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Dopamine binds to α_2 -adrenergic receptors in the song control system of zebra finches (*Taeniopygia guttata*)

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Abstract

A commonly held view is that dopamine exerts its effects via binding to D1- and D2-dopaminergic receptors. However, recent data have emerged supporting the existence of a direct interaction of dopamine with adrenergic receptors. Dopamine may also directly bind adrenergic receptors but this interaction has been poorly investigated. In this study, the pharmacological basis of possible *in vivo* interactions between dopamine and α_2 -adrenergic receptors was investigated in zebra finches. A binding competition study showed that dopamine displaces the binding of the selective α_2 -adrenergic ligand, [³H]RX821002, in the brain. The affinity of dopamine for the adrenergic sites does not differ between the sexes and is 10-28-fold lower than that for norepinephrine. To assess the anatomical distribution of this interaction, binding competitions were performed on brain slices incubated in 5nM [³H]RX821002 in the absence of any competitor or in the presence of norepinephrine or dopamine. Both norepinephrine and dopamine displaced the binding of the radioligand though to a different extent in most of the regions studied (e.g., Area X, the lateral part of the magnocellular nucleus of anterior nidopallium, HVC, arcopallium dorsale, ventral tegmental area and substantia grisea centralis) but not in the robust nucleus of the arcopallium. Together these data provide evidence for a direct interaction between dopamine and adrenergic receptors in songbird brains albeit with regional variation.

Keywords

binding competition; autoradiography; songbird; norepinephrine; catecholamine

Introduction

Dopaminergic and noradrenergic systems are usually presented as distinct entities with regard to their anatomy, receptors, transporters and functions. Neuronal cell bodies of the major dopaminergic and adrenergic nuclei are located in distinct areas and some of their target areas are innervated almost exclusively by either dopaminergic or noradrenergic neurons. Different functions are also ascribed to the two systems (Aston-Jones and Cohen, 2005, Berridge and

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Waterhouse, 2003, Bylund, et al., 1994, Callier, et al., 2003, Civelli, et al., 1993, Docherty, 1998, Harrison, et al., 1991b, Hoffman and Lefkowitz, 1996, Jaber, et al., 1996, Kuhar, et al., 2006, Missale, et al., 1998, Philipp, et al., 2002, Ruffolo, et al., 1993, Smeets and Gonzalez, 2000). Despite the anatomical and functional differences attributed to them, these two systems do not always operate distinctly from each other and there is overlap in target regions that receive inputs from both (e.g. prefrontal cortex, bed nucleus of the stria terminalis, the shell of the nucleus accumbens, preoptic/hypothalamic area). In these regions, the most common interaction, that have been reported in mammalian species, is of the sort that involves the action of noradrenergic cells that results in modulations of the firing of dopaminergic neurons and the associated release of dopamine and vice versa (Gresch, et al., 1995, Misu, et al., 1985, Pan, et al., 2004, Ueda, et al., 1983, Xu, et al., 1993). However, evidence is also accumulating that there are interactions between these monoamines and their receptors and transporters. Studies using selective transporter blockers and transporter knock-out mice demonstrated dopamine reuptake by the norepinephrine transporter (Carboni et al., 1990; Carboni et al., 2001; Moron et al., 2002; Valentini et al., 2004) and several studies support the existence of a direct action of dopamine on different subtypes of adrenergic receptors and vice-versa (Aguayo and Grossie, 1994, Cornil, et al., 2002, Lanau, et al., 1997, Newman-Tancredi, et al., 1997, Rey, et al., 2001, Zhang, et al., 1999, Zhang, et al., 2004). Most *in vitro* studies published so far have investigated the existence of an interaction between dopamine and noradrenergic receptors in cell lines transfected with cloned receptors. Only a few of these reports examined the actual binding of dopamine to adrenergic receptors *in vitro* (Nyronen, et al., 2001, Ruuskanen, et al., 2005, Zhang, et al., 1999). Therefore, the occurrence and the distribution of this phenomenon *in vivo* remains unknown.

Catecholamines are known to influence motor responses involved in motivated behaviors including sexual behaviors (Robbins and Everitt, 1996). Singing in songbirds is a good example of a sexually motivated behavior. In zebra finches, an Australian gregarious songbird species, singing is mainly used to attract mates (Zann, 1996). In a reproductive context, male birds must not only coordinate song production with other reproductive behaviors (such coordination is mediated by gonadal steroid hormones), but also use song appropriately in response to the presence and behavior of relevant conspecifics (social context). The social context in which song is produced can thus have profound effects on ongoing physiological activity as measured by electrophysiological recordings or immediate early gene expression in the song control nuclei (Hessler and Doupe, 1999, Jarvis, et al., 1998, Kao and Brainard, 2006, Kao, et al., 2005, Williams and Mehta, 1999). Several pieces of evidence suggest that this latter task is mediated by catecholamines (see below).

Birdsong is controlled by a neural circuit comprising several interconnected nuclei that represents a neural specialization unique to oscine songbirds (Brenowitz, et al., 1997, Kroodsma and Konishi, 1991). This circuit consists of two pathways: an efferent motor pathway required for song production and an anterior forebrain pathway implicated in the auditory feedback necessary for song learning, recognition and maintenance (Fig. 1A; for review, see Ball and Hulse, 1998, Brainard and Doupe, 2002, Brenowitz and Beecher, 2005, Brenowitz, et al., 1997, Konishi, 2004, Nottebohm, et al., 1990). The existence of a well-developed catecholaminergic innervation of the song nuclei is well documented. High concentrations of catecholamines are found in the song nuclei of zebra finches (Barclay and Harding, 1988) and immunocytochemical studies have revealed the presence of synthesizing enzymes such as tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DBH; Appeltants, et al., 2001, Bottjer, 1993, Mello, et al., 1998), and several subtypes of catecholaminergic receptors were detected by receptor autoradiography (Ball, 1994, Casto and Ball, 1996, Revilla, et al., 1999, Ritters and Ball, 2002, Ritters, et al., 2002; for review, see Ball and Balthazart, 2007) in the song nuclei of various species (Fig. 1B). Major sources of catecholaminergic inputs have been identified in the ventral tegmental area (VTA), the

periacqueductal gray (sometimes referred to as substantia grisea centralis gray in birds; GCt) and in the locus coeruleus (Fig. 1B; Appeltants, et al., 2000, Appeltants, et al., 2002, Castelino, et al., 2007, Lewis, et al., 1981). Finally, recent findings support a role of catecholamines in the regulation of neural processes controlling context-dependent singing behaviors (Castelino and Ball, 2005, Hara, et al., 2007, Heimovics and Ritters, 2005, Maney and Ball, 2003). Interestingly, HVC, RA and LMAN express high densities of adrenergic receptors and there is little evidence of high densities of dopaminergic receptors (Ball and Balthazart, 2007) but these nuclei receive substantial dopaminergic inputs (Appeltants, et al., 2000, Appeltants, et al., 2002, Barclay and Harding, 1988; Fig. 1B). Of particular interest, are the studies of Hara *et al.* (2007) and Heimovics *et al.* (2005) that suggest an involvement of dopamine in the regulation of context-dependent changes of gene expression in these nuclei. Although it can be hypothesized that new projections are still to be identified, an alternative would be to consider the existence of an interaction of dopamine with adrenergic receptors.

Therefore, the song system appears as a very useful system to study the existence of an interaction between dopamine and adrenergic receptors. The present study was designed to investigate the neuropharmacological basis for such an interaction in the song system as well as the main catecholaminergic nuclei of the zebra finch using autoradiographic methods. We chose the tritiated α_2 -adrenergic antagonist, [^3H]RX821002, as a marker of α_2 -adrenergic receptors and measured the ability of dopamine and norepinephrine to displace its binding. In the first study, we compared the binding properties of dopamine, norepinephrine and various catecholaminergic agonists and antagonists on homogenous whole brain tissue. Then, we investigated whether this interaction occurs everywhere adrenergic receptors are found or if it is localized to specific nuclei within the songbird brain. We also tested whether α_2 -adrenergic receptors are pre- or postsynaptic using the noradrenergic neurotoxin DSP-4 administered prior to the autoradiography procedure. Overall, this study provides the first demonstration of a direct interaction of dopamine with adrenergic receptors in the brain of a songbird.

Methods

Experimental animals

Sexually mature male (n = 17) and female (n=5) zebra finches (*Taeniopygia guttata*) were used for this study. The animals were purchased from a local breeder (Murad Ishmail breeders) and housed in same sex groups. All animals were provided with finch food and water *ad libitum* and maintained at 14h light : 10 h dark. Prior to treatment all birds were housed in same sex group cage (49 × 95 × 51 cm; four to six birds per cage) in the Ames Hall vivarium at Johns Hopkins University. All experimental protocols were approved by and carried out under the guidelines laid down by the Johns Hopkins University Animal Care and Use Committee.

DSP-4 Treatment

Twelve males were weighed just before the injection and were randomly sorted into two groups. All solutions were prepared fresh just before administration. The animals received two injections of DSP-4 or its vehicle (0.9% saline) one week apart. The animals were pretreated with an i.p. injection of a serotonin reuptake blocker (Zimelidine dihydrochloride; Sigma-Aldrich; 20 mg/kg) followed by an i.p. injection of DSP-4 (*N*-(2-chloroethyl)-*N*-2-bromobenzylamine hydrochloride; 50 mg/kg; Sigma-Aldrich; n = 6) or 0.9% saline (n = 6) one hour later. After the second set of injections, subjects were allowed to recover for 2 weeks before they were sacrificed.

Drugs

[^3H]RX821002 (45.0 – 67.0 Ci/mmol; Amersham Biosciences, Piscataway, NJ, USA) was the ligand selected to label α_2 -adrenergic receptors. All the other chemicals were purchased

from Sigma-Aldrich Inc., USA: R(-)-Apomorphine HCl Hemihydrate (non-selective dopaminergic agonist), clonidine hydrochloride (α_2 -agonist with affinity for I1 imidazoline receptors), clozapine (atypical antipsychotic, antagonist at D4, 5-HT₂, and muscarinic receptors), dopamine, epinephrine (non-selective α -adrenergic agonist), haloperidol (antipsychotic, nonselective D₂-receptor antagonist), idazoxan hydrochloride (α_2 -adrenoceptor antagonist; I₂ imidazoline receptor agonist; I₁ imidazoline receptor antagonist), L-745,870 hydrochloride (selective D₄ receptor antagonist), ($\pm$)-norepinephrine (+)-bitartrate salt, *N*-(2-chloroethyl)-*N*-2-bromobenzylamine hydrochloride (DSP-4), oxymetazoline hydrochloride (partial α_{2A} -adrenoceptor agonist; agonist at 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} serotonin receptors; mixed agonist-antagonist at 5-HT_{1C} serotonin receptors), phentolamine hydrochloride (non-selective α -adrenergic antagonist), prazosin hydrochloride (non-selective α_1 -adrenergic antagonist with some affinity for α_{2b} and α_{2C} -adrenergic receptors), ($\pm$) propranolol hydrochloride (β -adrenoceptor antagonist; 5-HT₁/5-HT₂ serotonin receptor antagonist; cardiac depressant), (-)-quinpirole hydrochloride (D₂ agonist with some selectivity for D₃ sites), S(-)-raclopride (+)-tartrate salt (non-selective D₂ receptor antagonist), RX821002 hydrochloride (selective α_2 -adrenergic antagonist lacking affinity for I₁ and I₂ imidazolin receptors but with a relatively high affinity for 5HT_{1A} receptors), 5-hydroxytryptamine (serotonin) hydrochloride (5-HT), R-(+)-SCH-23390 hydrochloride (selective non-selective D₁ antagonist), ($\pm$)-SKF-38393 hydrochloride (non-selective D₁-receptor agonist), spiperone (D₂ antagonist with an higher affinity for D₄), (S)-(-)-Sulpiride (antipsychotic, D₂ dopamine receptor antagonist) and yohimbine hydrochloride (non-selective α_2 -adrenergic antagonist).

Tissue preparation

The brains were collected from each subject via rapid decapitation. For the specificity experiments, the brains of intact male or female finches were rapidly dissected out of the skull. The white matter was removed from the brains. The remaining gray matter from each brain was then minced and molded into a cylindrical plastic mold ($\approx$ 5 mm high with a radius of $\approx$ 5 mm). Different molds were made for female and male tissue. Five brains were used per homogenate. Molded minced brain homogenates were then immediately frozen on powdered dry ice and stored at -70°C until use.

For α_2 -adrenergic receptor autoradiography, brains of males treated with saline or DSP-4 (see above) were also removed rapidly from the skull and frozen immediately on powdered dry ice and stored at -70°C until use.

[³H]RX821002 binding

Our 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. This general approach to the study of receptor properties prior to autoradiographic studies has been widely employed since *in vitro* autoradiographic procedures were first established (e.g., Young and Kuhar, 1979) and it was successfully applied to studies of the α_2 -adrenergic receptor in mammalian species (Unnerstall, et al., 1984). Conclusions concerning receptor properties based on this approach as compared to suspended cell membrane methods have been similar (e.g., Unnerstall, et al., 1984). Molded brain homogenates were cut into 16 μm sections using a cryostat and thaw mounted onto gelatin-coated microscope slides (one section 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 MgCl₂) for 30 min at room temperature. Slides were then incubated for

one hour at room temperature in 5nM [^3H]RX821002 buffer with different concentrations (two to thirteen) of several unlabeled compounds. Each condition/concentration was tested in a single mailer containing five slides. Total binding was determined by incubation without any competitor. Non-specific binding was determined by addition of a saturating concentration of a non-selective α -adrenergic ligand, phentolamine (10 μM). After one hour, slides were washed twice for five min in ice-cold buffer. Then each slice was wiped immediately from the slide with filter paper (Whatman, Cat. No 1001042). The filter paper was then placed into a scintillation vial. Vials were filled with scintillation fluid and binding was estimated using a scintillation counter.

[^3H]RX821002 autoradiography

The brains of male zebra finches treated with DSP-4 or saline were cut into 16 μm sections using a cryostat and thaw mounted onto gelatin 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 could fit on a slide. The slides were dried and stored at -20°C until use.

Four series of slides were used for this autoradiography: one series for the total binding, one for the non-specific binding, one for the competition with norepinephrine and another for the competition with dopamine. 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 [^3H]RX821002 buffer with no competitor (total binding), phentolamine (10 μM , non-specific), norepinephrine (0.1 μM , NE) or dopamine (1 μM , DA). After one hour, 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) along with standards (ART-123; American Radiolabeled Chemicals Inc., St Louis, MO) containing concentrations of tritium ranging from 0.00 to 489.1 $\mu\text{Ci/g}$. The films were developed after 9 weeks. The density of α_2 -adrenoceptor binding was analyzed in 4 telencephalic song control nuclei (Area X, the lateral portions [core and shell as described in Riters and Ball, 2002] of the magnocellular nucleus of the anterior nidopallium [LMAN], HVC and the robust nucleus of the arcopallium [RA] as well as the hook of RA [arcopallium dorsale; Ad]), the region just ventral to Area X within the medial striatum and the two catecholaminergic nuclei (the ventral tegmental area [VTA; A10] and the posterior part of the substantia grisea centralis [GCT; A11] or midbrain central gray). In some cases, the analysis could not be performed in every brain due to either damage or in some cases parts of nuclei were missing. 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. Mean density was calculated from the individual densities taken from each nucleus (average from the right and the left side) for each male. The optical densities of the tritium standards that had been apposed to film along with the sections were converted into approximate fmol/mg tissue of bound [^3H]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. The experimenter was blind to the treatment of the animals. The location of each song control nucleus was determined based on reference to the canary brain atlas (Stokes, et al., 1974) and previous studies in which α_2 -binding was used to identify nuclei of the song control system (Casto and Ball, 1996, Riters and Ball, 2002, Riters, et al., 2002). The shell and core of LMAN were identified using the description of this region by Johnson et al. (Johnson, et al., 1995) and the previous work by Riters and Ball (2002) that identified these sub-regions in sections labeled with the use of autoradiographic procedures for α_2 -receptors.

Data analysis

Competition binding curves and subsequent calculations (IC_{50} and K_i) were analyzed using non-linear 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: $Y = Y_{max} / (1 + 10^{(X - LogIC_{50})})$ in which Y is the percentage of [3H]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. The K_D value used was the K_D obtained by Ritters and Ball 2002. 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. In order to compare the mean K_i of the two catecholamines for α_2 -adrenergic sites in males and females, the mean of the K_i found in separate experiments was computed and compared using a two-way ANOVA.

Differences in the density of α_2 -adrenergic receptors between DSP-4 and saline treated males for each brain region were analyzed using Student's t -tests. Data from competition experiments were analyzed by one- or two-way ANOVA. When appropriate, these analyses were followed by Fisher's PLSD post-hoc tests. 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 [3H]RX821002 at α_2 -adrenergic receptors

RX821002 is an α_2 -adrenergic antagonist that does not select between the different α_2 -receptor subtypes. An advantage of using RX821002 is that it lacks binding at I_1 and I_2 imidazolin receptors. However, it has a relatively high affinity for 5HT $_1A$ receptors (Clarke and Harris, 2002, Newman-Tancredi, et al., 1998). Initial competition binding experiments were performed to ensure and compare the specificity of [3H]RX821002 for α_2 -adrenergic sites in male and female zebra finches. The analysis of the first set of competitions revealed no sex difference ($p > 0.1867$) as well as no interaction between the sex and the concentrations ($p > 0.3651$) for any of these unlabeled ligands. However, as will be discussed in detail for each ligand later in this section, concentration effects were observed with adrenergic ligands and, in some cases, with dopaminergic ligands (See Fig. 2 for Post-hoc comparisons). As expected, the non-selective α -adrenergic antagonist, phentolamine ($F_{3,8} = 38.3411$, $p = 0.0001$), and the non-selective α -adrenergic agonist, epinephrine ($F_{2,6} = 54.8605$, $p = 0.0001$), displaced the binding of [3H]RX821002 in a concentration-dependent fashion as indicated by the post-hoc analyses (Fig. 2A). Likewise, RX821002 and yohimbine, two non-selective α_2 -adrenergic antagonists, also concentration-dependently inhibited [3H]RX821002 binding ($F_{3,8} = 63.8188$, $p = 0.0001$ and $F_{3,8} = 15.1697$, $p = 0.0001$; Fig. 2A). The highest concentration of the α_1 -adrenergic antagonist, prazosin, reduced significantly, but by 50% only, the binding of [3H]RX821002 ($F_{2,6} = 7.0365$, $p = 0.0267$; Fig. 2A). Significant displacement of [3H]RX821002 binding was also observed after incubation with some dopaminergic drugs (Fig. 2B). When present, this displacement was only observed at the highest concentration of the unlabeled competitor. Indeed, at a concentration of 10 μM , the non-selective dopaminergic agonist, apomorphine, as well as SCH-23390, a non-selective D1 antagonist, almost completely inhibited the binding of the labeled α_2 -adrenergic ligand ($F_{2,6} = 35.1059$, $p = 0.0001$ and $F_{2,6} = 15.6419$, $p = 0.0001$, respectively), while the same drugs, at a concentration 100 fold lower, produced almost no binding displacement (Fig. 2B). No significant effect was observed following the co-incubation with the unlabeled D2 antagonist, sulpiride, at both low and high doses ($F_{2,6} = 2.8540$, $p = 0.1346$).

These results suggest that, at high concentrations, dopaminergic compounds can interact with the binding of [³H]RX821002. In order to further investigate this hypothesis, a set of other adrenergic and dopaminergic ligands was tested for their ability to interfere with [³H]RX821002 binding. Since no sex difference was observed in the first set of competitions, the subsequent competitions were run only on male tissue. Concentration effects were observed in some of the cases (Figure 3). The three additional α_2 -adrenergic ligands tested (idazoxan, oxymetazoline and clonidine) significantly inhibited the binding of [³H]RX821002 ($F_{3,3} = 2076.2819$, $p = 0.0001$; $F_{3,4} = 1157.5563$, $p = 0.0001$ and $F_{4,5} = 193.3464$, $p = 0.0001$, respectively). As indicated by the post-hoc analyses, clonidine and oxymetazoline displaced the binding of the adrenergic ligand at concentrations as low as the nanomolar range, while concentrations up to 10 μ M of idazoxan effectively shifted this binding (Fig. 3A). On the other hand, the β -adrenoceptor antagonist, propranolol, did not affect [³H]RX821002 binding ($F_{2,3} = 3.7345$, $p = 0.1534$; Fig. 3B). Likewise, although RX821002 is known to interact to some degree with serotonin receptors (Clarke and Harris, 2002), its binding was not modified by 5-HT ($F_{2,3} = 6.1703$, $p = 0.0865$; Fig. 3B). On the other hand, significant effects of the incubation with dopaminergic drugs were also observed (SKF-38393, a non-selective D1-receptor antagonist, $F_{3,4} = 11.2445$, $p = 0.0203$; Haloperidol, a non-selective D2-receptor antagonist, $F_{3,4} = 10.6912$, $p = 0.0222$; Spiperone, a D2 antagonist with a higher affinity for D4, $F_{3,4} = 73.6465$, $p = 0.0006$; quinpirole, a D2 agonist with some selectivity for D3 sites, $F_{2,3} = 204.2077$, $p = 0.0006$; L 745,870, a selective D4 receptor antagonist, $F_{4,5} = 163.6749$, $p = 0.0001$; clozapine, a selective agonist for D4 receptors, $F_{4,5} = 344.4272$, $p = 0.0001$). Only the non-selective D2 receptor antagonist, raclopride, did not interact at all with the binding of [³H]RX821002 ($F_{2,3} = 2.1065$, $p = 0.2682$). The post-hoc analyses indicate that these effects result primarily from an effect of the highest concentrations with the exception of the D4 selective drugs, clozapine and L 745,870, that displaced [³H]RX821002 binding at all concentrations even the lowest (Fig. 3C). It should be noted that the range of concentrations used for some dopaminergic drugs is not the same as the range used for adrenergic drugs. As a consequence, the inhibition curves of [³H]RX821002 by the dopaminergic drugs, SKF-38393, haloperidol, clozapine and L 745,870, are actually steeper than α_2 -adrenergic drugs curves.

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

Now that we have tested the specificity of [³H]RX821002 in our preparation, we can compare the ability of norepinephrine and dopamine to displace the binding of [³H]RX821002. Inhibition curves of [³H]RX821002 binding by norepinephrine and dopamine were generated to compute affinities of these agonists for α_2 -adrenergic receptors expressed in brain tissue from male and female finches. Inhibition curves were generated in two to three separate experiments (see Table 1, for details) and all data were analyzed by non-linear regression analysis. As illustrated in figure 4, [³H]RX821002 binding was inhibited in a concentration-dependent manner by coinubation with increasing concentrations of norepinephrine and dopamine. As illustrated in Table 1, dopamine K_i was found to be about 10-fold higher than norepinephrine's K_i in males and about 28-fold higher in females indicating that dopamine has an affinity 10 to 28-fold lower than norepinephrine. The comparison of these K_i values between the sexes reveals no significant difference between the incubation with norepinephrine or dopamine ($F_{1,6} = 3.9365$, $p = 0.0945$), no sex difference ($F_{1,6} = 0.0018$, $p = 0.9680$) and no interaction between the sex and the incubation conditions ($F_{1,6} = 0.0079$, $p = 0.9319$). These results thus indicate that in this preparation dopamine does interact with the binding of the α_2 -adrenergic radioligand.

[³H]RX821002 autoradiography – Distribution and DSP-4 effect

As a validation for the autoradiography assay, the density of α_2 -adrenergic receptors was investigated in the song system and in the main catecholaminergic nuclei of male zebra finches

and, in order to determine the synaptic localization of these receptors, this distribution was compared with that in birds treated with the neurotoxin DSP-4 which is known to deplete forebrain norepinephrine in male zebra finches (Barclay, et al., 1992, Barclay, et al., 1996). As described previously by Ritters and Ball (2002), relatively high densities of α_2 -adrenergic receptors were detected in Area X, RA, the dorsal arcopallium (Ad; a hook-like structure lateral and medial to RA; (Johnson, et al., 1995), the core and the shell of LMAN and HVC (Fig. 5–7). High densities were also measured in the ventral tegmental area (VTA) and the substantia nigra pars reticulata (SNr). As illustrated in figure 5, slight reductions of the density of α_2 -adrenergic receptors were detected in the RA of DSP-4 treated males as compared to untreated males. However, a Student's t-test revealed no significant effect ($t > 1.484$, $p > 0.1762$). Similarly, no treatment effect was detected in the other regions ($t > 1.029$, $p > 0.3302$).

[³H]RX821002 autoradiography – Anatomical localization of competition sites

The competition binding assays showed that dopamine displaces the binding of RX821002 to α_2 -adrenergic receptors with an affinity slightly lower than that of norepinephrine but not significantly different. In order to assess whether this interaction of dopamine with the α_2 -receptors occurs in an intact brain and determine whether it is ubiquitous or limited to certain areas, binding competitions were performed on the brain slices collected from the untreated and DSP-4 treated males whose total binding data are already presented above. The concentrations of norepinephrine (0.1 μ M) and dopamine (1 μ M) for these competitions were chosen to be close to the IC₅₀ values given by the inhibition curves described previously (see table 1), the aim being to observe a binding reduction of about 50% compared to the total binding. The density of binding measured in the absence of competitor (total) or in the presence of norepinephrine or dopamine was compared between untreated and DSP-4 treated finches using a mixed ANOVA with the treatment (DSP-4 vs. Saline) 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 treatment was found ($F < 2.9466$, $p > 0.1298$) suggesting that DSP-4 treatment did not affect the ability of norepinephrine and dopamine to bind α_2 -receptors. On the other hand, significant effects of the incubation condition were detected in all the nuclei studied ($F > 3.7177$, $p < 0.0472$), but RA ($F = 2.4712$, $p = 0.1099$). As illustrated in figure 6 and 7, co-incubation with norepinephrine resulted in a reduction of binding above 30% in all the nuclei with the exception of LMAN core (25.2%), RA (12.6%), GCt (19.6%) and VTA (9.2%), while the binding reduction resulting from co-incubation with dopamine was above 15% in all nuclei excepted in the medial striatum (9.9%), in RA (7.5 %), in Ad (13.3%), and in GCt (7.0%). HVC showed the maximal displacement for both amines (42.8 % for NE and 24.5% for DA, Fig. 6F). An increase in binding was also observed in VTA following incubation with dopamine (Fig. 6I). In a given nucleus, the difference in the percentage of binding between sections incubated with norepinephrine or dopamine ranges between 5 % in RA to 24 % in the medial striatum with an average of about 15%. Therefore, it appears that, at the concentrations chosen, both norepinephrine and dopamine substantially displaced [³H]RX821002 binding, but that norepinephrine reduced the binding to a greater extent than dopamine. This is supported by the post-hoc analyses which indicate that the significant effects of the incubation conditions result mostly from an effect of co-incubation with norepinephrine that is found to be significantly different from the total in all nuclei but VTA (Fig. 6). Co-incubation with dopamine resulted in a significant reduction of binding compared with total in Area X (Fig. 6A and Fig. 7A–C) and the core of LMAN only (Fig. 6C and Fig. 7A–C). In these nuclei, incubation with norepinephrine produced a significantly bigger reduction of the binding than incubation with dopamine. On the other hand, the treatment with dopamine resulted in a level of binding different from norepinephrine treated sections but not different than the total. Together, these data indicate that, at concentrations that were chosen to reduce the binding to 50% in both NE and DA conditions, norepinephrine displaced [³H]RX821002 binding more than dopamine. However, dopamine displaced substantially, even

though not always significantly, the binding of the radioligand in all song nuclei – especially area X and the core of LMAN – but not in RA. In VTA and GCt, both amines were not very effective in displacing the binding at these concentrations. In conclusion, the observation that dopamine did not yield a significant displacement of [³H]RX821002 binding in all regions but that norepinephrine did significantly alter this binding suggests that the interaction of dopamine with α_2 -adrenergic receptors may not occur in all areas of the songbird brain.

Discussion

The present study demonstrates that dopamine displaces the binding of the selective α_2 -adrenergic ligand, [³H]RX821002, in the brain of zebra finches. The affinity of dopamine for the adrenergic site does not differ between males and females and is 10 to 28 fold lower than that for norepinephrine. [³H]RX821002 binding appears specific for α_2 -adrenergic receptors, however, as will be discussed later, some binding to alpha 1 and D1 receptors cannot be ruled out. The distribution and the relative density of [³H]RX821002 binding in the song system of zebra finches is consistent with previous reports of α_2 -adrenergic receptor distribution in songbirds (Casto and Ball, 1996, Ritters and Ball, 2002, Ritters, et al., 2002, Ball, 1994). The lack of an effect of norepinephrine depletion on the density of α_2 -adrenergic receptors suggests that these receptors are not located primarily on presynaptic membranes of noradrenergic neurons as has been previously suggested in other avian based on studies in quail (Balthazart and Ball, 1989) and also seems to be the case in mammalian species (Greenberg, et al., 1976). Finally, both norepinephrine and dopamine substantially displace the binding of the radioligand in most song nuclei. The two catecholamines do not reduce the binding to the same extent in the regions studied. This observation is consistent with the idea that, *in vivo*, dopamine interacts with α_2 -adrenergic receptors in the zebra finch song system but suggests that this interaction may not be ubiquitous. However, several aspects relevant to these findings need to be discussed further.

Characterization of [³H]RX821002 pharmacology

Previous pharmacological studies have established [³H]RX821002 as a suitable radioligand to label the total population of α_2 -adrenergic receptors in various tissues and species (Diez-Alarcia, et al., 2006, Halme, et al., 1995, O'Rourke, et al., 1994, Ruuskanen, et al., 2005). This ligand does not select between different α_2 -receptor subtypes and lacks binding at I₁ and I₂ imidazoline receptors but displays a relatively high affinity for 5HT_{1A} receptors (Clarke and Harris, 2002). Accordingly, the present binding competitions indicate that α_2 -adrenergic drugs strongly compete with [³H]RX821002 binding at concentrations lower than the nanomolar range, while a micromolar concentration of the α_1 -adrenergic competitor is needed to moderately displace this binding. Based on the pharmacology of mammalian α_2 -adrenergic receptors, such a moderate binding displacement associated with a high displacement by oxymetazoline could indicate a predominance of the $\alpha_{2A/D}$ -receptor subtype in the zebra finch brain (Harrison, et al., 1991a). However, since only prazosin was tested here, it cannot be ruled out that this slight binding displacement at high concentration may also reflect a weak interaction with α_1 -adrenergic receptors. The β -adrenoceptor antagonist, propranolol, did not affect [³H]RX821002 binding at all. In addition, although RX821002 is known to interact with serotonin receptors (Newman-Tancredi, et al., 1998), its binding was not modified by 5-HT suggesting that the binding observed in the present conditions is unlikely to represent binding to serotonin receptors.

Because the aim of this study was to assess the ability with which dopamine binds adrenergic receptors, competitions were run with dopaminergic ligands to ensure that the binding displacement induced by dopamine did not result from an interaction of the radioligand with dopaminergic sites. Surprisingly, high concentrations (1 or 10 μ M) of most dopaminergic drugs

tested interfered with RX821002 binding. There are several ways to interpret this finding. On the one hand, RX821002 may bind to dopaminergic receptors. An interaction with dopaminergic receptors has indeed been documented for some adrenergic drugs (Johnston and File, 1989, Rey, et al., 2001, Scatton, et al., 1980) and norepinephrine binds and activates D4 receptors (Lanau, et al., 1997, Newman-Tancredi, et al., 1997). Alternatively, dopaminergic drugs could interact with adrenergic receptors and compete with RX821002 binding. Such an interaction has been observed for the non-selective dopaminergic agonist, apomorphine, and the D2/3 agonist, bromocriptine, with peripheral α -adrenoceptors (Gibson and Samini, 1979). These two hypotheses seem equally probable. However, in our opinion, several observations support the idea that it is less probable that our findings could be explained by the fact that RX821002 binds to dopaminergic receptors than that it binds to adrenergic receptor. First, high concentrations of dopaminergic drugs are needed to displace [3 H]RX821002 binding suggesting that these drugs have a lower affinity than adrenergic drugs at the binding sites of RX821002, so that there is a low probability that it binds to dopaminergic rather than adrenergic sites. Moreover, the present distribution of [3 H]RX821002 binding shows a clear pattern that is characteristic of the distribution of alpha-adrenergic receptors (see below). If a substantial fraction of the radioligand bound dopaminergic receptors it is likely that some binding would have been detected in dopaminergic regions such as the striatum. Finally, to our knowledge, there has been no report of an interaction of RX821002 with dopaminergic receptors. Overall, we thus think that the displacement of RX821002 binding by micromolar concentrations of dopaminergic compounds is likely to represent an interaction of these ligands with adrenergic sites but further investigations are needed to properly test this hypothesis.

Binding distribution and Effects of norepinephrine depletion on α_2 -adrenergic receptor density

In the present study, dense [3 H]RX821002 binding defined the boundaries of area X, RA, LMAN and HVC. A relatively high density of binding was also observed in the dorsal arcopallium (Ad), a structure adjacent to RA (Johnson, et al., 1995). These observations are consistent with the distribution of α_2 -adrenergic receptors in the song system reported previously (Ball, 1994, Casto and Ball, 1996, Ritters and Ball, 2002, Ritters, et al., 2002). High densities of receptors were also detected in the nuclei containing dopaminergic cell bodies. High densities of α_2 -adrenergic receptors have been described in the periaqueductal gray (referred to as GCt in this paper) of birds and mammals (Dermon and Kouvelas, 1989, Diez-Alarcia, et al., 2006, Talley, et al., 1996, Unnerstall, et al., 1984) as well as in the rat ventral tegmental area (Boyajian, et al., 1987).

In rodents, the neurotoxin DSP-4 specifically destroys noradrenergic axons via reuptake of norepinephrine (Dudley, et al., 1990) and targets the neurons projecting from the locus coeruleus (Fritschy and Grzanna, 1991). The central effects of systemic injections of DSP-4 are long lasting (Hallman, et al., 1984, Jaim-Etcheverry and Zieher, 1980). This neurotoxin selectively destroys noradrenergic projections while sparing most of the dopaminergic system. It has also been showed that DSP-4 treatment also results in a substantial depletion of norepinephrine synthesizing fibers (DBH-immunoreactive fibers) and norepinephrine in the song system (Barclay, et al., 1992, Castelino and Ball, 2005).

In the present study, no effect of DSP-4 treatment was found on α_2 -adrenergic receptors density arguing against previous claims that α_2 -adrenergic receptors are located primarily on presynaptic membranes of noradrenergic neurons (Starke, 2001). A similar experiment conducted in quail with [3 H]*p*-amino-clonidine, another selective α_2 -adrenergic ligand, led to the same conclusion (Balthazart and Ball, 1989). Although α_2 -adrenergic receptors density was not affected by DSP-4, the present data do not allow us to definitely assert that these receptors are exclusively postsynaptic. The neurotoxin only destroys a fraction of the

adrenergic neurons. Consequently, it can be hypothesized that the receptors observed here are located on presynaptic membranes of a population of neurons that have been spared by the treatment. Alternatively, as it was alluded to in the introduction, presynaptic α_2 -adrenergic receptors may also be found on non-adrenergic neurons, which are presumably not affected by DSP-4. For instance, recent data suggest that presynaptic α_2 -adrenergic receptors located on dopaminergic terminals participate in the regulation of dopamine concentration in area X (Gale and Perkel, 2005).

Binding competition in slices

The binding competitions performed in homogenates strongly support the idea that dopamine binds α_2 -adrenergic receptors with an affinity 10 to 28 fold lower than norepinephrine. In slices, co-incubation of the radioligand with dopamine resulted in a substantial binding reduction in some regions. However, although the amine concentrations were chosen in order to yield a 50% reduction of [3 H]RX821002 binding, the binding density was never reduced by 50% following co-incubation with norepinephrine or dopamine. One explanation is that the concentrations used for the competitions on slices were slightly lower (6x) than the actual IC₅₀ determined in the whole brain assays (see table 1). Given the steepness of the inhibition curves at concentrations close to the IC₅₀, slight variations in the concentration of the competitors relative to the real IC₅₀ may result in marked changes of binding. A higher concentration of both dopamine and norepinephrine should probably have been used. These differences can also be due to other factors than the affinity for the receptors *per se*, such as ligand accessibility and availability. In particular, dopamine or norepinephrine can also be taken up by terminals or metabolized before binding any receptor. Re-uptake may thus be incriminated in the weak binding displacement measured in the present study as well as a difference between the effect of norepinephrine and dopamine if one amine is preferentially re-uptaken over the other in some regions. However, the conditions of the autoradiography assays probably do not guarantee the metabolism required to sustain optimal re-uptake levels. The uptake process is dependent on temperature and requires energy (Cooper, et al., 2003a, Cooper, et al., 2003b). *In vitro*, catecholamine reuptake is measured at body temperature and in an oxygenated extracellular milieu containing glucose (Izenwasser, et al., 1990), three conditions that are not satisfied in the present experiment. Therefore we would like to argue that although the binding reductions induced by dopamine did not meet our predictions, yet the addition of dopamine resulted in a substantial and sometimes significant binding displacement of the radioligand in several brain regions supporting the hypothesis that dopamine binds α_2 -adrenergic receptors in zebra finches.

The difference between norepinephrine and dopamine effects may reflect the existence of receptor sub-types with differential affinities for the two catecholamines. Norepinephrine displays a higher affinity for the α_{2C} -receptor sub-type than for the α_{2a} -receptor subtype (Bunemann, et al., 2001, Zhang, et al., 1999). Dopamine also shows differential affinities for the two sub-types, but its selectivity for the two receptors is not as pronounced as for norepinephrine, indicating that both ligands might behave differently towards the two receptor sub-types (Zhang, et al., 1999). Although comparable data on the α_{2B} -receptor sub-type would be helpful to fully discuss this matter, this supports the idea that a differential expression of receptor sub-types with different affinities for the two amines may help interpret our observations. A differential distribution of sub-types with differential affinities for the two amines may also account for the regional variation in binding reduction observed here. If these explanations are true, they may explain why co-incubation with dopamine did not yield statistically significant effects on RX821002 binding in all brain regions. However, before further support is provided in favor of this hypothesis, the absence of significant displacement of RX821002 binding by dopamine in all regions where norepinephrine displaced it leads us to conclude that this interaction between dopamine and α_2 -adrenergic receptors seems

anatomically specialized. The functional underpinning of such specialization is not clear at this point and should be further investigated in future studies.

Anatomical localization of the interaction of dopamine with α_2 -adrenergic receptors

Dopamine significantly displaces [^3H]RX821002 binding in area X. Dopamine concentration and turnover in area X is higher than that of norepinephrine (Barclay and Harding, 1990, Barclay, et al., 1992, Gale and Perkel, 2005). In European starlings, area X also expresses a high density of D1 (Casto and Ball, 1994) and D2 receptors (Heimovics and Cornil, unpublished observations). In zebra finches, dopamine has been shown to modulate excitability of spiny neurons as well as the firing rate of the non-spiny neurons of area X through both D1 and D2 receptors (Ding and Perkel, 2002, Ding, et al., 2003, Perkel and Wendel, 2006). Our data suggest that dopamine could also influence neurotransmission in area X through the activation of α_2 -adrenergic receptors. Whereas there is currently no data supporting a modulation of electrical activity in area X by these receptors, recent evidence indicates that dopamine partially inhibits its own release by activating α_2 -adrenergic receptors (Gale and Perkel, 2005).

Similarly, dopamine significantly reduce adrenergic binding in LMAN core and, even though this difference was not significant, a high degree of binding reduction was also achieved in HVC and LMAN shell following incubation with dopamine. This suggests that dopamine may interact with α_2 -adrenergic receptors in these regions as well. Along with the interaction in area X, this indicates that such an interaction could influence the function of the two pathways implicated in the control of singing behavior (See fig. 1).

Physiological significance of the dopaminergic interaction with adrenergic receptors

The present data show that dopamine binds to α_2 -adrenergic receptors but does this binding trigger any intracellular event? And if so, does dopamine activate the same cascades as norepinephrine? Does this interaction carry any functional significance *in vivo*?

Previous *in vitro* studies reported that dopamine can activate via its action on adrenergic receptors various physiological responses such as intracellular calcium flow (Rey, et al., 2001), intracellular cAMP concentrations (Zhang, et al., 1999, Zhang, et al., 2004) and neuronal firing rate (Cornil, et al., 2002, Malenka and Nicoll, 1986). Dopamine inhibits the firing rate of neurons recorded from quail brain slices through α_2 -receptors at concentrations 10 times higher than norepinephrine (Cornil, et al., 2002) suggesting that dopamine activates these receptors at concentrations close to its affinity for the receptors. However, some studies argued that the potency of dopamine at adrenergic sites is actually lower than its affinity. For instance, while they measured a 3-fold lower affinity of dopamine for the human α_{2A} -adrenergic receptor expressed in Chinese Hamster Ovary (CHO) cells as compared to norepinephrine, Nyrönen and colleagues observed that the potency (EC_{50}) of dopamine was 93% less than that of norepinephrine as determined by functional [^{35}S]GTP γ S assay (Nyrönen, et al., 2001). In addition, although dopamine was shown to bind and activate α_{2C} -receptors expressed in normal rat kidney (NRK) cells at relatively low concentrations (Zhang, et al., 1999), another report indicated that much higher concentrations had to be added in order to modulate intracellular cAMP concentration through α_{2C} -receptors transfected in CHO cells (Zhang, et al., 2004). Dopamine also binds to α_{2A} -receptors expressed in NRK cells but, whereas norepinephrine facilitated forskolin-stimulated cAMP accumulation, dopamine inhibited it (Zhang, et al., 1999). Together, these observations indicate that dopamine when it binds to adrenergic receptors can activate them. However, the potency of this activation seems variable and also depends on the system in which the receptors were expressed. Finally, the molecular mechanisms of α_{2A} -adrenoceptors activation by norepinephrine and dopamine may also differ. More work is thus needed to clarify this issue and, in particular, future studies should investigate

whether physiological concentrations of dopamine are able to activate adrenergic receptors in the song system. The recent work from Gale and Perkel (2005) suggests that this might be true in area X at least (see above).

In conclusion, the data presented here suggest that dopamine interacts with α_2 -adrenergic receptors in zebra finch. This interaction occurs in the song system but exhibits a regional variation. Along with other studies, the results presented in this article support the broad distribution of such an interaction across vertebrate species. This observation may prove to be important for the design and interpretation of future experiments as well as the design of new pharmacological drugs. In particular, future research would be useful to provide insights on the specific role of such interaction between dopamine and adrenergic receptors for the control of singing behavior.

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Figure 1. Generalized view of the songbird vocal system

A. Schematic representation of song control pathways. The nuclei of the efferent motor pathway (colored in *black*) required for song production required are connected by *black arrows*. Nuclei of the anterior forebrain pathway (colored in *white*) implicated in the auditory feedback necessary for song learning during ontogeny and song recognition and maintenance in adulthood are connected by *dashed arrows*. For review, see Ball and Hulse, 1998, Brainard and Doupe, 2002, Brenowitz and Beecher, 2005, Brenowitz, et al., 1997, Konishi, 2004, Nottebohm, et al., 1990. B. Schematic representation of known catecholaminergic inputs to the song control circuit and of the distribution of catecholaminergic receptors (based on substantial binding relative to background). Nuclei containing dopaminergic cells bodies are

colored in *white* and nuclei containing noradrenergic cell bodies are *black*. Known noradrenergic inputs are represented by *black arrows*. Dopaminergic inputs are represented by *dashed arrows*. The width of arrows is indicative of the extent of the input (relative number of cells projecting to the nuclei). This schematic representation is based on data collected in canary (tract-tracing: Appeltants, et al., 2000, Appeltants, et al., 2002), European starlings (α_2 -receptors: Casto and Ball, 1996, Ritters, et al., 2002; D1- and D2-receptors: Casto and Ball, 1994, Casto and Ball, 1996, Ritters, et al., 2002, Heimovics and Cornil, unpublished observations), goldfinch ($\beta_{1/2}$ receptors: Revilla, et al., 1999) and zebra finch (α_2 -receptors: Ritters and Ball, 2002; tract-tracing: Castelino, et al., 2007, Lewis, et al., 1981). Abbreviations: GCt, substantia grisea centralis (midbrain central gray); HVC, HVC of the nidopallium; LMAN, lateral magnocellular nucleus of anterior nidopallium; LoC, locus coeruleus; RA, robust nucleus of the arcopallium; VTA, ventral tegmental area; X, area X of the medial striatum.

Figure 2. Characterization of the α₂-adrenergic specificity of [³H]RX21002 in male and female zebra finches

A. Competitions with increasing concentrations of α-adrenergic ligands, B. Competitions with increasing concentrations of dopaminergic ligands. Each bar represents the results of two separate experiments, each performed in 5 replicates. Note that the range of concentrations used for adrenergic and dopaminergic drugs is not the same. Error bars represent S.E.M. * p < 0.05 vs. Total; † p < 0.05 vs. 10⁻⁹ M; ‡ p < 0.05 vs. 10⁻⁷ M.

Figure 3. Further characterization of the α_2 -adrenergic specificity of [3 H]RX21002 in zebra finch
 A. Competitions with increasing concentrations of α_2 -adrenergic ligands; B. Competitions with non α -adrenergic ligands; C. Competitions with increasing concentrations of dopaminergic ligands. Each bar represents the results of two separate experiments, each performed in 5 replicates. Error bars represent S.E.M. * $p < 0.05$ vs. Total; # $p < 0.05$ vs. 10^{-11} M; † $p < 0.05$ vs. 10^{-9} M; ‡ $p < 0.05$ vs. 10^{-8} M; Δ $p < 0.05$ vs. 10^{-7} M; $\diamond$ $p < 0.05$ vs. 10^{-6} M.

Figure 4. Inhibition of $[^3\text{H}]\text{RX21002}$ binding to α_2 -adrenergic receptors in male (A) and female (B) zebra finch by increasing concentrations of norepinephrine (■) or dopamine (▲). The results are representative of two to three similar experiments, each performed in 5 replicates. Error bars represent S.E.M.

Figure 5. NE depletion does not affect the density of α_2 -adrenergic receptors in the song system and in the main catecholaminergic nuclei

Numbers in the bars refer to the number of subjects tested. Ad, arcopallium dorsale (hook of RA); GCt, substantia grisea centralis; LMANc, core of the lateral portion magnocellular nucleus of the anterior nidopallium [LMAN]; LMANs, shell of LMAN; RA, robust nucleus of the arcopallium; VTA, ventral tegmental.

Figure 6. Competitions of norepinephrine (NE) and dopamine (DA) for the binding of [³H]RX21002 in the song system and the main catecholaminergic nuclei
 Numbers in the bars refer to the number of subjects tested. * p < 0.05 vs. Total; † p < 0.05 vs. DA.

Figure 7. Autoradiograms illustrating norepinephrine and dopamine effects

Adjacent sections have been incubated in the absence of competitor (Total; A, D and G) or in the presence of norepinephrine (B, E and H) or dopamine (C, F and I).

Table 1**Norepinephrine and dopamine IC₅₀ and K_i for α_2 -adrenergic receptors**

Results are means $\pm$ S.E.M. of two to three separate experiments, each performed in 5 replicates. The number under brackets indicates the number of experiments performed for each condition.

	IC ₅₀ (nM)		
	NE	DA	Ratio DA/NE
Male (3)	604.9 $\pm$ 431.9	6145.8 $\pm$ 3798.1	10.17
Female (2)	214.8 $\pm$ 130.3	6009.7 $\pm$ 4485.7	28.32
	K _i (nM)		
	NE	DA	Ratio DA/NE
Male (3)	86.07 $\pm$ 61.45	874.99 $\pm$ 540.68	10.17
Female (2)	31.59 $\pm$ 17.52	894.69 $\pm$ 599.42	28.32