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RESEARCH PAPER

Catecholamine synthesis and metabolism in the central nervous system of mice lacking α_2-adrenoceptor subtypes

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Background and purpose: This study investigates the role of α_2-adrenoceptor subtypes, α_{2A}, α_{2B} and α_{2C}, on catecholamine synthesis and catabolism in the central nervous system of mice.
Experimental approach: Activities of the main catecholamine synthetic and catabolic enzymes were determined in whole brains obtained from α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor knockout (KO) and C56Bl\7 wild-type (WT) mice.
Key results: Although no significant differences were found in tyrosine hydroxylase activity and expression, brain tissue levels of 3,4-dihydroxyphenylalanine were threefold higher in α_{2A}- and α_{2C}-adrenoceptor KO mice. Brain tissue levels of dopamine and noradrenaline were significantly higher in α_{2A} and α_{2C}KOs compared with WT [WT: 2.8 ± 0.5, 1.1 ± 0.1; α_{2A}KO: 6.9 ± 0.7, 1.9 ± 0.1; α_{2B}KO: 2.3 ± 0.2, 1.0 ± 0.1; α_{2C}KO: 4.6 ± 0.8, 1.5 ± 0.2 nmol·(g tissue)$^{-1}$, for dopamine and noradrenaline respectively]. Aromatic L-amino acid decarboxylase activity was significantly higher in α_{2A} and α_{2C}KO [WT: 40 ± 1; α_{2A}: 77 ± 2; α_{2B}: 40 ± 1; α_{2C}: 50 ± 1, maximum velocity (V_{max}) in nmol·(mg protein)$^{-1}$·h^{-1}], but no significant differences were found in dopamine β-hydroxylase. Of the catabolic enzymes, catechol-*O*-methyltransferase enzyme activity was significantly higher in all three α_2KO mice [WT: 2.0 ± 0.0; α_{2A}: 2.4 ± 0.1; α_{2B}: 2.2 ± 0.0; α_{2C}: 2.2 ± 0.0 nmol·(mg protein)$^{-1}$·h^{-1}], but no significant differences were found in monoamine oxidase activity between all α_2KOs and WT mice.
Conclusions and implications: In mouse brain, deletion of α_{2A}- or α_{2C}-adrenoceptors increased cerebral aromatic L-amino acid decarboxylase activity and catecholamine tissue levels. Deletion of any α_2-adrenoceptor subtypes resulted in increased activity of catechol-*O*-methyltransferase. Higher 3,4-dihydroxyphenylalanine tissue levels in α_{2A} and α_{2C}KO mice could be explained by increased 3,4-dihydroxyphenylalanine transport.
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Keywords: α_2-adrenoceptors; L-DOPA; noradrenaline; dopamine; tyrosine hydroxylase; aromatic L-amino acid decarboxylase; catechol-*O*-methyltransferase

Abbreviations: 3-MT, 3-methoxytyramine; 5-HT, 5-hydroxytryptamine; 6-BH_4, 6-methyl-5,6,7,8-tetrahydropterin; AAAD, aromatic L-amino acid decarboxylase; COMT, catechol-*O*-methyltransferase; DβH, dopamine β-hydroxylase; DHPG, 3,4-dihydroxyphenylglycol; DOPAC, 3,4-dihydroxyphenylacetic acid; JP-1302, acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine; K_M, Michaelis–Menten constant; L-DOPA, 3,4-dihydroxyphenylalanine; LAT, L-type amino acid transporter; MAO, monoamine oxidase; NMN, normetanephrine; TH, tyrosine hydroxylase; V_{max}, maximum velocity; WT, wild-type

Introduction

Pharmacological and biochemical research has led to a subdivision of α_2-adrenoceptors into three distinct subtypes: α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors (Bylund *et al.*, 1994; nomenclature follows Alexander *et al.*, 2008). In the mouse central nervous system (CNS) the α_{2A}- and α_{2C}-adrenoceptor subtypes have been identified as the main presynaptic inhibitory receptors regulating noradrenaline release (Bucheler *et al.*, 2002). A similar functional overlap between α_{2A}- and α_{2C}-adrenoceptors has also been reported for 5-hydroxytryptamine (5-HT) release from the hippocampus (Scheibner *et al.*, 2001) and for dopamine release in the basal ganglia (Bucheler *et al.*, 2002).

Catecholamine biosynthesis starts with the conversion of the amino acid L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) a step mediated by tyrosine hydroxylase (TH; EC 1.14.16.2), the rate limiting step enzyme for catecholamine

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synthesis (Kumer and Vrana, 1996). L-DOPA in brain may also derive from plasma after having crossed the blood–brain barrier, originating from the sympathoneural system or from synthesis by tyrosinase in non-neuronal cells (Goldstein *et al.*, 2003). At the level of brain capillary endothelium, L-DOPA and other large neutral amino acids are transported by the L-type amino acid transporter (LAT) (del Amo *et al.*, 2008). L-DOPA is then converted to dopamine by aromatic L-amino acid decarboxylase (AAAD; EC 4.1.1.28), an enzyme with a wide-ranging cellular distribution. Dopamine is translocated into storage vesicles by the vesicular monoamine transporter (Henry *et al.*, 1998). The presence of the enzyme dopamine β-hydroxylase (DβH; EC 1.14.17.1) as a constituent of storage vesicles leads to intravesicular conversion of dopamine to noradrenaline (Ahn and Klinman, 1989) and ensures the appropriate noradrenergic phenotype of sympathetic nerves and CNS noradrenergic neurons.

Catecholamines are metabolized by several enzymes including monoamine oxidase (MAO; EC 1.4.3.4) and catechol-*O*-methyltransferase (COMT; EC 2.1.1.6). There are two distinct MAO isoenzymes, type A (MAOA) and type B (MAOB) and two molecular forms of COMT, a soluble isoform (S-COMT) and a membrane-bound isoform (MB-COMT). Noradrenaline and adrenaline are metabolized by the combination of MAO and aldose or aldehyde reductase to the deaminated glycol metabolite, 3,4-dihydroxyphenylglycol (DHPG) and dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC). The pathway of catecholamine metabolism also involves COMT-catalysed *O*-methylation of noradrenaline to normetanephrine (NMN), adrenaline to metanephrine and dopamine to 3-methoxytyramine (3-MT).

Although the physiological role of α_2-adrenoceptor control over monoamine release in the CNS is well defined, a more precise definition of the influence of these receptors on monoamine synthesis and metabolism is still required. In this view, the present study was designed to evaluate the influence of the α_2-adrenoceptor on catecholamine synthesis and metabolism in the CNS of mice. For this purpose we evaluated the activity of each of the enzymes involved in catecholamine biosynthesis – TH, AAAD and DβH, and the two key enzymes in monoamine metabolism – COMT and MAO, in the brain of wild-type (WT) and knockout (KO) mice for each of the α_2-adrenoceptor subtypes: α_{2A}, α_{2B} and α_{2C}. To further support the data obtained with KO mice, the activity of enzymes found altered in the KO mice was also evaluated in mice treated with a single dose of the selective α_2-adrenoceptor antagonist, yohimbine, or the selective α_{2C}-adrenoceptor antagonist acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine (JP-1302). Finally, given that L-DOPA may also originate from other sources besides synthesis via TH (Goldstein *et al.*, 2003), we also evaluated the influence of the α_2-adrenoceptor in the transport system responsible for its uptake in a human neuroblastoma cell line (SK-N-SH).

Methods

Animals

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the experiments were performed according to the Portuguese law on animal welfare. In all experiments, brains from 4–6-month-old male WT (C57BL/6) and α_{2A}, α_{2B} and α_{2C}KO mice were used. The generation of the mouse lines lacking α_2-adrenoceptor subtypes has been described previously (Link *et al.*, 1996; Altman *et al.*, 1999). Animals were kept under controlled environmental conditions (12:12 h light/dark cycle and room temperature 22 ± 2°C). In the study to evaluate the effect of the α_2-adrenoceptor antagonists yohimbine (3 $mg \cdot kg^{-1}$) and JP-1302 (3 $\mu mol \cdot kg^{-1}$), drug treatments or vehicle (saline), as three i.p. injections, were given at 12 h intervals. The selection of doses was based on previous *in vivo* studies (Haapalinna *et al.*, 2003; Sallinen *et al.*, 2007). Experiments were carried out 1 h after the last injection. After decapitation under anaesthesia (sodium pentobarbital 60 $mg \cdot kg^{-1}$, i.p.), the brains were immediately removed and placed on ice. In experiments carried out to evaluate enzyme activity or Western blot analysis, whole brain was homogenized in the appropriate buffer (see Appendix S1). In experiments carried out to evaluate L-DOPA, the biogenic amines (noradrenaline, 5-HT and dopamine) and their metabolites, whole brain was placed in vials containing 1 mL of perchloric acid (0.2 M).

Tyrosine hydroxylase (TH)

Tyrosine hydroxylase activity and expression were measured as previously described (Moura *et al.*, 2005) (See Appendix S1 for more details).

Aromatic L-amino acid decarboxylase (AAAD)

L-amino acid decarboxylase activity was evaluated as previously described (Vieira-Coelho and Soares-Da-Silva, 1998) (See Appendix S1 for more details).

Dopamine β-hydroxylase (DβH)

Dopamine β-hydroxylase activity was evaluated as previously described (Matsui *et al.*, 1981) (See Appendix S1 for more details).

Monoamine oxidase (MAO)

Monoamine oxidase activity was determined in cell homogenates, as previously described (Vieira-Coelho *et al.*, 1999) (See Appendix S1 for more details).

Catechol-O-methyltransferase

Catechol-*O*-methyltransferase activity was evaluated by the methylation of adrenaline to metanephrine, as previously described (Soares-da-Silva *et al.*, 1996). The soluble and membrane-bound fractions of COMT from brain and peripheral tissues were obtained by the method previously described (Vieira-Coelho and Soares-da-Silva, 1999) (See Appendix S1 for more details).

Cell culture

SK-N-SH cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in a

humidified atmosphere of 5% CO2/95% air at 37°C. SK-N-SH cells were grown in minimum essential medium adjusted to contain 1.0 mM sodium pyruvate (Sigma). Culture medium was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma), 100 $U \cdot mL^{-1}$ penicillin G, 0.25 $mg \cdot mL^{-1}$ amphotericin B, 100 $mg \cdot mL^{-1}$ streptomycin (Sigma) and 25 mM HEPES (Sigma). Twenty-four hours before each experiment, the cell medium was changed to medium free of fetal bovine serum. Experiments were generally performed 3–5 days after the initial seeding.

Transport studies

On the day of the experiment, the growth medium of SK-N-SH monolayers was aspirated, and the cells were washed with Hanks' medium. Then, the cell monolayers were pre-incubated for 30 min in Hanks' medium at 37°C. The Hanks' medium had the following composition (in mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 0.25 $CaCl_2$, 1.0 $MgCl_2$, 0.15 Tris-HCl and 1.0 sodium butyrate, pH 7.4. The incubation medium also contained benserazide (1 mM) and tolcapone (1 mM) to inhibit the enzymes AAAD and COMT respectively. Saturation experiments were performed in cells incubated for 6 min with increasing concentrations of L-DOPA (25–5000 μM). Test substances were applied from the apical side and were present during the pre-incubation and incubation periods. To test the effect of medetomidine, this compound was added at different concentrations (0.1–1000 nM) 15 min before L-DOPA addition, in the presence and in the absence of a single concentration of yohimbine (300 nM) that was present during the 30 min of pre-incubation. During pre-incubation and incubation, the cells were continuously shaken and maintained at 37°C. Apical uptake was initiated by the addition of 250 μL of Hanks' medium with a single concentration of L-DOPA (2500 μM) for 6 min. Uptake was terminated by the rapid removal of this solution by means of a vacuum pump connected to a Pasteur pipette, followed by a rapid wash with cold Hanks' medium and the addition of 250 mL of 0.2 mM perchloric acid. The acidified samples were stored at 4°C before being injected into the high-pressure liquid chromatograph for the assay of L-DOPA.

Monoamine assay

The assay of the L-DOPA and catecholamines in tissues, plasma and samples was performed by high-performance liquid chromatography with electrochemical detection (HPLC-ED) as described previously (Moura *et al.*, 2005) (See Appendix S1 for more details). The assays for NMN, metanephrine, 3-MT and 5-HT were performed by means of high-pressure liquid chromatography, as previously described (Vieira-Coelho and Soares-da-Silva, 1996). In brief, aliquots of 50 μL of the filtered perchloric acid extract of tissues or from enzymatic assay samples were injected into an HPLC-ED system (Gilson Model 141, Gilson Medical Electronics, Villiers, Le Bel, France). The lower limit of detection of NMN, metanephrine, 3-MT and 5-HT ranged from 350 to 1000 fmol.

Protein assay

Protein concentration in brain homogenates was measured as previously described (Bradford, 1976).

Statistical analysis

Results are expressed as mean ± SEM from n mice, as shown. The kinetic parameters, V_{max} (maximum velocity) and K_M (Michaelis–Menten constant) values for the activity of enzymes were calculated from non-linear regression analysis by using the Graphpad Prism Software package (version 5.0). The significance of differences between means was evaluated using one way ANOVA followed by Newman-Keuls multiple comparison test. Mean values were considered statistically different when $P < 0.05$.

Materials

L-3,4-dihydroxyphenylalanine, dopamine hydrochloride, (–)-adrenaline (+)-bitartrate salt bitartrate, L-(–)-noradrenaline (+)-bitartrate salt monohydrate, 3,4-dihydroxybenzylamine hydrobromide, L-tyrosine, D-tyrosine, 3-iodo-L-tyrosine, ferrous sulphate heptahydrate and 6-methyl-5,6,7,8-tetrahydropterin (6-BH_4) dihydrochloride, phentolamine hydrochloride were from Sigma Chemical Company (St. Louis, MO, USA). Other compounds used were as follows: S-adenosyl-homocysteine (Sigma, St. Louis, MO, USA), S-adenosyl-methionine (Sigma), DL-metanephrine hydrochloride (Sigma), pargyline hydrochloride (Sigma), yohimbine (Tocris, Ellisville, MO, USA), JP-1302 (Tocris). Ro-407592 (tolcapone) was synthesized at Laboratory of Chemistry, Department of Research and Development, BIAL. Pefabloc was obtained from Boehringer Mannheim (Germany).

Results

Tyrosine hydroxylase (TH) activity and protein expression

To determine the kinetic parameters of TH, saturation curves using the substrate (L-tyrosine) (Figure 1A) and the cofactor 6-BH_4 (Figure 1B) were performed. Incubation of the TH assay mixture prepared from brain of WT and each of the α_2-adrenoceptor KO mice in the presence of increasing concentrations of either L-tyrosine or 6-BH_4 resulted in a concentration-dependent formation of L-DOPA. The values of the kinetic parameters, V_{max} and K_M, obtained from the corresponding saturation curves are given in Table S1. As shown in this table, the V_{max} and K_M values for brain TH activity were similar between WT and all three α_2-adrenoceptor KO mouse strains. Results obtained from WT mice treated with the α_2-adrenoceptor antagonists JP-1302 and yohimbine show that the kinetic parameters for brain TH were similar between control and treated mice (Table S2), in both the substrate (Figure 2A) and cofactor (Figure 2B) assay.

Western blot analysis revealed that the levels of both TH total protein (Figure 1C) and the phosphorylated form of TH (TH-p-Ser40) (Figure 1D) were also similar between all three α_2-adrenoceptor KO mice and the WT. In agreement with the result obtained in the KOs, in mice treated with the selective α_2-adrenoceptor antagonists, TH total protein (Figure 2C) and TH-p-Ser40 (Figure 2D) expression values were similar to the control mice.

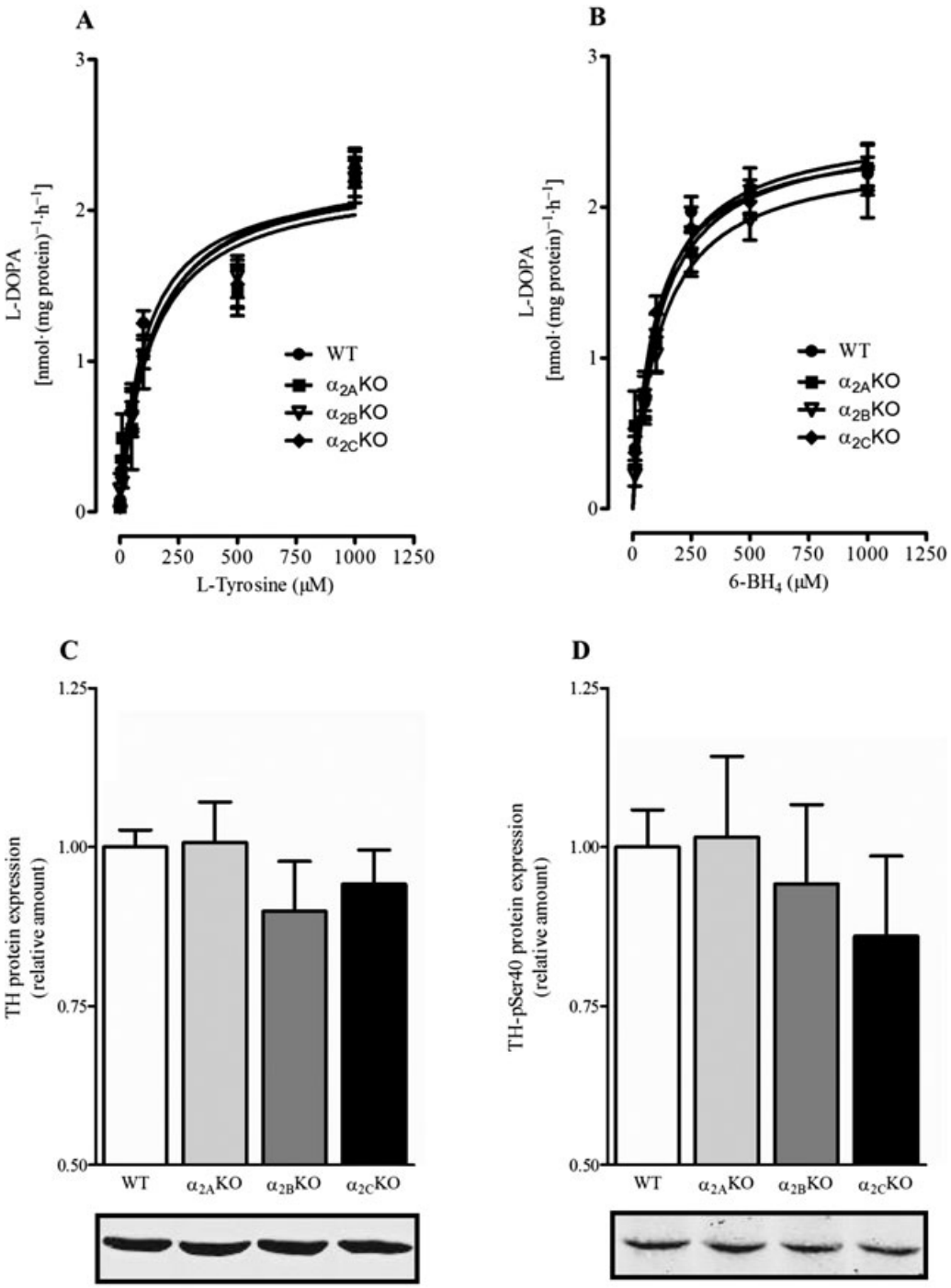

Figure 1 Tyrosine hydroxylase (TH) activity and expression. In (A) and (B), saturation curves of TH in brain homogenates of wild-type (WT) and knockout (KO) mice for each of the three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}) using increasing concentrations of the substrate l-tyrosine (A) or the cofactor 6-methyl-5,6,7,8-tetrahydropterin (6-BH$_4$) (B). In (C) and (D), representative Western blots with densitometric analysis of TH total protein (C) and TH protein phosphorylated at residue Ser40 (TH-pSer40; D), performed on protein extracts from whole brain of WT and α_{2A}, α_{2B} and α_{2C}KO mice. TH activity is expressed as the levels of 3,4-dihydroxyphenylalanine (L-DOPA) [in nmol·(mg protein)$^{-1}$·h^{-1}] formed during a given incubation period. Densitometric analysis of total TH and TH-pSer40 normalized to β-actin levels is expressed as fraction of control. Symbols, columns and vertical lines represent mean ± SEM of $n = 5$.

Aromatic L-amino acid decarboxylase (AAAD)

In order to determine the kinetic parameters of the enzyme, saturation curves using the substrate L-DOPA were performed. Incubation of the AAAD mixture prepared from brain of WT and each of the α_2-adrenoceptor KO mice in the presence of increasing concentrations of L-DOPA resulted in a concentration-dependent formation of dopamine (Figure 3). The kinetic parameters V_{max} and K_M are given in Table 1. As shown in this table, the V_{max} values were significantly higher in α_{2A} and α_{2C}KO mice compared with WT, with no significant differences in the α_{2B}KO mice. V_{max} values for α_{2A}KO mice were significantly higher compared with α_{2B} and α_{2C}KO mice. The

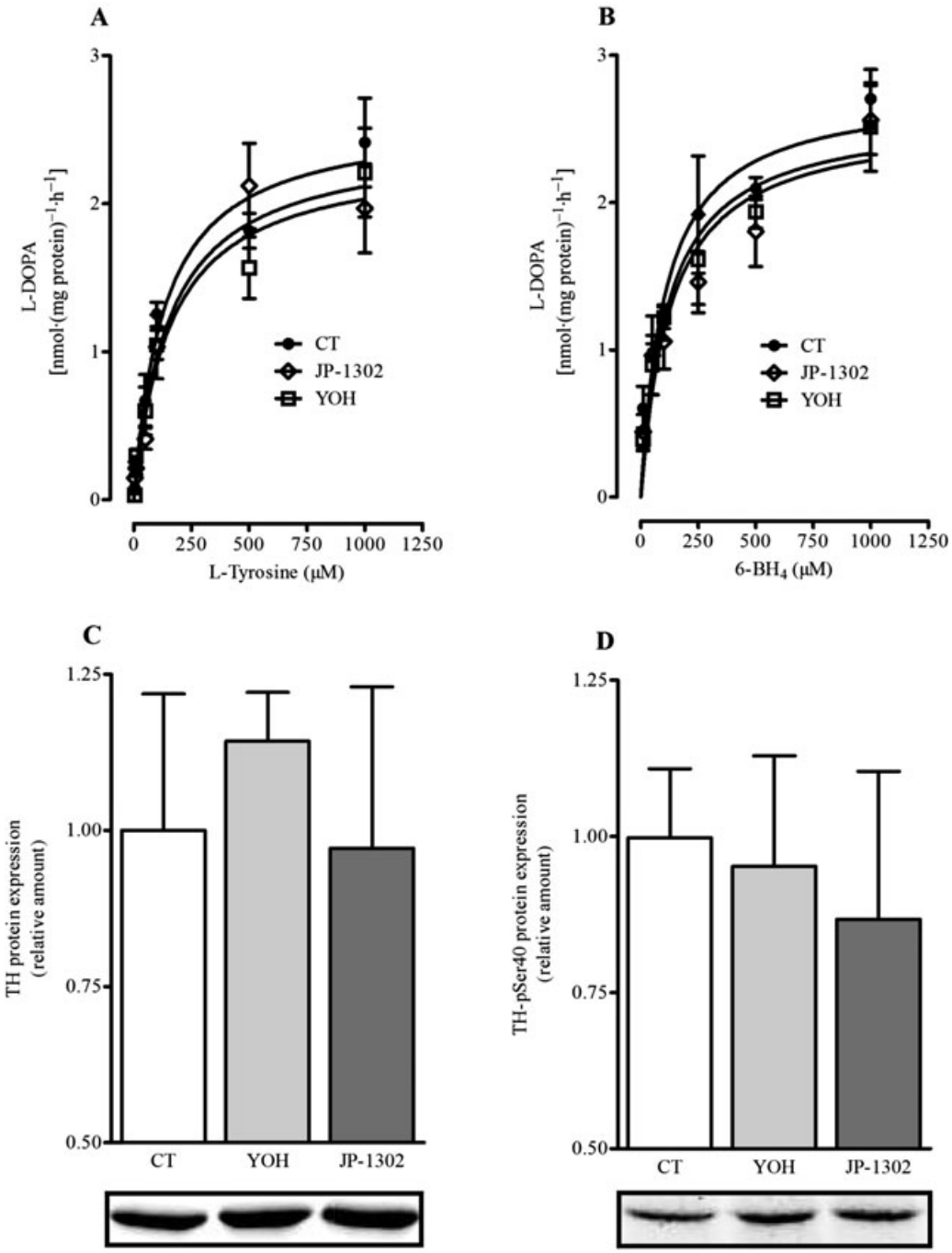

Figure 2 Tyrosine hydroxylase (TH) activity and expression. In (A) and (B), saturation curves of TH in brain homogenates of control (CT) mice and mice treated with yohimbine (YOH) (3 mg·kg^{-1}) or acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine (JP-1302) (3 μg·kg^{-1}) using increasing concentrations of the substrate L-tyrosine (A) or the cofactor 6-methyl-5,6,7,8-tetrahydropterin (6-BH$_4$) (B). In (C) and (D), representative Western blots with densitometric analysis of TH total protein (C) and TH protein phosphorylated at residue Ser40 (TH-pSer40; D), performed on protein extracts from whole brain from CT mice and mice treated with YOH (3 mg·kg^{-1}) or JP-1302 (3 μg·kg^{-1}). TH activity is expressed as the levels of 3,4-dihydroxyphenylalanine (L-DOPA) [in nmol·(mg protein)$^{-1}$·h^{-1}] formed during a given incubation period. Densitometric analysis of total TH and TH-pSer40 normalized to β-actin levels is expressed as fraction of CT. Symbols, columns and vertical lines represent mean ± SEM of $n = 6$.

K_M values were similar between all three α_2-adrenoceptor KO mice and the WT mice. In the study with the α_2-adrenoceptor, results show that AAAD activity was similar between control mice and the mice treated with either of the α_2-adrenoceptor antagonists (Table S3).

Dopamine β-hydroxylase (DβH)

To determine the kinetic parameters of the enzyme, saturation curves using the substrate (dopamine) were performed. Incubation of the DβH assay mixture prepared from brains of WT and each of the α_2-adrenoceptor KO mice in the presence

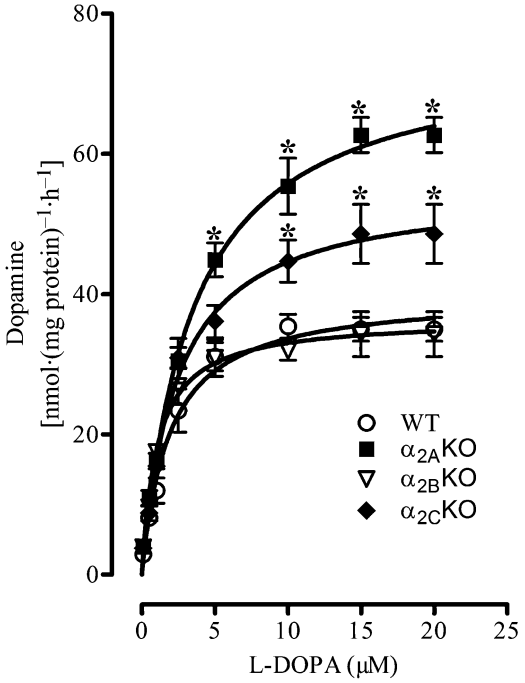

Figure 3 Saturation curves of AAAD activity in brain homogenates of WT and KO mice for each of the three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}). AAAD activity is expressed as the levels of dopamine [in nmol·(mg protein)$^{-1}$·h^{-1}] formed during a given incubation period. Symbols and vertical lines represent mean ± SEM of n = 5–8. *Significantly different from corresponding values in WT ($P < 0.05$). AAAD, aromatic L-amino acid decarboxylase; KO, knockout; L-DOPA, 3,4-dihydroxyphenylalanine; WT, wild-type.

of increasing concentrations of dopamine resulted in a concentration-dependent formation of noradrenaline. The values of the kinetic parameters, V_{max} and K_M, obtained from the corresponding saturation curves are given in Table S4. As shown in this table, the V_{max} and K_M values for DβH activity in the brain were similar between WT and all three α_2-adrenoceptor KO mouse strains.

Monoamine oxidase (MAO)

To determine the kinetic parameters of the enzyme, saturation curves using the substrate (dopamine) were performed. Incubation of the MAO assay mixture prepared from brains of WT and each of the α_2-adrenoceptor KO mice in the presence of increasing concentrations of dopamine resulted in a concentration-dependent formation of DOPAC. The values of the kinetic parameters, V_{max} and K_M, obtained from the corresponding saturation curves are given in Table S5. As shown in this table, the V_{max} and K_M values for MAO activity in the brain were similar between WT and all three α_2-adrenoceptor KO mouse strains.

*Catechol-*O*-methyltransferase (COMT)*

To determine the kinetic parameters of the enzyme, saturation curves using the substrate adrenaline were performed for the two isoenzymes of COMT (MB- and S-COMT) and for the combination of both – total COMT. Incubation of the total COMT assay mixture prepared from brains of WT and each of the α_2-adrenoceptor KO mice in the presence of increasing concentrations of adrenaline resulted in a concentration-dependent formation of metanephrine. The values of the kinetic parameters, V_{max} and K_M, obtained from the corresponding saturation curves are given in Table 2. Total COMT activity was significantly higher in all three α_2-adrenoceptor subtype KO mice compared with WT. COMT activity was significantly higher in α_{2A}KOs compared with α_{2B} and α_{2C}KOs. Incubation of an enzyme assay mixture containing S- or MB-COMT also resulted in a concentration-dependent increase in the *O*-methylation of adrenaline. There was no significant difference between α_2KO mice and WT in V_{max} values for the S-COMT (Table 2). The V_{max} values for the MB-COMT were significantly higher in all three α_2-adrenoceptor KO mice compared with WT and for α_{2A} and α_{2C}KO compared with α_{2B}KO (Figure 4 and Table 2). The K_M values were similar between the KO mice and the WT for the two isoforms and total COMT. Mice treated with either yohimbine or JP-1302 also presented a significant increase in total COMT activity compared with control mice (Figure 5 and Table 3)

Monoamine levels

The brain tissue levels of catecholamines and their metabolites for all three α_2-adrenoceptor KO mice and the WT mice are presented in Table 4. L-DOPA, dopamine and noradrenaline levels were significantly higher in the α_{2A} and α_{2C}KO mice compared with WT and α_{2B}KO mice. The α_{2A}KO presented higher levels of noradrenaline and dopamine compared with α_{2C}KO mice. Higher levels of NMN, DHPG and DOPAC were found in the brain of α_{2A}KO mice compared with α_{2B}KO, α_{2C}KO and WT mice. Treatment with the α_2-adrenoceptor antagonists also produced a significant increase in L-DOPA brain tissue levels, but not in dopamine or noradrenaline tissue levels (Table 5). Whereas DOPAC tissue levels were significantly increased only in mice treated with yohimbine, in mice treated with either yohimbine or JP-1302, NMN tissue levels were significantly increased (Table 5).

L-DOPA uptake

SK-N-SH cells took up L-DOPA in a concentration-dependent manner. Non-linear analysis of the saturation curves revealed for L-DOPA a K_M of 623 ± 76 μM and a V_{max} of 5.4 ± 0.2 nmol·(mg protein)$^{-1}$·(6 min)$^{-1}$ (Figure 6A). Incubation with different concentrations of the selective α_2-adrenoceptor agonist medetomidine (Figure 6B) produced a concentration-dependent decrease of L-DOPA uptake (IC_{50}, 7.3 ± 2.0 nM; E_{max}, 53 ± 10% control uptake). Pre-incubation with the selective α_2-adrenoceptor antagonist yohimbine (300 nM; Figure 6B) abolished the reduction produced by incubation with a range of doses of medetomidine. Incubation with different concentrations (0.1–1000 nM) of yohimbine alone did not produce a significant effect on L-DOPA uptake (Figure 6B).

Discussion and conclusions

The results presented here show that, in the CNS of mice, the increased spillover of catecholamines due to lack of α_{2A}- or

Table 1 Kinetic parameters maximum velocity (V_{max}) and Michaelis–Menten constant(K_M) of aromatic L-amino acid decarboxylase activity

	WT	*α_{2A}KO*	*α_{2B}KO*	*α_{2C}KO*
$V_{max.}$ [nmol·(mg protein)$^{-1}$·h^{-1}]	39.5 ± 0.3	76.9 ± 1.5*	40.3 ± 0.5$^{\#}$	49.9 ± 0.6*$^{\#}$
K_M (mM)	1.6 ± 0.1	3.7 ± 1.2	1.3 ± 0.1	1.9 ± 0.2

Enzyme assay was performed with brain homogenates obtained from wild-type (WT) mice and knockout (KO) mice for each of the three α_2-adrenoceptor subtypes (α_{2A}, α_{2A} and α_{2C}). Values are presented as mean ± SEM of n = 5–8.
*Significantly different from corresponding values in WT ($P < 0.05$).
$^{\#}$Significantly different from corresponding values in α_{2A}KO ($P < 0.05$).

Table 2 Kinetic parameters V_{max} and K_M for total (T), membrane-bound (MB) and soluble (S) COMT activity

	T-COMT		*MB-COMT*		*S-COMT*	
	V_{max} *[nmol·(mg protein)$^{-1}$·h^{-1}]*	K_M *(μM)*	V_{max} *[nmol·(mg protein)$^{-1}$·h^{-1}]*	K_M *(μM)*	V_{max} *[nmol·(mg protein)$^{-1}$·h^{-1}]*	K_M *(μM)*
WT	2.00 ± 0.04	2.02 ± 0.14	4.69 ± 0.12	2.20 ± 0.21	1.82 ± 0.07	156 ± 24
α_{2A}KO	2.35 ± 0.05*	1.64 ± 0.28	7.31 ± 0.06*	1.80 ± 0.07	1.84 ± 0.11	145 ± 35
α_{2B}KO	2.17 ± 0.01*$^{\#}$	1.95 ± 0.05	5.85 ± 0.10*$^{\#}$	1.90 ± 0.10	1.86 ± 0.08	175 ± 31
α_{2C}KO	2.16 ± 0.05*$^{\#}$	2.15 ± 0.16	6.69 ± 0.10*	1.65 ± 0.06	1.87 ± 0.09	185 ± 34

Enzyme assay was performed with brain homogenates obtained from WT mice and KO mice for each of the three α_2-adrenoceptor subtypes (α_{2A}, α_{2A} and α_{2C}). Values are presented as mean ± SEM of n = 5–8.
COMT, catechol-*O*-methyltransferase; K_M, Michaelis–Menten constant; KO, knockout; V_{max}, maximum velocity; WT, wild-type.
*Significantly different from corresponding values in WT ($P < 0.05$).
$^{\#}$Significantly different from corresponding values in α_{2A}KO ($P < 0.05$).

α_{2C}-adrenoceptors is accompanied by a higher uptake of the catecholamine precursor L-DOPA and by an increase in the activity of AAAD, an enzyme involved in monoamine synthesis, and of MB-COMT, an enzyme responsible for monoamine metabolism.

Brain tissue levels of L-DOPA, dopamine and noradrenaline were significantly higher in α_{2A} and α_{2C}KO mice compared with WT, an indication of increased catecholamine biosynthesis. The first step in catecholamine synthesis is the conversion of the amino acid L-tyrosine to L-DOPA, a reaction catalysed by TH. As this is usually taken to be the rate-limiting step of this pathway, TH is regulated by a large array of physiological mechanisms, both short- and long-term, including phosphorylation, feedback inhibition by all catecholamines and regulation of mRNA and protein synthesis (Kumer and Vrana, 1996). Therefore it would be expected that the observed changes in the pathway of synthesis would result primarily from changes in TH function with either increased activity (higher V_{max} values) or higher substrate affinity (lower K_M values). Surprisingly, no significant differences were found between α_2KO and WT mouse in TH activity or TH total protein expression. Nevertheless, TH activity may be increased by post-translational modifications such as phosphorylation that leads to an increase in affinity towards the substrate. Although TH can be phosphorylated at several serine (Ser) residues – Ser8, Ser19, Ser31 and Ser40, the most significant changes in TH activity occur when the protein is phosphorylated at Ser40 (Kumer and Vrana, 1996). Loss of endogenous α_2-adrenoceptors would result in a persistent increase in cAMP levels that could lead to enhanced phosphorylation of TH by protein kinase A and consequently to an increased activity. Because of this possibility, we assessed TH activity using different concentrations of the cofactor (6-BH_4),

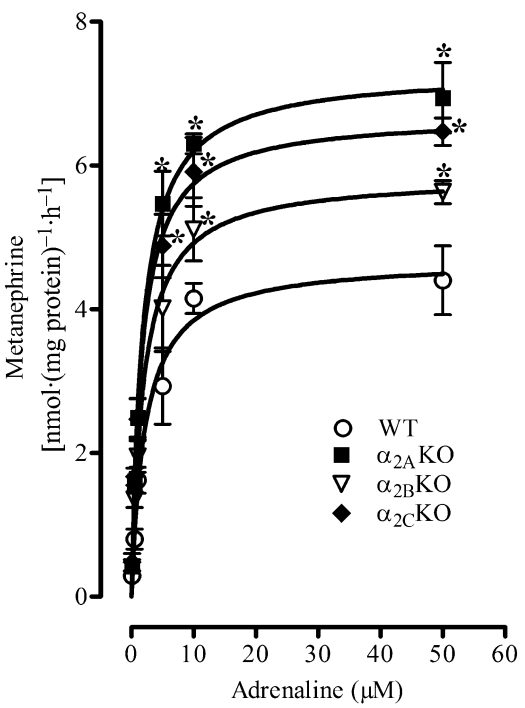

Figure 4 *O*-methylation of adrenaline by membrane-bound catechol-*O*-methyltransferase (MB-COMT) in brain homogenates of wild-type (WT) and knockout (KO) mice for each of the three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}). COMT activity is expressed as the levels of metanephrine [in nmol·(mg protein)$^{-1}$·h^{-1}] formed during a given incubation period. Symbols and vertical lines represent mean ± SEM of n = 5–8. *Significantly different from corresponding values in WT ($P < 0.05$).

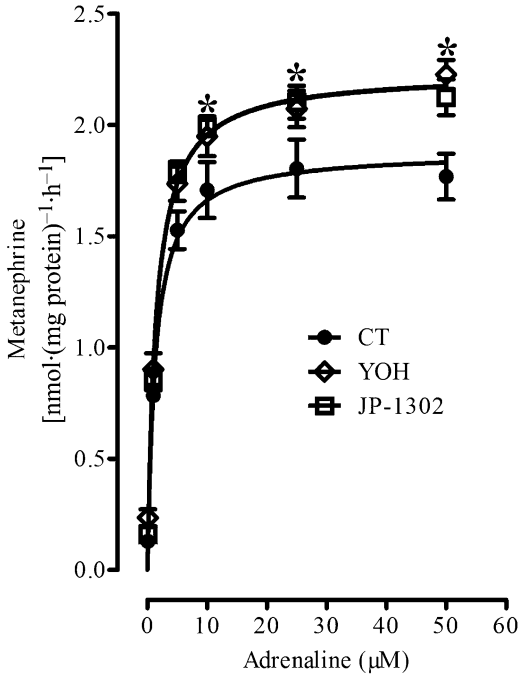

Figure 5 *O*-methylation of adrenaline by total catechol-*O*-methyltransferase (COMT) in brain homogenates from control (CT) mice and mice treated with yohimbine (YOH) (3 mg·kg^{-1}) or acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine (JP-1302) (3 μg·kg^{-1}). COMT activity is expressed as the levels of metanephrine [in nmol·(mg protein)$^{-1}$·h^{-1}] formed during a given incubation period. Symbols and vertical lines represent mean ± SEM of $n = 6$. *Significantly different from corresponding values in control ($P < 0.05$).

Table 3 Kinetic parameters V_{max} and K_M for total COMT activity

	CT	*YOH*	*JP-1302*
V_{max} [nmol·(mg protein)$^{-1}$·h^{-1}]	1.9 ± 0.1	2.3 ± 0.1*	2.2 ± 0.1*
K_M (μM)	1.3 ± 0.1	1.4 ± 0.2	1.5 ± 0.2

Enzyme assay was performed with brain homogenates obtained from CT mice and mice treated with YOH (3 mg·kg^{-1}) or JP-1302 (3 μg·kg^{-1}). Values are presented as mean ± SEM of $n = 6$.
COMT, catechol-*O*-methyltransferase; CT, control; JP-1302, acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine; K_M, Michaelis–Menten constant; V_{max}, maximum velocity; YOH, yohimbine.
*Significantly different from corresponding values in CT ($P < 0.05$).

for which affinity increases (K_M values decrease) when the enzyme is phosphorylated, as well as the levels of TH phosphorylated protein (TH-p-Ser40) by Western blot. The latter assay has been previously used to evaluate brain TH because it shows a good correlation between enzyme activity as measured by the endogenous levels of L-DOPA and the level of protein phosphorylation (Lew *et al.*, 1999; Lindgren *et al.*, 2000; Lindgren *et al.*, 2001). In agreement with the activity assay and the total protein expression, no significant differences were found in this form of phosphorylated TH between α_2KO mice and WT. However, given that our group has recently shown that TH activity and expression in rodents may be modulated by the α_2-adrenoceptor agonist, clonidine (Moura *et al.*, 2009), to test the lack of change in the α_2KO mice, the same experimental design was repeated in the brain of mice treated with an α_2-adrenoceptor antagonist (yohimbine) and a selective α_{2C}-adrenoceptor antagonist (JP-1302). These assays produced similar results to those obtained in the α_2KOs: no significant differences between the treated and the control group.

Previous studies have shown that chronic treatment with the α_2-adrenoceptor antagonists' yohimbine and atipamezole produces changes in central monoaminergic activity (Anden *et al.*, 1982; Haapalinna *et al.*, 1997) and that clozapine (20 mg·kg^{-1}, i.p.) produces an increase in TH activity 10 min after administration. However chronic administration (18 days) of imipramine was required to produce a change in TH activity (Rosin *et al.*, 1995). Although the data obtained for TH activity in mice treated with the α_2-adrenoceptor antagonists support the data from α_2KO mice, given that the latter have to cope with a life-long absence of the α_2-adrenoceptor, one should not exclude the idea that a longer period of drug treatment may be required to observe changes in TH activity or expression. Altogether, these findings are even more significant given that the endogenous levels of L-DOPA are usually taken as a measure of TH activity and that the latter is

Table 4 Brain tissue levels [in pmol·(mg tissue)$^{-1}$] of 3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), noradrenaline (NA), 5-hydroxytryptamine (5-HT), normetanephrine (NMN), 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylglycol (DHPG) and 3,4-dihydroxyphenylacetic acid (DOPAC) from wild-type (WT) and knockout (KO) mice for each of the three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C})

	WT	*α_{2A}KO*	*α_{2B}KO*	*α_{2C}KO*
L-DOPA	116 ± 17	301 ± 33*	129 ± 5	300 ± 13*
DA	2739 ± 509	6882 ± 711*	2309 ± 186#	4648 ± 861*#
NA	1087 ± 80	1851 ± 86*	1008 ± 46#	1526 ± 176*#
5-HT	1288 ± 76	1082 ± 34	1158 ± 63	1118 ± 119
NMN	111 ± 10	178 ± 15*	126 ± 12#	106 ± 24#
3-MT	136 ± 8	122 ± 2	95 ± 9	126 ± 8
DHPG	148 ± 12	156 ± 12	140 ± 23	143 ± 23
DOPAC	358 ± 52	653 ± 91*	247 ± 19#	317 ± 58#

Values are presented as mean ± SEM of $n = 8$–10.
*Significantly different from corresponding values in WT ($P < 0.05$).
#Significantly different from corresponding values in α_{2A}KO ($P < 0.05$).

Table 5 Brain tissue levels [in pmol·(mg tissue)$^{-1}$] of 3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), noradrenaline (NA), normetanephrine (NMN), 3-methoxytyramine (3-MT) and 3,4-dihydroxyphenylacetic acid (DOPAC) from control (CT) mice and mice treated with yohimbine (YOH) (3 mg·kg^{-1}) or acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine (JP-1302) (3 μg·kg^{-1})

	CT	*YOH*	*JP-1302*
L-DOPA	101 ± 12	180 ± 38*	208 ± 29*
DA	2821 ± 302	3870 ± 634	4326 ± 1060
NA	1244 ± 108	1209 ± 101	1257 ± 166
NMN	124 ± 9	206 ± 19*	183 ± 31
3-MT	155 ± 32	159 ± 57	189 ± 24
DOPAC	359 ± 52	652 ± 91*	389 ± 86#

Values are presented as mean ± SEM of $n = 6$.
*Significantly different from corresponding values in CT ($P < 0.05$).
#Significantly different from corresponding values in YOH ($P < 0.05$).

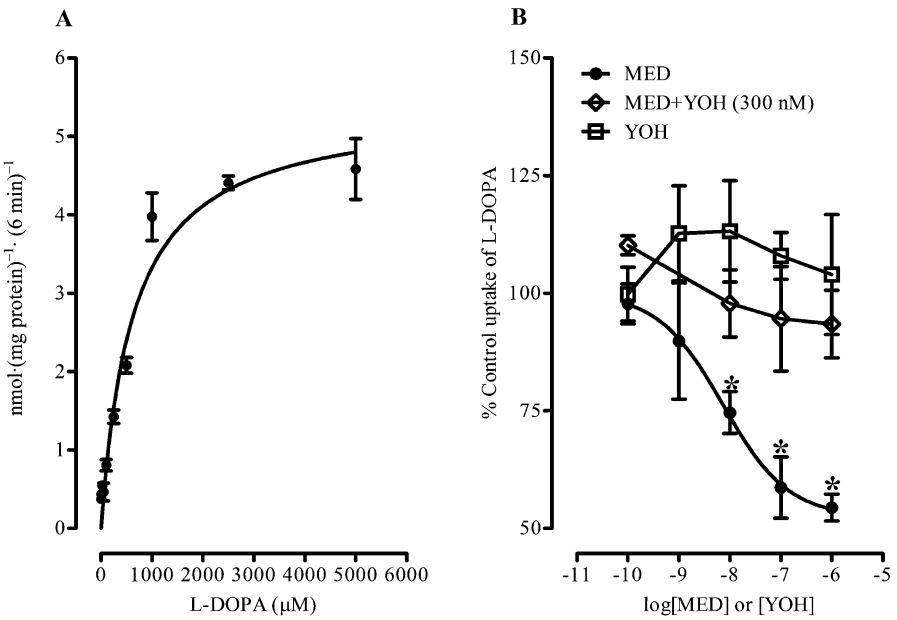

Figure 6 (A) Concentration-dependent accumulation of 3,4-dihydroxyphenylalanine (L-DOPA) in SK-N-SH cells. Cells were incubated for 6 min at 37 and 4°C, and increasing concentrations (25–5000 μM) of the substrate were applied from the apical cell side. (B) Effect of medetomidine (MED) and yohimbine (YOH) on L-DOPA uptake in SK-N-SH cells. Concentration–response curves were obtained by adding MED (0.1–1000 nM) 15 min before L-DOPA (2500 μM) in the presence or absence of a single concentration (300 nM) of YOH or by adding YOH (0.1–1000 nM) 30 min before L-DOPA (2500 μM). Symbols and vertical lines represent mean ± SEM of $n = 8$. *Significantly different from corresponding values in control ($P < 0.05$).

increased in the brain of α_{2A} and α_{2C}KO mice. In fact, higher L-DOPA tissue levels in the adrenal medulla of α_{2C}KO mice have been used as an indication of increased TH activity (Brede *et al.*, 2003), although they occur without changes in TH activity or in tissue levels of noradrenaline and dopamine in the adrenal medulla of α_{2C}KO mice (Moura *et al.*, 2006).

Given that TH activity is unchanged in α_{2A} and α_{2C}KO, one may exclude increased synthesis. Therefore, a possible explanation for the increase in L-DOPA levels could be increased uptake of the catecholamine precursor. To support this view we evaluated the effect of α_2-adrenoceptor activation, by the selective agonist medetomidine on L-DOPA transport in a neuronal cell line that expresses both the L-DOPA transporter (LAT) and α_2-adrenoceptors (Baron and Siegel, 1989; Hashimoto *et al.*, 2005). Our data show that activation of α_2-adrenoceptors decreased L-DOPA uptake in this human neuroblastoma cell line, giving further support to the idea that α_2-adrenoceptors may act to reduce cellular uptake of the catecholamine precursor. Moreover, treatment with yohimbine, a selective α_2-adrenoceptor antagonist, or JP-1302, a selective α_{2C}-adrenoceptor antagonist, significantly increased L-DOPA brain tissue levels. In fact, a previous report has shown that administration of the α_2-adrenoceptor antagonist atipamezole in combination with L-DOPA selectively increased dopamine overflow in dopaminergic presynaptic terminals of the rat (Yavich *et al.*, 2003). Furthermore, atipamezole improved the efficacy of L-DOPA and apomorphine in an animal model of Parkinson's disease and also reduced adverse dopaminergic effects on vigilance and on cardiovascular function (Haapalinna *et al.*, 2003). Finally, it is worth mentioning that α_2-adrenoceptors and the L-DOPA transporter are also expressed in astrocytes present at the blood brain barrier (Hansson and Ronnback, 1992) and that this mechanism may also contribute in part to the higher brain tissue levels of L-DOPA. Therefore, the effect of α_2-adrenoceptor antagonists at the pre-synaptic and post-synaptic level on L-DOPA uptake could be particularly relevant regarding the role of dopamine in Parkinson's disease and of L-DOPA in the treatment of this disorder.

The second step in catecholamine biosynthesis is the decarboxylation of L-DOPA to dopamine, a reaction catalysed by AAAD. In the CNS, AAAD is mainly localized in catecholamine and 5-HT containing neurons (Hökfelt *et al.*, 1973). Different mechanisms exist for the short- and long-term regulation of AAAD, similar to those for TH. However, the primary mechanism for regulation of this enzyme seems to be the long-term changes in RNA synthesis that result in changes of enzyme activity (Coge *et al.*, 1990). As AAAD is responsible for virtually all the decarboxylation of L-DOPA and that there are no other enzymes capable of converting L-DOPA into dopamine, the higher levels of dopamine could be accounted by the increased activity of AAAD found in the brain of α_{2A} and α_{2C}KO mice. In fact, the increase in AAAD activity and the increase in dopamine tissue levels are parallel: a twofold increase in V_{max} values in α_{2A}KO mice is accompanied by a 2.5-fold increase in dopamine tissue levels; a 1.3-fold increase in V_{max} values in α_{2C}KO mice is accompanied by a 1.7-fold increase in dopamine tissue levels. However, treatment with the α_2-antagonists did not produce significant changes in either dopamine tissue levels or in AAAD activity. A possible explanation for this lack of effect could be the that changes in AAAD activity may take longer to occur, with the observations in α_2KO representing a life-long adaptation to the lack of the receptor. An example of this late activation is the effect of the

promiscuous compound clozapine on AAAD activity in the mouse striatum: an early and transient activation is followed by a late and prolonged induction (Neff *et al.*, 2006). Although a previous study has shown that α_2-adrenoceptor activation reduces AAAD activity (Rossetti *et al.*, 1989), a longer period of treatment with the α_2-antagonists may be required to assess the possibility of targeting the α_2-adrenoceptors as modulators of AAAD activity in the CNS.

In noradrenergic neurons, DβH is the enzyme responsible for catalysing the final step in catecholamine synthesis, the conversion of dopamine to noradrenaline. Despite the increase in noradrenaline levels no significant differences in DβH activity were found between α_{2A} or α_{2C}KOs and WT mice. DβH is the enzyme with the highest K_M values, in the 40 mM range, of the catecholamine synthesis pathway. It is unlikely that an increase in DβH enzyme activity is required to match the increase in the levels of dopamine in order to produce more noradrenaline. Thus, and although DβH is required for noradrenaline synthesis, the increased activity in AAAD is more likely to account also for the increase in noradrenaline synthesis.

The synaptic action of catecholamines is terminated primarily by reuptake into presynaptic nerve terminals (uptake 1) where they are either returned into storage vesicles or metabolized by MAO-A. There is a less important route, uptake into glial cells (uptake 2), where catecholamines are metabolized by COMT and/or MAO-B (Eisenhofer *et al.*, 2004). With respect to catecholamine metabolism, our data show that whereas lack of any of the three α_2-adrenoceptor subtypes did not influence MAO activity, all three α_2KO mice presented an increased COMT-MB activity. Uptake by the dopamine transporter is the most effective mechanism for terminating the synaptic actions of dopamine. In fact, dopamine oxidation by MAO is the catabolic route preferred to dopamine methylation by COMT (Huotari *et al.*, 2002). The α_{2A}-adrenoceptor is the main subtype controlling the release of noradrenaline from noradrenergic neurons, acting as an auto-receptor (Trendelenburg *et al.*, 2003) and of dopamine from dopaminergic neurons, acting as a heteroreceptor (Bucheler *et al.*, 2002). So it is not surprising that despite the lack of change in MAO activity, that α_{2A}KO mice present higher levels of DOPAC, a product resulting from the metabolism of dopamine via MAO, but no significant differences in 3-MT levels, a product of dopamine metabolism via COMT. In good agreement, a similar result was obtained in mice treated with the α_2-antagonist yohimbine: an increase in DOPAC levels but not of 3-MT.

On the other hand, we here provide for the first time, evidence that COMT may be a more efficient pathway for noradrenaline catabolism than MAO-A and that this metabolic pathway is attuned to small changes in increased nerve activity. The role of α_{2B}-adrenoceptors in the control of noradrenaline release in the CNS has been shown *in vitro* (Trendelenburg *et al.*, 2003) but with little or no significance *in vivo* (Bucheler *et al.*, 2002; Lahdesmaki *et al.*, 2003). The fact that COMT activity is increased even in α_{2B}KO mice demonstrates that this catabolic pathway was responsive even to the small increase in noradrenaline release that occurs in the CNS of these mice. In fact, WT mice treated with either α_2-antagonist, yohimbine or JP-1302 showed a significant increase in total COMT activity and NMN levels. This idea is further supported by the finding that MB-COMT was increased without changes in S-COMT. The MB-COMT, despite representing the minor isoform of COMT in most tissues, is the most important isoenzyme for *O*-methylation of catecholamines in the brain, acting predominantly at low physiological concentrations of catecholamines (Roth, 1992; Lotta *et al.*, 1995). This is due to the difference in kinetic behaviour between S- and MB-COMT: MB-COMT has a 100-fold higher affinity for catechol substrates ($K_M \sim 2$ μM) (Vieira-Coelho and Soares-da-Silva, 1999).

The transmitter and related metabolite measurements were made in a whole brain homogenate, thus one observes only the net effect of the drugs on transmitter systems. The measurement of whole brain transmitter changes is probably not valid for studying the role of α_2-adrenoceptors for regional transmitter release. However, it could be claimed that the *in vivo* net effect reveals the difference between a specific and non-specific drug. Similarly it is the net effect, that is, the interaction of the drug and the endogenous state of an animal that is observed in behavioural studies. Changes in the expression of α_2-adrenoceptors have been related to behavioural changes in mice. Studies performed *in vivo* have shown that although α_{2A}-adrenoceptors mediate most of the classical responses to α_2-adrenoceptor agonists, such as hypotension, anti-nociception, sedative-hypnotic and anaesthetic sparing effects, the α_{2C}-adrenoceptors seem to have more subtle effects on behaviour in mice. α_{2C}KO mice demonstrated increased startle reactivity, impaired sensorimotor gating, impulsive aggression and a tendency for locomotor overactivity in stimulated conditions (Scheinin *et al.*, 2001). A polymorphism of the gene for the α_{2C}-adrenoreceptor, the deletion of four consecutive amino acids at codons 322–325 of the α_{2C}-adrenoceptor gene designated α_{2C}-Del322–325-AR (Small *et al.*, 2000), has been identified in humans. Results suggest that this polymorphism confers a change in brain function that may be associated with the pathophysiology of major depressive disorder (Neumeister *et al.*, 2006). Given that, the distribution of the α_{2C}-adrenoceptor subtype pattern appears to be conserved between rodents and humans (Fagerholm *et al.*, 2008), results obtained from studies of the α_{2C}-adrenoceptor in rodent models may be relevant for human diseases associated to neuropsychiatric disorders. Furthermore, given that α_{2A}-adrenoceptors mediate a large array of functions, pharmacological targeting of α_{2C}-adrenoceptors may be a more useful approach in disorders related to changes in α_2-adrenoceptors (Sallinen *et al.*, 2007).

In conclusion, in the CNS, a new mechanism for α_{2A}- or α_{2C}-adrenoceptors related to the control of catecholamine synthesis and release may be added to their well-known role as inhibitory feedback regulators of dopamine and noradrenaline release: inhibition of the uptake of the catecholamine precursor L-DOPA. Furthermore, lack of either α_{2A}- or the α_{2C}-adrenoceptor subtype results in a long-term increase in dopamine and noradrenaline synthesis that may be explained by an increase in AAAD activity. Finally, in α_{2A}KO mice the higher rate of synthesis and release is also accompanied by an increase in metabolism of the two monoamines via different pathways: the methylation pathway for noradrenaline (COMT-MB) and the oxidative pathway for dopamine (MAO) (Figure 7).

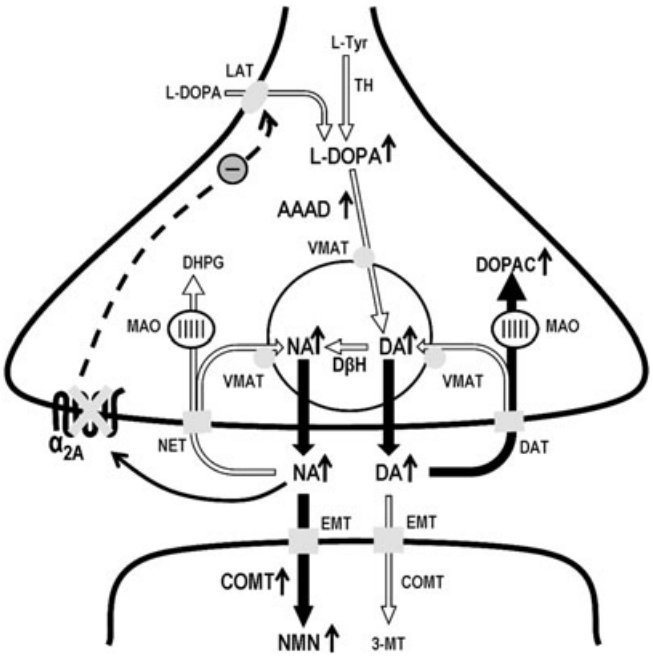

Figure 7 Scheme of changes observed in NA and DA turnover in the brain of α_{2A}KO mice. Open arrows indicate unaltered pathways; bold arrows and ↑ indicate increased enzyme activity or monoamine levels; dotted arrow indicates suggested mechanism; (-) indicates inhibitory mechanism. Deletion of the α_{2A}-adrenoceptor blunts the inhibitory mechanism over AAAD activity and L-DOPA transport into the neuron, resulting in an increase in DA and NA synthesis. This higher rate of synthesis and release is met by an increase in the metabolic pathways of oxidation via MAO for DA and of methylation via COMT for NA. 3-MT, 3-methoxytyramine; AAAD, aromatic L-amino acid decarboxylase; COMT, catechol-*O*-methyltransferase; DA, dopamine; DAT, dopamine transporter; DβH, dopamine β-hydroxylase; DHPG, 3,4-dihydroxyphenylglycol; DOPAC, 3,4-dihydroxyphenylacetic acid; EMT, extracellular monoamine transporter; L-DOPA, 3,4-dihydroxyphenylalanine; MAO, monoamine oxidase; NA, noradrenaline; NET, noradrenaline transporter; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter.

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Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Kinetic parameters maximum velocity (V_{max}) and Michaelis–Menten constant (K_M) of tyrosine hydroxylase activity for the substrate (L-tyrosine) and the cofactor 6-methyl-5,6,7,8-tetrahydropterin (6-BH_4)
Table S2 Kinetic parameters maximum velocity (V_{max}) and Michaelis–Menten constant (K_M) of tyrosine hydroxylase activity for the substrate (L-tyrosine) and the cofactor 6-methyl-5,6,7,8-tetrahydropterin (6-BH_4)
Table S3 Kinetic parameters maximum velocity (V_{max}) and Michaelis–Menten constant (K_M) of aromatic L-amino acid decarboxylase activity
Table S4 Kinetic parameters maximum velocity (V_{max}) and Michaelis–Menten constant (K_M) of dopamine β-hydroxylase activity
Table S5 Kinetic parameters maximum velocity (V_{max}) and Michaelis–Menten constant (K_M) of monoamine oxidase activity

Appendix S1 Detailed methods.

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