus of the hypothalamus, the anterior part of the lateral hypothalamus, the intercollicular nucleus, the ventral pallidum and the ventral tegmental area. Finally, weak binding (<10

fmol/mg protein) was identified in the presumed nucleus accumbens (based on (Balint and Csillag, 2007), the medial and lateral striatum, the globus pallidus, the ventromedial nucleus of the hypothalamus, the infundibulum, the substantia nigra and the periacqueductal gray (Central gray, GCt).

As illustrated in figures 10 and 11, [^{3}H]RX821002 binding shows the typical distribution of α_2-adrenergic receptors in rat. The densest binding was detected in the intermediate amygdala anterior. High binding density (from 20 to 35 fmol/mg tissue) was also observed in the lateral and medial septum, the medial preoptic area (medial preoptic nucleus excluded), the anterior bed nucleus of stria terminalis (medial and lateral parts), the posterior part of the medial bed nucleus of stra terminalis, the nucleus accumbens shell, the basolateral and basomedial amygdala (medial and lateral), the anterior part of the medial amygdala, the central nucleus of the amygdala, the cortical amygdala, the intermediate amygdala posterior, the stria terminalis and the striatal fundus. Intermediate binding (from 10 to 20 fmol/mg tissue) was observed in the nucleus accumbens core, the nucleus arcuate of the hypothalamus, the vendromedial nucleus of the hypothalamus, the medial preoptic nucleus, the supraoptic nucleus, the paraventricular nucleus of the hypothalamus, the anterior nucleus of the hypothalamus, the posterior part of the medial bed nucleus of the stria terminalis, the lateral amygdala and the posterior part of the medial amygdala. Finally, a weak binding density (<10 fmol/mg tissue) was measured in the caudate putamen and the globus pallidus.

[^{3}H]RX821002 autoradiography – Anatomical localization of the interaction of dopamine with α_2-adrenergic receptors in quail and rats

The previous results indicate that dopamine displaces the binding of RX821002 to α_2-adrenergic receptors with an affinity slightly lower than that of norepinephrine. These assays have been performed in brain homogenates, no anatomical data were thus available. To determine whether this cross-talk occurs in an intact brain and whether it is widespread or confined to specific regions, binding competitions were conducted on the brain slices collected from male and female quail and rats. Aiming at a binding reduction of about 50% of the total binding, we chose concentrations of cold norepinephrine and dopamine close to the IC_{50} values (see table 1). In each species, the density of binding measured in the absence of competitor (total) or in the presence of norepinephrine or dopamine was compared between males and females using a mixed ANOVA design with the sex as the independent factor and the incubation condition (absence of competitor, presence of norepinephrine or dopamine) as the repeated measure. No significant effect of the sex was observed (Rat: $F<3.594$, $p>0.0905$; Quail: $F < 4.266$, $p>0.0689$) with the exception of the rostral BSTM that showed a significant sex difference in quail ($F_{1,11} = 5.239$, $p = 0.0429$). No significant interaction between the two factors was found (Rat: $F<2.529$, $p>0.1077$; Quail: $F<2.702$, $p>0.097$). However, significant effects of the incubation condition were detected in all nuclei studied in both species (Rat: $F > 17.675$, $p<0.0001$; Quail: $F>8.233$, $p<0.0043$). Even though the main effect seems identical between quail and rat, the pattern of the effect is somewhat different. As illustrated in figure 10, co-incubation of the radioligand with either norepinephrine or dopamine yielded a comparable displacement of [^{3}H]RX821002 binding in rat. The difference between norepinephrine and dopamine induced displacements ranging between 0 (medial septum, posterior part of the paraventricular nucleus of the hypothalamus and the nucleus anterior of the hypothalamus) and 10% only (nucleus accumbens core and stria terminalis anterior). This observation is corroborated by the post-hoc analyses. Both norepinephrine and dopamine significantly inhibited [^{3}H]RX821002 binding. Although the difference between norepinephrine and dopamine effects is relatively small, it is significant in 21 out of the 36 examined regions (See Figure 10 for details). In addition, this binding was displaced by more than 50% (from 60 to 85%), that is norepinephrine and dopamine

effects exceeded the 50% binding reduction that was aimed at. In quail, dopamine yielded a substantial reduction of the binding (from 16 to 49%) in most regions, but norepinephrine was more potent with a binding reduction of 60 to 84% (Fig. 8). As a consequence the difference between norepinephrine and dopamine effects is higher and range between 17 (Lateral striatum) and 67% (paraventricular nucleus of the hypothalamus; PVN) with an average of 50.08 ± 2.26%. The brain regions showing the smaller difference between the effect of dopamine and the effect of norepinephrine all belong to the dopaminergic system: presumptive nucleus accumbens (47%), medial striatum (46%), lateral striatum (17%), globus pallidus (22%), ventral tegmental area (41%), substantia nigra (42%), central gray anterior (41%) and posterior (48%). These observations are reflected in the post-hoc analyses (see figures for details). Norepinephrine significantly decreased [^{3}H]RX821002 binding relative to total binding in all regions studied. Dopamine also significantly decreased [^{3}H]RX821002 binding in all regions with the exception of the rostral part of the preoptic nucleus, the paraventricular nucleus of the hypothalamus and the substantia nigra. But, given the relatively large difference between norepinephrine and dopamine effects, it is not surprising to find these treatments significantly different in almost all regions (22 out of 25; Fig. 8). Together, the results indicate that dopamine does interact with α_2-adrenergic receptors in both species. This interaction does not seem anatomically specific in rat. In quail, although statistical significance was not reached in all areas examined, incubation of the radioligand with dopamine resulted in a substantial reduction of the binding. The pattern difference observed between both rat and quail suggests that there may be species differences in dopamine affinity for α_2-adrenergic receptors relative to norepinephrine.

Discussion

Several lines of evidence have converged over the past few years supporting the existence of a direct interaction of dopamine with α_2-adrenergic receptors. Yet, such interactions have rarely been studied in unmodified cells and/or in an intact system. The present results provide further support for the existence of such a direct interaction *in vivo* in different vertebrate species.

Specificity of [^{3}H]RX821002 binding

All results obtained on quail and rat brain homogenates are similar to what has been described previously for zebra finches (Cornil et al., 2008) and support the specificity of the present binding for α_2-adrenergic receptors. As in the previous study, it is difficult to establish definitively whether the displacement of RX821002 binding by most dopaminergic drugs results from the binding of these ligands to adrenergic receptors or from the binding of RX821002 to dopaminergic sites. Data supporting both hypothesis have been documented (Johnston and File, 1989; Rey et al., 2001; Scatton et al., 1980); (Gibson and Samini, 1979). However, the competitions performed on Spodoptera frugiperda (Sf9) membranes expressing the human α_{2A}-adrenergic subtype shed light on this issue. Indeed, most ligands display comparable properties when tested in brain homogenates and on Sf9 membranes expressing human α_{2A}-adrenoceptors. The fact that these ligands do not lose their capacity to reduce [^{3}H]RX821002 binding in an environment expressing a high concentration of α_{h2A}-adrenergic receptors and most probably devoid of dopaminergic receptors (Knight et al., 2003) indicates that these drugs probably bind the α_{h2A}-adrenergic receptors. Based on these observations, we think that this conclusion can be extended to those made on quail and rat material. Therefore, the fact that RX821002 binding is displaced by high concentrations of most dopaminergic drugs tested in the competition assays using zebra finch, quail and rat tissue should be attributed to their binding to α_2-adrenergic receptors.

Distribution of the binding

In quail, dense [^{3}H]RX821002 binding defines the boundaries of the medial preoptic nucleus (POM), supraoptic nucleus (SO) and in the posterior part of the medial bed nucleus of the stria terminalis (BSTMp). A relatively high density of binding was also observed in the anterior part of the medial bed nucleus of the stria terminalis (BSTMa), the lateral septum (LS), the nucleus paraventricular of the hypothalamus (PVN), the lateral preoptic area (POA) and the lateral hypothalamus (LHy). These observations are consistent with the distribution of α_2-AR in the avian brain reported previously (Ball et al., 1989; Diez-Alarcia et al., 2006; Fernandez-Lopez et al., 1997). The binding observed in the ventromedial nucleus of the hypothalamus (VMN) is lower than reported before in quail (Ball et al., 1989). Actually the VMN stands out because of its low density contrasting with the higher binding density of the LHy (see Fig 9G). This could be due to the radioligand used as supported by the fact that a study using [^{3}H]RX821002 found a similar pattern in chicken (Fernandez-Lopez et al., 1997). In general, the distribution of this binding is typical of the α_2-AR and fits with the distribution of noradrenergic terminals identified by NE- or DBH-immunoreactive fibers (Bailhache and Balthazart, 1993; Moons et al., 1995).

In rats, high binding levels are observed in the amygdaloid complex, with the higher densities detected in the anterior parts of the intermediate (IAa), central (CeAa) and medial nuclei (MeAa), the bed nucleus of the stria terminalis and the lateral septum whose boundaries are outlined by this high binding density. Relatively dense binding is also detected in the medial preoptic area with the preoptic nucleus standing out with a lower binding density than the surrounding structures. In the hypothalamus, the ventromedial nucleus (VMH) is also well defined based on the pattern of binding as compared to its surrounding. Overall, although there are some minor variations as compared to previous studies, the binding distribution is consistent with previous reports of α_2-adrenergic receptors in rats (Happe et al., 2004; Unnerstall et al., 1984). As extensively documented by Smeets and Gonzalez (2000), this distribution matches the distribution of noradrenergic terminals described in rodents.

Both norepinephrine and dopamine displace [^{3}H]RX821002 binding in brain slices

The results obtained from the assays conducted on homogenates show that DA binds α_2-AR with an affinity 10 to 20 fold lower than NE in quail and rats. The results obtained from competitions run on intact brain sections do show that both NE and DA significantly displace the binding of the α_2-adrenergic ligand. Certain aspects of the data also suggest the existence of a complex concentration-response relationship: co-incubation with the chosen concentration of NE yielded a binding displacement exceeding the expectation in both species, while the addition of DA produced similar binding as NE in rats but not in quail where it never reached the 50% binding inhibition. These observations probably result from a combination of different factors such as a slight deviation of the effective concentration between the homogenate and the brain slice condition, differential ligand accessibility or active re-uptake, etc (For further discussion of these aspects, see Cornil et al., 2008). In addition, it has been suggested that the two catecholamines have different affinities for the different receptor sub-types (Bunemann et al., 2001; Zhang et al., 1999). Since the same effect is observed in all regions studied, it is unlikely that a differential binding to separate subtypes is sufficient to explain the pattern of response observed here. Moreover, the different receptor subtypes display different cellular localizations, α_{2A}-AR and α_{2B}-AR subtypes are membrane bound receptors while α_{2C}-AR is primarily found intracellularly (Daunt et al., 1997; Rosin et al., 1996; Saunders and Limbird, 1999). This feature could explain differences in affinity measured between the homogenate (where the radioligand has access to all three subtypes) and the slice preparation (where the radioligand binds predominantly membrane bound receptors: α_{2A}-AR and α_{2B}-AR). In conclusion, not

surprisingly, a variety of factors may account for these displacement discrepancies between homogenates and slices. In any case, even if the actual IC_{50} differs slightly from our predictions, the present results provide strong evidence that dopamine binds α_2-adrenergic receptors and that this interaction does not show an anatomical specialization.

Where does dopamine interact with α_2-adrenergic receptors?

In quail, DA interferes in a statistically significant manner with RX821002 binding in all regions investigated with the exception of the anterior part of the medial preoptic nucleus, the paraventricular nucleus of the hypothalamus and the substantia nigra. Yet, the co-incubation with DA resulted in a substantial reduction of binding in these three regions (17, 18 and 26%, respectively). The lack of significance observed in these regions is probably explained by the various factors discussed in a previous paragraph. In addition, results collected from the rat sections strongly suggest that dopamine binds α_2-AR everywhere where these receptors occur in the brain.

In order for this interaction to have functional significance, DA should be released in areas containing α_2-AR. So an important question to ask is whether dopaminergic terminals co-exist with α_2-AR. In rodents, specific brain areas expressing α_2-AR receive dopaminergic inputs in conjunction or not with noradrenergic inputs e.g., the nucleus accumbens (NAc) shell, the bed nucleus of stria terminalis (BST), the lateral septum (LS), the amygdaloid nuclei and the preoptic/hypothalamic nuclei, locus coeruleus (Berridge et al., 1997; Bjorklund et al., 1975; Fallon et al., 1978; Glowinski et al., 1984; Kozicz, 2001; Moore, 1978; Phelix et al., 1992; Risold and Swanson, 1997; Sawchenko and Swanson, 1983; Smeets and Gonzalez, 2000). In many of these regions, DA release occurs in response to stimuli as various as stress, conditioned cues associated with positive (arousing or rewarding) or negative (stressful) outcomes, amphetamine administration or sexual interaction (Coco et al., 1992; Feenstra et al., 2001; Harmer and Phillips, 1999; Hull et al., 1995; Inglis and Moghaddam, 1999; McKittrick and Abercrombie, 2007; Mingote et al., 2004; Yokoyama et al., 2005). These are thus potential areas where interaction between dopamine and α_2-AR may occur. The present data confirm that the α_2-AR expressed in these regions show a substantial affinity for dopamine. Also, because DA is part of the synthesis pathway of NE, one would expect that noradrenergic terminals would always express DA (however exceptions to this rule have been reported, see Smeets and Gonzalez, 2000 for discussion). Interestingly, Devoto and colleagues demonstrated that electrical stimulation of the locus coeruleus elicits release of both NE and DA in several cortical areas suggesting that DA may be released from noradrenergic terminals. If such co-release of DA with NE proved to be a common feature of all noradrenergic terminals, DA could virtually be released in any brain region receiving noradrenergic inputs thus enabling its interaction with adrenergic receptors (Devoto et al., 2003; Devoto et al., 2005; Feenstra et al., 2001; Mingote et al., 2004). There are no data available on the co-release of DA and NE in birds. However, the distribution of TH, DBH and α_2-AR as well as the connectivity of most catecholaminergic nuclei is quite similar between the two taxa (Smeets and Gonzalez, 2000; Smeets and Reiner, 1994). The present data also indicates that, as in rats, such an interaction of dopamine to α_2-AR can occur wherever these receptors are present. Therefore, it seems that, at least in some regions, DA can cross-talk with α_2-AR in birds. Such a conclusion is supported by the functional data that will be discussed in the penultimate paragraph of this discussion. Overall, we thus believe that the probability of cross-talk is similar in the two taxa.

To date, only a few studies have demonstrated an interaction of DA with adrenergic receptors in the central nervous system. Zhang and colleagues identified such an interaction with α_{2C}-AR in mouse striatum, a region that receive only sparse noradrenergic inputs but surprisingly contains a relatively high quantity of these receptors (Zhang et al., 1999). More

recently, an interaction of dopamine with α_1-adrenergic receptors has also been reported in the lateral amygdala (Yamamoto et al., 2007). The latter piece of data is particularly interesting given that although this region receives a dopaminergic input involved in fear conditioning it displays very low densities of dopaminergic receptors but high densities of adrenergic receptors (Charuchinda et al., 1987; Dawson et al., 1986; Rosin et al., 1996; Talley et al., 1996; Unnerstall et al., 1984; Wamsley et al., 1989). The present results bring further evidence that such phenomena not only takes place at these sites, but could potentially occur at any adrenergic site provided that dopamine is released. Altogether there are thus numerous regions that express α_2-AR and where DA release has been documented. Of course, the fact that adrenergic receptors possess the capacity to bind DA does not mean that this property is exploited everywhere the receptors is present. In the present study, we assessed the presence of this interaction in brain regions displaying relatively high binding densities of [^{3}H]RX821002 binding in order to take advantage of the optimal density window to quantitatively assess this binding. But the fact that this interaction was observed in virtually all areas studied indicates that this could be a common feature to all α_2-AR independent of whether or not they receive DA inputs.

Physiological significance of a possible cross-talk between dopamine and a_2-adrenergic receptors

Dopamine interacts with α_2-adrenergic receptors. Other molecules, called trace amines (namely octopamine, L-DOPA and tyramine), also bind these receptors (Airriess et al., 1997; Zhang et al., 2004). It is well-known that epinephrine which directly derives from norepinephrine strongly binds them as well (Hieble and Ruffolo, 1996; Peltonen et al., 2003). Does this mean that α_2-adrenergic receptors perhaps constitute some sort of non-specific receptors for catecholamines? We argue strongly that this is not the case. The classification of catecholaminergic receptors is based on pharmacological and morphological features. Pharmacological criteria are not absolute, they rely on the differential potency of various ligands that bind and activate a given receptor (Bylund et al., 1994; Hieble and Ruffolo, 1996). The notion that DA binds α_2-adrenergic receptors with a lower affinity than NE does not change fact that these receptors are adrenergic and are examples of the α_2-subtype as defined by their molecular structures and pharmacological properties. What is interesting is that the affinity of DA for these receptors is actually quite close to that of NE, as close as 3 fold if specific subtypes are considered (present study, (Nyronen et al., 2001; Peltonen et al., 2003) suggesting that it could be functionally relevant.

As a matter of fact, the activation of adrenergic receptors by dopamine can trigger intracellular cascades involving changes in calcium and/or cAMP concentrations or influence neuronal excitability (Cornil et al., 2002; Malenka and Nicoll, 1986; Zhang et al., 1999; Zhang et al., 2004). However, the notion that dopamine modulates functional responses with the same affinity as norepinephrine has been questioned (Nyronen et al., 2001). It has also been suggested that DA might activate different intracellular cascades than NE or that the system in which the receptor is expressed influences the response measured (Zhang et al., 2004). Together, these observations indicate that dopamine when it binds to adrenergic receptors can activate them, but many questions remain regarding the efficient concentration, the mechanism of activation as well as the type of cascade triggered.

Data collected in quail provide evidence supporting a functional implication of an action of dopamine mediated through α_2-AR. Indeed, DA inhibits the firing rate of neurons recorded from the posterior part of the medial preoptic nucleus (POM) through α_2-AR at concentrations 10-fold higher than NE (Cornil et al., 2002) as observed in the present study. The preoptic area (POA) is a critical site for the control of male sexual behavior and studies conducted in rats and quail have identified dopamine as a major player in this control (Absil et al., 1994; Balthazart et al., 1997; Castagna et al., 1997; Dominguez and Hull, 2005;

Panzica et al., 1996). However, there seem to be considerably more adrenergic receptors in this region than dopaminergic receptors (Charuchinda et al., 1987; Dawson et al., 1986; Rosin et al., 1996; Talley et al., 1996; Unnerstall et al., 1984; Wamsley et al., 1989). A role for NE in the control of this behavior has also been proposed but its nature remains unclear (For review see (Hull et al., 2002) and to our knowledge there has been no report of a release of NE during sexual activity. The present results thus indicate that it is possible that the interaction of DA with α_2-AR in the POA participates in the control of sexual behavior. Given the high concentrations required for DA to bind α_2-AR, its action would probably occur when its concentration culminates at ejaculation. Another *in vivo* manipulation implicated such a dopaminergic activation of adrenergic receptors in the regulation of sleep (Crochet and Sakai, 2003). These observations thus indicate that a direct interaction of dopamine with adrenergic receptors may be physiologically significant in living animals. Given the number of regions in which it may take place (see above), this interaction may participate in processes as varied as responses to stress, learning, memory consolidation, drug addiction and sexual behavior.

In conclusion, this interaction between dopamine and α_2-adrenergic receptors may represent an example of the maintenance of an ancestral condition when several catecholamines could bind to a common catecholaminergic receptor. Although selective interactions with specific and pharmacologically distinct receptor subtypes have emerged more recently, these older more general interactions could still be physiologically significant. Together with other studies, the results presented herein indicate that this interaction of dopamine with adrenergic receptors constitute a common feature shared among vertebrate species. Along with the observation that dopaminergic drugs bind to some degree to adrenergic receptors, this conclusion should open new avenues in the design and the interpretation of new studies.

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Abbreviations

ACB	Nucleus accumbens
ACBc	Nucleus accumbens core
ACBs	Nucleus accumbens shell
AHN	Nucleus anterior of the hypothalamus
ARH	Arcuate nucleus of the hypothalamus
BLAa	Anterior Basolateral amygdala
BLAp	Posterior Basolateral amygdala
BMAa	Anterior Basomedial amygdala
BMAp	Posterior Basomedial amygdala
BSTald	laterodorsal portion of the anterior bed nucleus of the stria terminalis

BSTM	Medial bed nucleus of the stria terminalis
BSTMa	Anterior portion of the medial bed nucleus of the stria terminalis
BSTMp	Posterior portion of the medial bed nucleus of the stria terminalis
BSTL	Lateral bed nucleus of the stria terminalis
BSTp	anterior portion of the bed nucleus of the stria terminalis
CA	commissura anterior
CC	Corpus callosum
CO	optic chiasma
CPu	Caudate-putamen
FS	Striatal fundus
FPL	Fasciculus prosencephali lateralis (lateral forebrain bundle)
GCt	Central gray
GCta	Anterior portion of the central gray
GCtp	Posterior portion of the central gray
GP	Globus pallidus
ICo	Intercollicular nucleus
LA	Lateral amygdala
LHy	Lateral hypothalamus
LHy ant	Lateral hypothalamus, anterior part
LHy post	Lateral hypothalamus, posterior part
LPOA	lateral preoptic area
LPOA ant	lateral preoptic area, anterior part
LPOA post	lateral preoptic area, posterior part
LS	lateral septum
L.St	Lateral Striatum
MeAa	anterior portion of the Medial amygdala
MeAp	posterior portion of the Medial amygdala
MS	Medial septum
M.St	Medial Striatum
OM	Tractus occipitomesenchephalicus
POA	preoptic area
POMa	anterior portion of the preoptic medial nucleus
POMp	posterior portion of the preoptic medial nucleus
PVN	Paraventricular nucleus of the hypothalamus
SO	Supraoptic nucleus
STa	Stria terminalis anterior

STp	Stria terminalis posterior
VMN	ventromedial nucleus of the hypothalamus
VP	ventral pallidum

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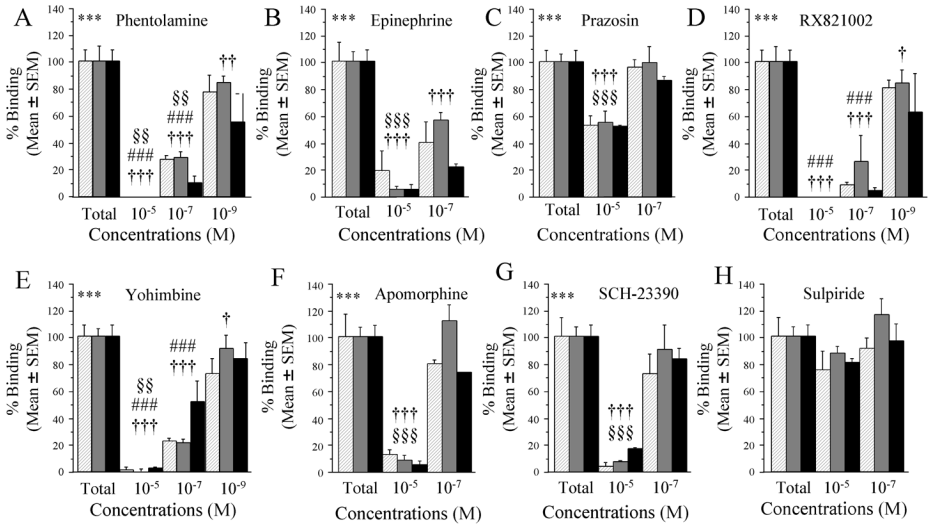

Figure 1. Characterization of α_2 specificity of [^{3}H]RX821002 in quail, zebra finch and rat whole brain homogenate

Competitions with non-selective α-adrenergic ligands (A, B), a α_1-adrenergic ligand (C), α_2-adrenergic ligands (D–E) and with a non-selective dopaminergic ligand (F), a D1 dopaminergic ligand (G) and a D2 dopaminergic ligand (H). Striped, gray and black represent quail, zebra finch and rat tissue, respectively. Each bar represents the results of two to three separate experiments, each performed in 5 replicates of brain homogenates containing 3, 4 or 5 brains depending on the species (see methods). Main effect for concentration (in the left corner of each graph): ***p>0.001. No species difference was found. No sex difference was found so male and female data were pooled. Post-hoc analysis: single, double and triple signs represent respectively p<0.05, 0.01 and 0.001 with † vs. Total; # vs. 10^{-9} M; § vs. 10^{-7} M. The Zebra finch data have been reported previously (Cornil et al., 2008) but are included here in this more comprehensive inter-species comparison.

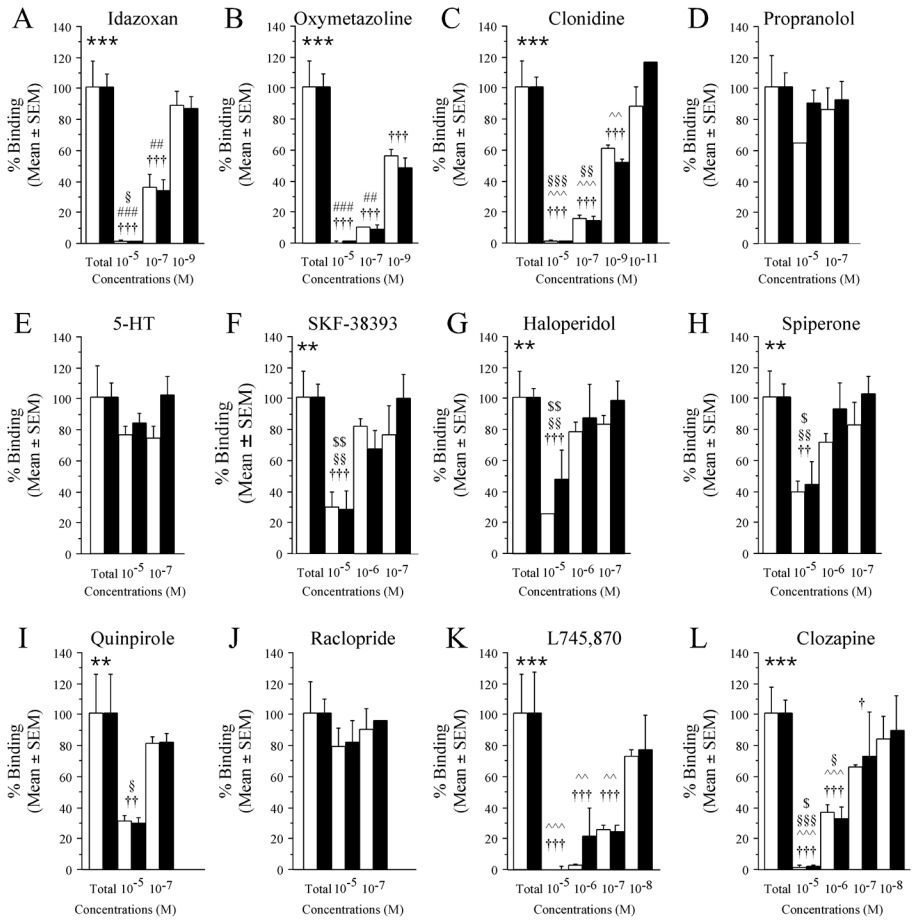

Figure 2. Further characterization of α_2 specificity of [^{3}H]RX821002 in male and female quail whole brain homogenate

A–C. Competitions with α_2-adrenergic ligands; D–E. Competitions with non α-adrenergic ligands; F–L. Competitions with dopaminergic ligands: D1 ligand (F), D2 ligands (G–J) and D4 ligands (K–L). White bars represent male tissue whil black bars represent female tissue. Each bar represents the results of two to three separate experiments, each performed in 5 replicates of brain homogenates containing 4 brains. Main effect for concentration (in the left corner of each graph): *, **, *** p> 0.05, 0.01, 0.001. No sex effect or interaction between sex and concentration was found. Post-hoc analysis: single, double and triple signs represent respectively p<0.05, 0.01 and 0.001 with † vs. Total; # vs. 10^{-9} M; ^ vs. 10^{-8} M ; § vs. 10^{-7} M; $ vs. 10^{-6} M.

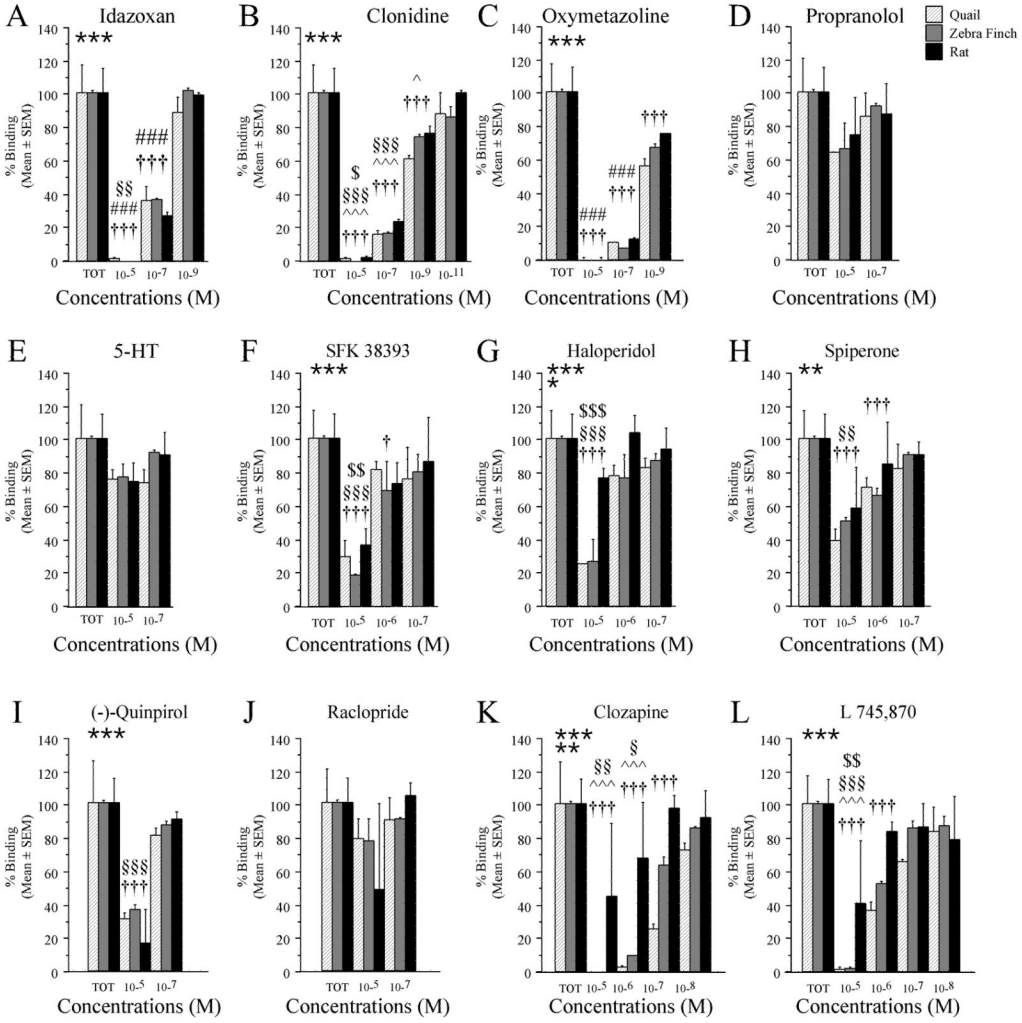

Figure 3. Further characterization of α_2 specificity of [^{3}H]RX21002 in male quail, zebra finch and rat whole brain homogenate

A–C. Competitions with α_2-adrenergic ligands; D–E. Competitions with non α-adrenergic ligands; F–L. Competitions with dopaminergic ligands: D1 ligand (F), D2 ligands (G–J) and D4 ligands (K–L). Each bar represents the results of two to three separate experiments, each performed in 5 replicates of brain homogenates containing 4 or 5 brains depending on the species (see methods). In the left corner of each graph, the results of the two-way ANOVA with the ligand concentrations and the species as independent factors are represented by stars (*, **, *** p> 0.05, 0.01, 0.001): the first line indicates the concentration effect while the second line indicates the species effect. Post-hoc analysis: single, double and triple signs represent respectively p<0.05, 0.01 and 0.001 with † vs. Total; # vs. 10^{-9} M; ^ vs. 10^{-8} M ; § vs. 10^{-7} M; $ vs. 10^{-6} M. The Zebra finch data have been reported previously (Cornil et al., 2008) but are included here in this more comprehensive inter-species comparison.

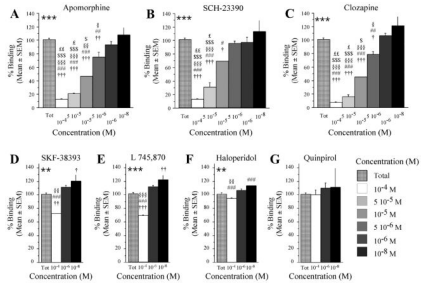

Figure 4. Characterization of α_{2A} specificity of [^{3}H]RX821002 at human α_{2A}-receptor subtype

Each bar represents the results of two to three separate experiments, each performed in 5 replicates. Main effect for concentration (in the left corner of each graph): *, **, *** p> 0.05, 0.01, 0.001. Post-hoc analysis: single, double and triple signs represent respectively p<0.05, 0.01 and 0.001 with † vs. Total; # vs. 10^{-8} M; § vs. 10^{-6} M; $ vs. 5 10^{-6} M; £ vs. 10^{-5} M.

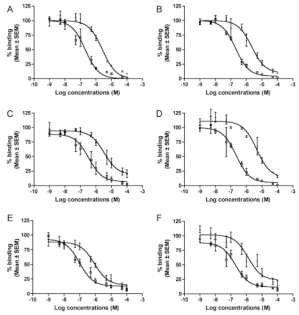

Figure 5. Inhibition of [^{3}H]RX821002 binding to α_2-adrenergic receptors in the three different species studied by increasing concentrations of norepinephrine (□) or dopamine (▲)

quail (A, male; B, female), zebra finch (C, male; D, female) and rat (E, male; F, female). Data points are means ± S.E.M. of two to six experiments, each performed in 5 replicates of brain homogenates containing 4 or 5 brains depending on the species (see methods). The zebra finch data have been reported previously (Cornil et al., 2008) and are presented here to enhance the comparison among species.

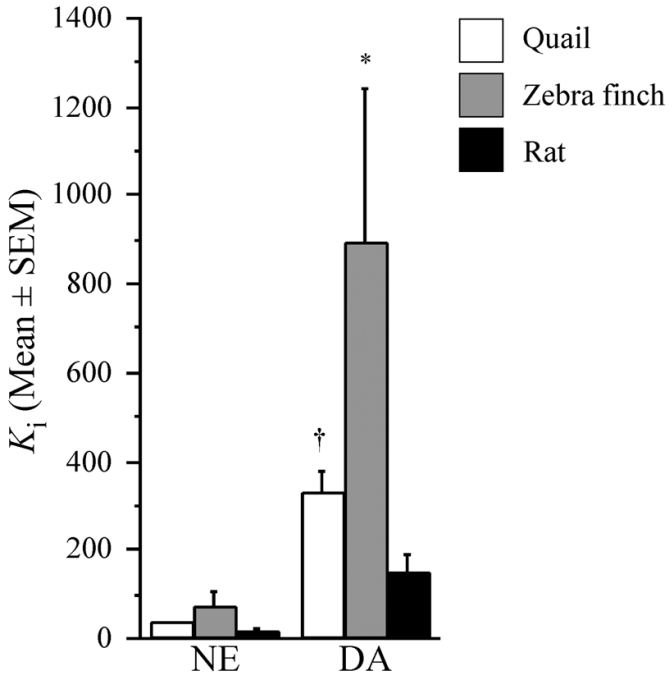

Figure 6. Norepinephrine (NE) and dopamine (DA) affinities for α_2-adrenergic receptors in quail, zebra finch and rat brain tissue sections

Results are the same as these presented in Table 1 whose male and female data have been pooled to illustrate the statistical results. Post-hoc analysis: $p<0.05$ with †vs. Rat-NE and § vs. All-NE and Quail-DA and Rat-DA. The zebra finch data have been reported previously (Cornil et al., 2008) and are presented here to enhance the comparison among species.

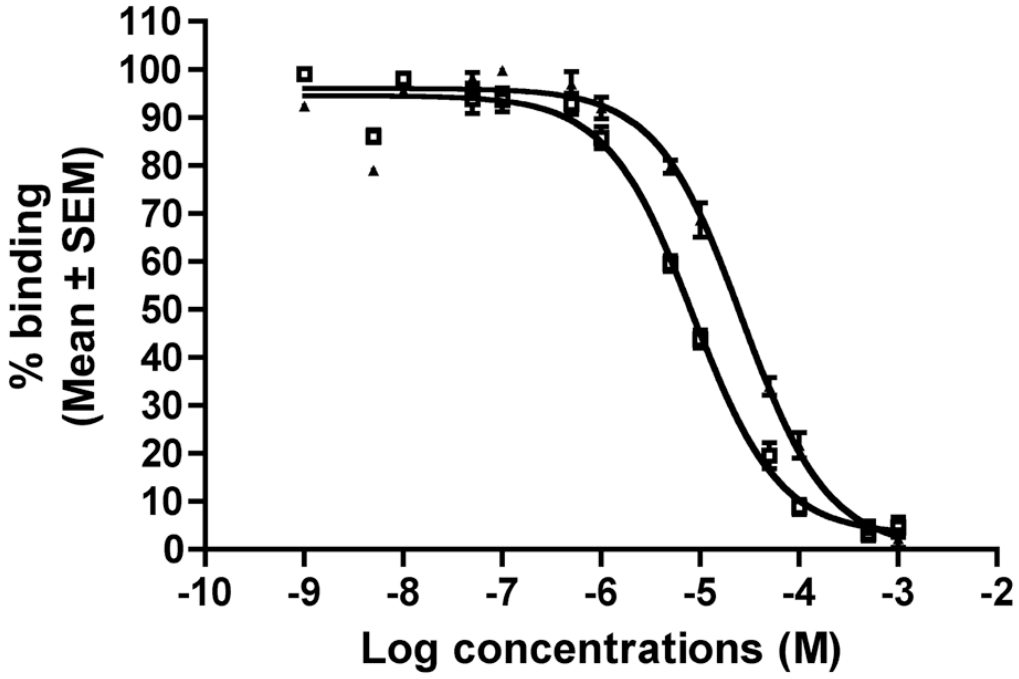

Figure 7. Inhibition of [^{3}H]RX821002 binding at human α_{2A}-receptor subtype by increasing concentrations of norepinephrine (□) or dopamine (▲)

Data points are means ± S.E.M. of three experiments, each performed in triplicates.

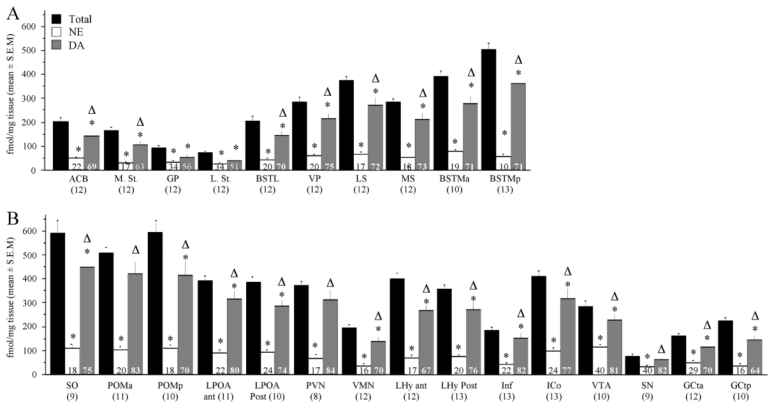

Figure 8. Competitions of norepinephrine (NE) and dopamine (DA) for the binding of [^{3}H]RX821002 on quail brain sections

A. Basal ganglia, septal region and subdivisions of bed nucleus of the stria terminalis. B. Preoptic/hypothalamic area and midbrain dopaminergic regions. No sex difference was detected so the graphs present only the main effect of treatment with males and females pooled. Numbers in the graph bars represent the percentage of the total binding left after co-incubation with NE or DA. Numbers in parenthesis indicate the number of subjects included in the analysis of a given nucleus. * $p < 0.05$ vs. Total; Δ $p < 0.05$ vs. NE.

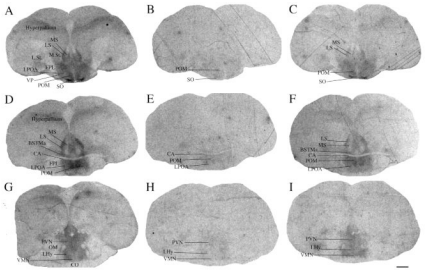

Figure 9. Autoradiograms illustrating norepinephrine and dopamine induced displacement of [^{3}H]RX821002 binding in the quail brain

Adjacents sections have been incubated in the absence of competitor (Total binding; A, D and G) or in the presence of norepinephrine (B, E and H) or dopamine (C, F and I), respectively. The first row shows sections containing the anterior portion of the POM. The second row shows sections containing the posterior portion of the POM. Finally, the third row shows sections containing the anterior portion of the VMN. Scale bar = 1mm.

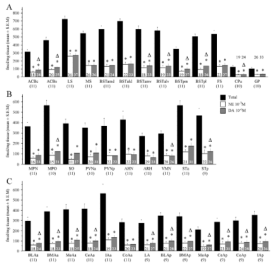

Figure 10. Competitions of norepinephrine (NE) and dopamine (DA) for the binding of [^{3}H]RX821002 on rat brain sections

A. Basal ganglia, septal region and subdivisions of the bed nucleus of the stria terminalis. B. Preoptic/hypothalamic area. C. Amygdaloid nuclei. No sex difference was detected so the graphs present only the main effect of treatment with males and females pooled. Numbers in the graph bars represent the percentage of the total binding left after co-incubation with NE or DA. Numbers in parenthesis indicate the number of subjects included in the analysis of a given nucleus. * $p < 0.05$ vs. Total; Δ $p < 0.05$ vs. NE.

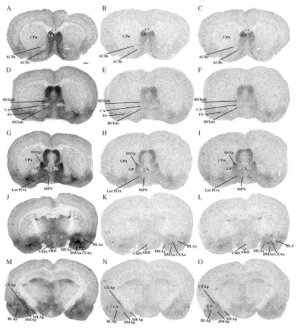

Figure 11. Autoradiograms illustrating norepinephrine and dopamine induced displacement of [^{3}H]RX821002 binding in the rat brain

Adjacents sections have been incubated in the absence of competitor (Total binding; A, D, G, J and M) or in the presence of norepinephrine (B, E, H, K and N) or dopamine (C, F, I, L and O), respectively. The first row shows sections containing the anterior portion of the POM. The successive rows show from top to bottom sections containing the nucleus accumbens (level 14 in Swanson Atlas), BSTa, MPN, VMH and the anterior amygdaloid complex and finally the posterior amygdaloid complex. Scale bar = 1mm.

Table 1

Norepinephrine and dopamine affinities for α_2-adrenergic receptors in the four systems considered

Results are means ± S.E.M. of two to six separate experiments, each performed in 5 replicates. The number under brackets indicates the number of experiments performed for each condition. The zebra finch data have been reported previously (Cornil et al., 2008) and are presented here to enhance the comparison among species.

	K_i (nM)		
QUAIL	**NE**	**DA**	**Ratio DA/NE**
Male (3)	26.37 ± 6.97	297.25 ± 54.86	11.27
Female (3)	28.85 ± 2.73	338.00 ± 106.06	11.71
ZB			
Male (3)	86.07 ± 61.45	874.99 ± 540.68	10.17
Female (2)	31.59 ± 17.52	894.69 ± 599.42	28.32
RAT			
Male (6)	8.65 ± 2.67	100.55 ± 32.65	11.62
Female (4)	12.03 ± 4.45	263.67 ± 101.19	21.92
Membrane			
Human α_{2A}-R (3)	1285.93 ± 93.04	4094.23 ± 583.33	3.18