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[Ethyl-^{3}H]RS-79948-197 α_2-adrenoceptor autoradiography validation in α_2-adrenoceptor knockout mice

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Abstract

[Ethyl-^{3}H][8*aR*,12*aS*,13*aS*]-5,8,8*a*,9,10,11,12,12*a*,13,13*a*-decahydro-3-methoxy-12-(ethylsulfonyl)-6*H*-isoquino[2,1-*g*][1,6]naphthyridine ([ethyl-^{3}H]RS-79948-197) was evaluated for α_2-adrenoceptor autoradiography in brain sections from wild-type mice and α_{2A}- and α_{2ABC}-adrenoceptor knockout mice. Receptor numbers were 83% lower in cortex and 28% lower in caudate putamen of α_{2A}-knockout mice than in wild-type mice. No specific binding was seen in α_{2ABC}-knockout mice. [Ethyl-^{3}H]RS-79948-197 saturation binding parameters were compared to those of [^{3}H]2-(2,3-dihydro-2-methoxy-1,4-benzodioxan-2-yl)-4,5-dihydro-1*H*-imidazoline ([^{3}H]RX821002) and [methyl-^{3}H]17α-hydroxy-20α-yohimban-16β-carboxylic acid methyl ester ([methyl-^{3}H]rauwolscine). [Ethyl-^{3}H-]RS-79948-197 detected a larger number of both α_{2A}- and $\alpha_{2B/C}$-adrenoceptors than [^{3}H]RX821002, while [methyl-^{3}H]rauwolscine only underestimated the number of α_{2A}-adrenoceptors. Oxymetazoline and prazosin competed for [ethyl-^{3}H]RS-79948-197 binding with the expected rank order of affinities. Higher than necessary [ethyl-^{3}H]RS-79948-197 concentrations resulted in a rapid increase in non-specific binding. Slow dissociation kinetics, high specific radioactivity and high α_2-adrenoceptor affinity (slightly lower for the α_{2A}-adrenoceptor than for the other subtypes) confer [ethyl-^{3}H]RS-79948-197 distinct advantages compared to [^{3}H]RX821002 for detection of α_2-adrenoceptor subtypes in a mixed α_2-adrenoceptor population.

Keywords: Receptor autoradiography; α_{2A}-Knockout; α_{2ABC}-Knockout; RX821002; Rauwolscine

1. Introduction

α_2-Adrenoceptors mediate many of the physiological effects of adrenaline and noradrenaline both in the central nervous system and in peripheral tissues. There are three α_2-adrenoceptor subtypes, α_{2A}, α_{2B} and α_{2C}, with distinct tissue distributions. Hypotension, sedation, analgesia and hypothermia are centrally mediated effects of α_{2A}-adrenoceptor activation. Little is known about the functions of the other α_2-adrenoceptor subtypes in brain. Receptor autoradiography is a quantitative method to localise receptors in tissue sections, and is especially useful when receptors are heterogeneously distributed, such as in the central nervous system. The conclusions drawn from autoradiographic studies rely on the assumption that the employed ligands are specific for the receptors of interest, wherefore thorough ligand characterisation is of great importance. The binding profiles of new drugs are often first examined in cell lines with high receptor expression levels. Native tissues contain many components, including receptors or receptor subtypes, not present in cell lines, and interactions with such binding sites may result in misinterpretation of the binding results.

[Ethyl-^{3}H][8*aR*,12*aS*,13*aS*]-5,8,8*a*,9,10,11,12,12*a*,13,13*a*-decahydro-3-methoxy-12-(ethylsulfonyl)-6*H*-isoquino[2,1-*g*][1,6]naphthyridine ([ethyl-^{3}H]RS-79948-197) is the latest addition to the list of commercially available α_2-adrenoceptor antagonist radioligands, which includes tritiated 2-(2,3-dihydro-2-methoxy-

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1,4-benzodioxan-2-yl)-4,5-dihydro-1*H*-imidazoline (RX821002), 17α-hydroxy-20α-yohimban-16β-carboxylic acid methyl ester (rauwolscine), 17-hydroxyyohimban-16-carboxylic acid methyl ester (yohimbine) and (2*S*, 12*bS*)dimethylspiro(1,3,4,5′ ,6,6′ ,7,12*b*-octahydro-2*H*-benzo[*b*]furo[2,3-*a*]quinazoline)-2,4′ -pyrimidin-2′ one (MK-912). RX821002 has relatively high affinity for all three α_2-adrenoceptor subtypes, while rauwolscine and yohimbine have clear preference for the α_{2C}-adrenoceptor and show significantly lower affinity for the mouse and rat α_{2A}-adrenoceptor (Michel et al., 1989; Lanier et al., 1991; Link et al., 1992; O'Rourke et al., 1994; Renouard et al., 1994). MK-912 is the most α_{2C}-selective of these compounds, with an approximately 20-fold preference for the α_{2C}- over α_{2A}- and α_{2B}-adrenoceptors in the rat (Uhlén et al., 1998). Previous studies on native and recombinant α_2-adrenoceptors in membrane preparations have shown [ethyl-^{3}H]RS-79948-197 to possess both high α_2-adrenoceptor affinity, with somewhat higher affinity for α_{2C}- and α_{2B}- than for α_{2A}-adrenoceptors, and high selectivity relative to α_1, 5-HT_{1A}, 5-HT_2, D_1, D_2, I_2, M_1, M_2 and dihydropyridine binding sites (pK_i<6) (Milligan et al., 1997; Uhlén et al., 1998). This suggested that [ethyl-^{3}H]RS-79948-197 would be useful for autoradiographic detection of α_2-adrenoceptors in brain sections. [^{3}H]RX821002 and [methyl-^{3}H]rauwolscine binding in rat and mouse brain sections has been previously described (Boyajian and Leslie, 1987; Boyajian et al., 1987; Hudson et al., 1992; Wamsley et al., 1992; Holmberg et al., 2003), and the binding properties of [^{125}I]17α-hydroxy-20α-yohimban-16β-(*N*-4-hydroxyphenethyl)carboxamide ([^{125}I]rauwolscine-OHPC) in mouse brain sections were also recently reported (Dossin et al., 2000).

In this study, [ethyl-^{3}H]RS-79948-197 binding in mouse brain sections was characterised in the cerebral cortex, which is rich in α_{2A}-adrenoceptors, and in the caudate putamen, which contains mainly α_{2C}-adrenoceptors. As a control for radioligand binding, we utilised α_2-adrenoceptor knockout mice. The binding of [ethyl-^{3}H]RS-79948-197 was compared to that of [^{3}H]RX821002 and [methyl-^{3}H]rauwolscine in wild-type control (WT) and in α_{2A}-adrenoceptor knockout (α_{2A}-KO) mice. The binding of [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 to non-α_2-adrenoceptor sites (non-specific binding) was further investigated in a mouse line lacking all three α_2-adrenoceptor subtypes (α_{2ABC}-KO).

2. Materials and methods

2.1. Drugs

[Ethyl-^{3}H]RS-79948-197, specific radioactivity 3.2–3.3 GBq/μmol, and [^{3}H]RX821002, specific radioactivity 2.1 GBq/μmol, were purchased from Amersham Biosciences (Buckinghamshire, UK). [Methyl-^{3}H]rauwolscine, specific radioactivity 2.6 GBq/μmol, was purchased from Perkin-Elmer (Boston, MA, USA). 2-[*N*-(*m*-hydroxyphenyl)-*p*-toluidinomethyl]imidazoline hydrochloride (phentolamine), 2-[3-hydroxy-2,6-dimethyl-4-*t*-butylbenzyl]-2-imidazoline hydrochloride (oxymetazoline) and 1-[4-amino-6,7-dimethoxy-2-quinazolinyl]-4-[2-furanylcarbonyl]-piperazine hydrochloride (prazosin) were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental animals and tissue preparation

Animal care was in accordance with the guidelines of the International Council of Laboratory Animal Science (ICLAS). The study was approved by the laboratory animal welfare committee of the University of Turku. WT C57Bl/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained for at least 7 days at the local animal facility before they were used. Congenic α_{2A}-KO mice on a C57Bl/6J genetic background (Altman et al., 1999) were bred locally. α_{2ABC}-KO mice were generated from single knockout lines as described previously (Philipp et al., 2002). α_{2ABC}-KO mice were previously reported to die during embryonic development due to an extraembryonic defect in the yolk sac and placenta (Philipp et al., 2002). However, the penetrance of this genotype was incomplete, and a few surviving mice deficient in all three α_2-adrenoceptor subtypes could be recovered. The genotypes of the α_2-adrenoceptor deficient mouse lines were determined by polymerase chain reaction (PCR) with allele specific primers (Philipp et al., 2002). WT and α_{2A}-KO male mice, 12–14 weeks old, were anaesthetised with CO_2 and decapitated. The brains were frozen by immersion in 2-methyl butane (isopentane) chilled on frozen CO_2. For the experiments on α_{2ABC}-KO brain sections, one female mouse, 6 months old, was used. Coronal 14 μm brain sections at the level of the caudate putamen were thaw-mounted onto gelatin-coated glass slides, and stored frozen until used.

2.3. Radioligand binding

Mouse brain sections were incubated in 50 mM potassium phosphate buffer, pH 7.4, with radioligand and unlabelled ligands as appropriate. Non-specific binding, defined as binding in the presence of 10 μM phentolamine, was determined from consecutive sections at each radioligand concentration or time point. To determine [ethyl-^{3}H]RS-79948-197 dissociation rates, sections were incubated with 1 nM [ethyl-^{3}H]RS-79948-197 for 1 h, then washed in buffer on ice for various times up to 4 h, or at room temperature for up to 6 h. Association rates were determined by incubating with 1 nM [ethyl-^{3}H]RS-79948-197 for various times up to 4 h, followed by a 20-min wash in buffer on ice. For saturation and competition binding experiments, radioligand incubation times were 20 min for [^{3}H]RX821002, and 1 h for [ethyl-^{3}H]RS-79948-197 and [methyl-^{3}H]rauwolscine.

Washes were performed on ice, and were 2 s+20 min for [ethyl-^{3}H]RS-79948-197, 2×30 min for [methyl-^{3}H]rauwolscine and 2×2 min for [^{3}H]RX821002. All slides were subsequently dipped in ice-cold deionised water, and dried in an ambient-temperature stream of air. The dry slides, together with tritium standards (ARC, St. Louis, MO, USA), were apposed to autoradiographic film; ^{3}H-Hyperfilm (Amersham) for 6 weeks (WT and α_{2A}-KO), or Kodak BioMax MR (Kodak, Rochester, NY, USA) for 18 weeks (α_{2ABC}-KO).

2.4. Quantitative autoradiography and data analysis

Densitometric analysis was performed with the AIS image analysis station (Imaging Research, St. Catharines, Ontario, Canada) connected to a charge-coupled device (CCD) camera. From each animal, bilateral readings from two to three sections per ligand concentration or time point were collected from the dorsolateral cerebral cortex (motor and somatosensory cortex) and from the caudate putamen, as outlined in Fig. 1A. Relative optical density values were transformed into an estimate of femtomoles of bound radioligand per milligram tissue by reference to tritium standards co-exposed with the brain sections (Geary and Wooten, 1983; Geary et al., 1985).

The binding data were analysed with non-linear regression analysis using the computer programme GraphPad Prism, version 2.01 (GraphPad Software, San Diego, CA, USA). Saturation binding group means were compared using one-way analysis of variance (one-way ANOVA) followed by Tukey's test for multiple comparisons. Pharmacological variables were calculated separately from each animal and reported as means±S.E.M. The level of statistical significance was established at $P=0.05$.

3. Results

3.1. Anatomical distribution of binding sites

Consecutive WT and α_{2A}-KO mouse brain sections labelled with 2 nM [ethyl-^{3}H]RS-79948-197, [^{3}H]RX821002 and [methyl-^{3}H]rauwolscine are illustrated in Fig. 1. The binding patterns seen with [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 were similar, revealing substantial region-specific reductions in binding in α_{2A}-KO, as compared to WT mouse brain sections. In contrast, genotype differences in [methyl-^{3}H]rauwolscine binding were less evident, and the [methyl-^{3}H]rauwolscine binding pattern in both WT and α_{2A}-KO mouse brain sections

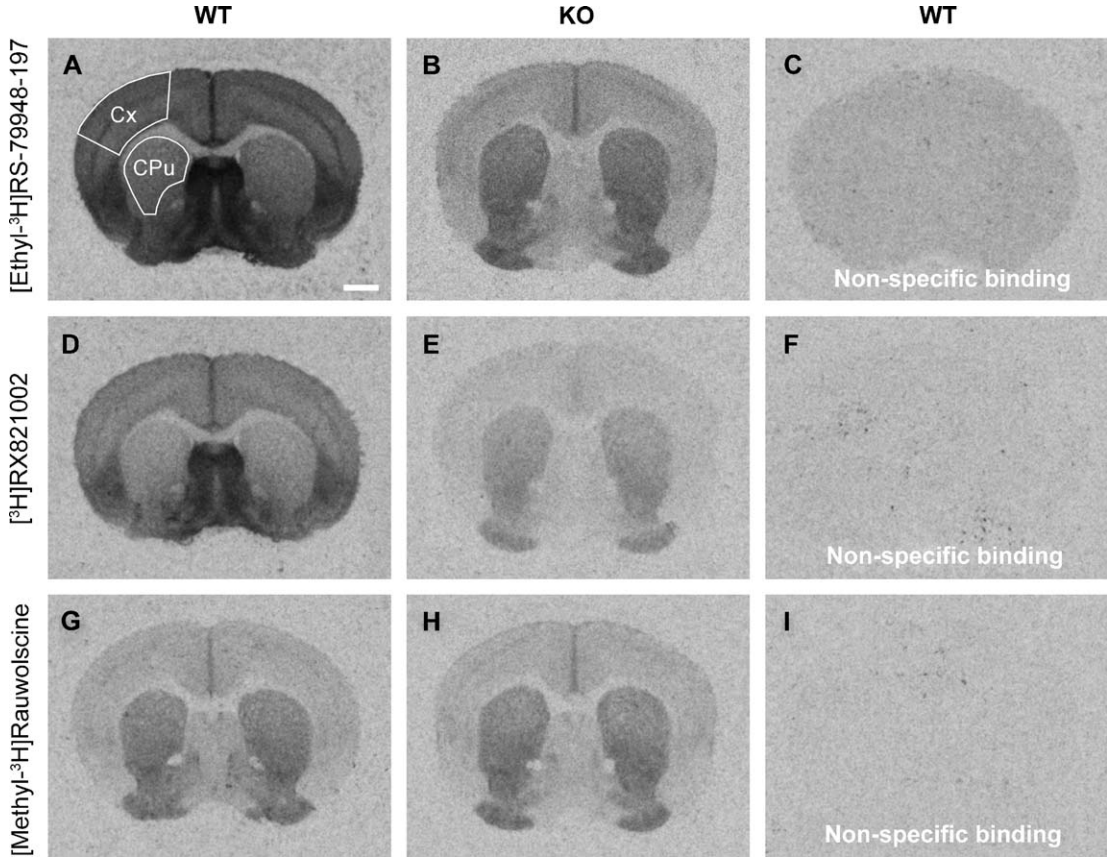

Fig. 1. Autoradiographic images of WT (A, C, D, F, G, I) and α_{2A}-KO (B, E, H) coronal mouse brain sections at the level of the caudate putamen, labelled with 2 nM [ethyl-^{3}H]RS-79948-197 (A, B, C), [^{3}H]RX821002 (D, E, F) and [methyl-^{3}H]rauwolscine (G, H, I). Phentolamine (10 μM) was used to define non-specific binding (C, F, I). The analysed brain regions (Cx, cortex; CPu, caudate putamen) are outlined in A. Scale bar=1 mm.

resembled the binding of [ethyl-^{3}H]RS79948-197 and [^{3}H]RX821002 in α_{2A}-KO mouse brain sections. Radioligand binding in the presence of 10 μM phentolamine, defined as non-specific binding, and radioligand binding in α_{2ABC}-KO mouse brain sections were low and homogenous at radioligand concentrations sufficient to label most α_2-adrenoceptors. Excessive amounts (>2.5 nM) of [ethyl-^{3}H]RS-79948-197 rapidly increased the level of non-specific binding, which was non-specific binding was higher in the rhombencephalon, especially in the cerebellum, than in the telencephalon.

3.2. Association and dissociation of [ethyl-^{3}H]RS-79948-197 binding

Kinetic binding experiments with [ethyl-^{3}H]RS-79948-197 were performed on WT and α_{2A}-KO mouse brain sections from three animals of each genotype, in order to determine incubation and washing times for the equilibrium binding experiments, and to validate the equilibrium dissociation constants (K_d) obtained from saturation binding experiments. Specific binding reached an apparent steady state within 30 min and remained stable until 100 min, whereafter a slow decline in binding was seen (Fig. 2A,B). When dissociation of binding was investigated in ligand-free buffer on ice, there was very little reduction of specific binding during 4 h, and dissociation rates could not be determined. At room temperature, specific binding was eliminated in 6 h (Fig. 2C,D). Based on these results, a 1-h radioligand incubation time at room temperature, and a 20-min wash time on ice was employed in subsequent [ethyl-^{3}H]RS-79948-197 saturation and competition binding experiments. Association and dissociation binding data were fit to one-phase exponential equations. The kinetic K_d values derived from the obtained dissociation and apparent association rate constants were in the pM range, and indicated a slight $\alpha_{2B/C}$-adrenoceptor preference of [ethyl-^{3}H]RS-79948-197 (Table 1).

Table 1
[Ethyl-^{3}H]RS-79948-197 kinetic binding parameters in WT and α_{2A}-KO mouse brain sections

	WT Cx	WT CPu	α_{2A}-KO Cx	α_{2A}-KO CPu
k_{ob} (min^{-1})	0.16±0.01	0.37±0.05	0.51±0.15	1.24±0.20
k_{off} (min^{-1})	0.012±0.001	0.025±0.002	0.033±0.01	0.041±0.004
K_d (nM)	0.091	0.079	0.076	0.038

Cx, cortex; CPu, caudate putamen. Values are means±S.E.M. (n=3), except for the kinetic equilibrium dissociation constants (K_d), which were calculated from the experimentally determined observed association rate constants (k_{ob}) and dissociation rate constants (k_{off}). $K_d=k_{off}/k_{on}$, where $k_{on}=(k_{ob}-k_{off})$/[radioligand].

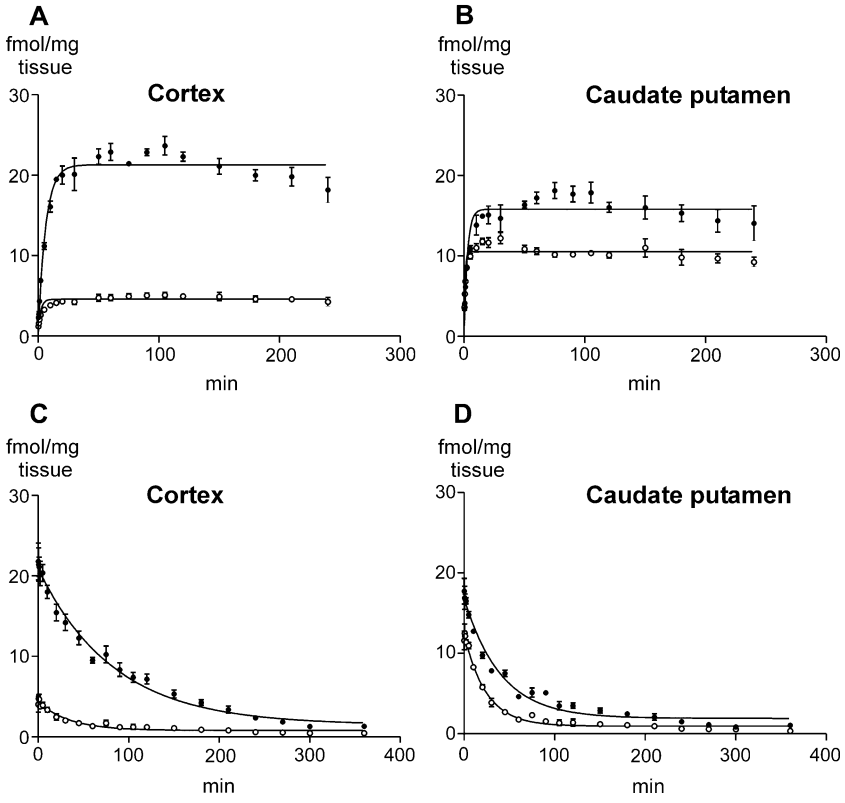

Fig. 2. Binding kinetics of 1 nM [ethyl-^{3}H]RS-79948-197 in WT (●) and α_{2A}-KO (○) mouse brain sections at room temperature. Association (A and B) and dissociation (C and D) of specific binding were fit to one-phase exponential models. Analysed data are mean values±S.E.M. from three animals. The means of the values from individual analysis of each animal are given in Table 1.

3.3. Saturation binding

Equilibrium dissociation constants (K_d) and receptor densities (B_{max}) were determined in five WT and five α_{2A}-KO mice for [ethyl-^{3}H]RS-79948-197, [^{3}H]RX821002 and [methyl-^{3}H]rauwolscine in saturation binding experiments. The saturation binding curves are shown in Fig. 3. For both genotypes, [^{3}H]RX821002 and [methyl-^{3}H]rauwolscine binding at the radioligand concentration ranges used was satisfactorily described by a one-site binding model. When performing a full-scale (up to 9.8 nM) [ethyl-^{3}H]RS-79948-197 saturation binding experiment, a seemingly non-saturable component was evident not only in WT, but also in α_{2A}-KO mice, and binding was best described by a two-site binding model.

In order to further characterise this apparently non-α_2-adrenoceptor binding, saturation binding experiments using high concentrations of [ethyl-^{3}H]RS-79948-197 (up to 26.1 nM) and [^{3}H]RX821002 (up to 29.9 nM) were performed on α_{2ABC}-KO brain sections. Radioligand binding increased with increasing concentrations of [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002, and appeared fairly linear over the concentration ranges used. In the presence of 10 μM phentolamine, [ethyl-^{3}H]RS-79948-197, and to a lesser degree [^{3}H]RX821002 binding was reduced, i.e., phentolamine interacted to some extent with low-affinity non-α_2-

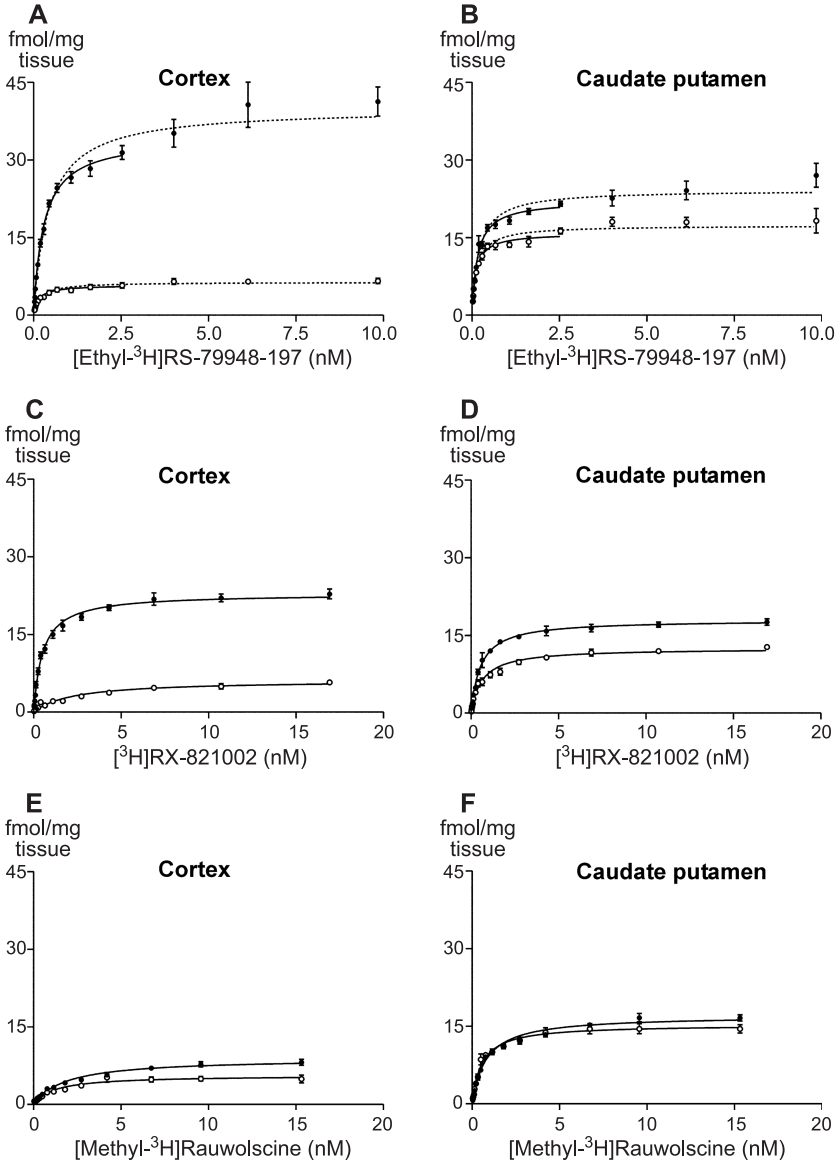

Fig. 3. [Ethyl^{3}H]RS-79948-197 (A, B), [^{3}H]RX821002 (C, D) and [methyl-^{3}H]rauwolscine (E, F) saturation binding in WT (●) and α_{2A}-KO (○) mouse brain sections. Specific binding data were fit to a one-site binding model. [Ethyl-^{3}H]RS-79948-197 K_d and B_{max} determinations were based on radioligand concentrations not exceeding 2.5 nM (solid lines, A and B), since a low-affinity component became evident at higher concentrations (dashed lines, A and B). Analysed data are mean values±S.E.M. from five animals. The means of the values from individual analysis of each animal are given in Table 2.

adrenoceptor binding sites recognised by [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 (Fig. 4). To minimise the interference of the non-specific low-affinity component, data from binding at higher concentrations than 2.5 nM were excluded from the analysis when determining K_d and B_{max} values for [ethyl-^{3}H]RS-79948-197 in α_{2A}-WT and α_{2A}-KO mice (Table 2). This resulted in binding to an apparently homogenous receptor population in both genotypes. At the estimated K_d concentrations, non-specific binding expressed as % of total binding was lowest for [ethyl-^{3}H]RS-79948-197, closely followed by [^{3}H]RX821002, while the non-specific binding of [methyl-^{3}H]rauwolscine was significantly higher (Table 2). Non-specific binding for [ethyl-^{3}H]RS-79948-197 in the cortex and caudate putamen at 2.5 nM ranged between 3.8 and 4.0 fmol/mg tissue.

[Ethyl-^{3}H]RS-79948-197 exhibited the highest affinity of the three radioligands, with slight preference for $\alpha_{2B/C}$- over α_{2A}-adrenoceptors, as indicated by the differences in K_d values between the α_{2A}-adrenoceptor-rich cortex and the α_{2C}-adrenoceptor-rich caudate putamen, as well as similar differences between WT and α_{2A}-KO mice. [^{3}H]RX821002, which otherwise appeared subtype non-selective, displayed an unexpectedly low affinity in α_{2A}-KO cortex, with a fivefold difference relative to α_{2A}-KO caudate putamen. For [methyl-^{3}H]rauwolscine, there were no statistically significant differences in K_d values between genotypes or brain regions. Compared to the maximal number of binding sites recognised by [ethyl-^{3}H]RS-79948-197, [^{3}H]RX821002 labelled 66% of the sites in cortex and 81% in caudate putamen of WT mice, and 105% in cortex and 79% in caudate putamen of α_{2A}-KO mice. The proportions of specific binding sites that [methyl-^{3}H]rauwolscine detected compared to [ethyl-^{3}H]RS-79948-197 in WT mice were 27% in cortex and 77% in caudate putamen, and in α_{2A}-KO mice 98% in cortex and 96% in caudate putamen.

Table 2
Comparison of [ethyl-^{3}H]RS-79948-197, [^{3}H]RX821002 and [methyl-^{3}H]rauwolscine K_d (nM), B_{max} (fmol/mg tissue), and non-specific binding (% of total binding at the K_d concentration) obtained from saturation binding studies in WT and α_{2A}-KO mouse brain sections

	WT Cx	WT CPu	α_{2A}-KO Cx	α_{2A}-KO CPu
[Ethyl-^{3}H] RS-79948-197				
K_d	0.28±0.02	0.16±0.01[a]	0.13±0.01[b]	0.11±0.01[c]
B_{max}	34.4±1.8	22.2±0.8[a]	5.8±0.5[b]	16.0±0.8[a,c]
% NSB	3.9	4.6	14.7	5.7
[^{3}H]RX821002				
K_d	0.53±0.06	0.56±0.09	2.41±0.43[b]	0.73±0.16[d]
B_{max}	22.8±0.9	17.9±0.5[a]	6.1±0.2[b]	12.7±0.3[a,b]
% NSB	4.9	6.4	19.0	8.7
[Methyl-^{3}H] rauwolscine				
K_d	2.15±0.50	0.80±0.08	1.45±0.43	0.57±0.09
B_{max}	9.3±0.7	17.0±0.6[a]	5.7±0.8[e]	15.3±0.9[a]
% NSB	30.6	12.6	37.8	12.2

Cx, cortex; CPu, caudate putamen; NSB, non-specific binding; K_d, equilibrium dissociation constant; B_{max}, number of specific binding sites. Values are means±S.E.M. ($n=5$).
[a] $P<0.001$; difference between brain regions within each genotype.
[b] $P<0.001$; difference between WT and α_{2A}-KO mice.
[c] $P<0.01$; difference between WT and α_{2A}-KO mice.
[d] $P<0.01$; difference between brain regions within each genotype.
[e] $P<0.05$; difference between WT and α_{2A}-KO mice.

3.4. Competition binding

Competition binding experiments (Fig. 5, Table 3) against 0.4 nM [ethyl-^{3}H]RS-79948-197 were performed on WT and α_{2A}-KO brain sections, three animals per genotype, with the α_{2A}- respectively $\alpha_{2B/C}$-adrenoceptor preferring ligands oxymetazoline and prazosin, as well as with phentolamine, since non-specific binding was assessed using this compound. Oxymetazoline and prazosin binding curves were not consistently best described by either one- or two-site binding models, but heterogeneity of the competition binding curves was evident for both ligands in the cortex of WT mice (Fig. 5A,C). Phentolamine recognised an apparently single receptor population in both genotypes and brain regions, with low nanomolar affinity (Fig. 5E,F). Apparent IC_{50} values were obtained by assuming one-site interactions for all compounds. The prazosin/oxymetazoline IC_{50} ratios were

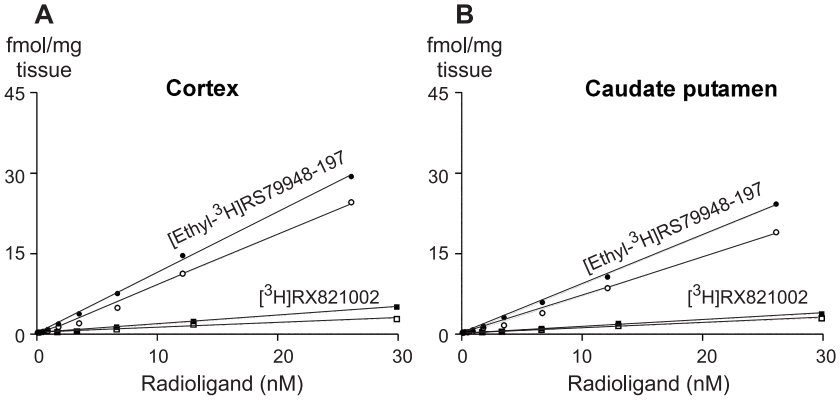

Fig. 4. [Ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 binding in the absence (●) and presence (○) of 10 μM phentolamine in α_{2ABC}-KO cortex (A) and caudate putamen (B). Data are from one animal with at least three sections, bilateral readings, analysed at each concentration point and brain region.

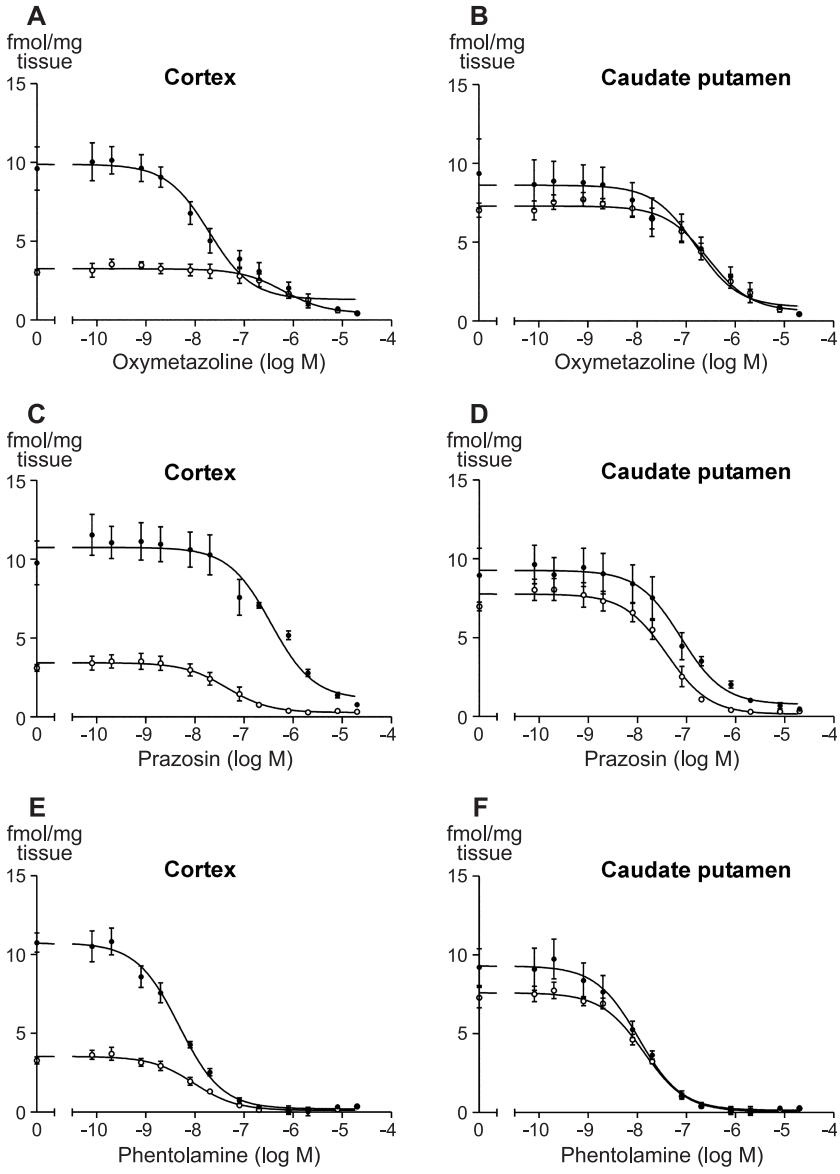

Fig. 5. Competition curves from one-site models depicting specific binding in the presence of 0.4 nM [ethyl-^{3}H]RS-79948-197 with the competing ligands oxymetazoline (α_{2A}-adrenoceptor preferring) (A, B), prazosin ($\alpha_{2B/C}$-adrenoceptor preferring) (C, D) or the α_2-adrenoceptor subtype non-selective antagonist phentolamine (E, F) in WT (●) and α_{2A}-KO (○) mouse brain sections. Presented results are means±S.E.M. of binding from three animals. The means of the values from individual analysis of each animal are given in Table 3.

18 in WT cortex, 0.46 in WT caudate putamen, 0.07 in α_{2A}-KO cortex and 0.13 in α_{2A}-KO caudate putamen, confirming the assumed α_2-adrenoceptor subtype identities of the binding sites recognised by [ethyl-^{3}H]RS-79948-197.

Table 3
[Ethyl-^{3}H]RS-79948-197 competition binding IC_{50} (nM) values for oxymetazoline, prazosin and phentolamine in WT and α_{2A}-KO mouse brain sections

	WT Cx	WT CPu	α_{2A}-KO Cx	α_{2A}-KO CPu
Oxymetazoline	22.2±7.7	160.1±50.9	646.1±294.1	301.8±77.9
Prazosin	400.0±94.0	74.2±7.9	44.6±12.5	39.7±6.2
Phentolamine	4.9±0.7	11.2±1.7	9.6±1.5	13.7±2.1

Cx, cortex; CPu, caudate putamen. IC_{50}, concentration of competitor that inhibited 50% of the specific binding of 0.4 nM [ethyl-^{3}H]RS-79948-197. Values are means±S.E.M. (n=3).

4. Discussion

4.1. Anatomical distribution of binding sites

From the autoradiographic images of WT and α_{2A}-KO mouse brain sections, it was evident that [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 labelled both α_{2A}- and $\alpha_{2B/C}$-adrenoceptors, while [methyl-^{3}H]rauwolscine labelled mainly $\alpha_{2B/C}$-adrenoceptors, at the moderate radioligand concentrations used. $\alpha_{2B/C}$-Adrenoceptor binding sites were

more readily detected with [ethyl-^{3}H]RS-79948-197 than with [^{3}H]RX821002.

4.2. Non-specific [ethyl-^{3}H]RS-79948-197 binding

Binding of [ethyl-^{3}H]RS-79948-197 in α_{2ABC}-KO brain sections revealed that phentolamine, which had been used to define non-specific binding in WT and α_{2A}-KO mice, interacted with a portion of the low-affinity non-α_2-adrenoceptor binding sites recognised by [ethyl-^{3}H]RS-79948-197. Specific [ethyl-^{3}H]RS-79948-197 binding in WT and α_{2A}-KO mice had thus been overestimated as a result of the true non-specific binding being underestimated. The low-affinity component of [ethyl-^{3}H]RS-79948-197 binding in WT and α_{2A}-KO mice at [ethyl-^{3}H]RS-79948-197 concentrations above 2.5 nM is therefore likely an artefact caused by the use of 10 μM phentolamine to determine non-specific binding. Using [ethyl-^{3}H]RS-79948-197 concentrations below 2.5 nM, at which non-specific binding is low, will avoid this problem, but at 2.5 nM, [ethyl-^{3}H]RS-79948-197 binding in WT mouse cortex did not appear to have saturated, so the pharmacological parameters especially in α_{2A}-adrenoceptor-rich brain regions will be estimates to some extent.

4.3. Saturation binding

The K_d values for [ethyl-^{3}H]RS-79948-197, obtained from saturation assays, were in accordance with the kinetic binding results in that [ethyl-^{3}H]RS-79948-197 was slightly $\alpha_{2B/C}$- over α_{2A}-adrenoceptor preferring, although the affinities consistently were lower compared to those determined in kinetic experiments. [^{3}H]RX821002 has been reported to have similar α_{2A}- and α_{2C}-adrenoceptor affinity in the rat (Uhlén et al., 1998), and our results supported this notion with the exception of α_{2A}-KO cortex, where the affinity of [^{3}H]RX821002 was unexpectedly low. In the rat, the affinity of [^{3}H]RX821002 for the α_{2B}-adrenoceptor is fourfold lower than for the other subtypes (Uhlén et al., 1998), and our preliminary receptor autoradiography results in a mouse line lacking both α_{2A}- and α_{2C}-adrenoceptors, suggest the presence of some amount of α_{2B}-adrenoceptors in the mouse cortex (Fagerholm, V., unpublished observations). The presence of α_{2B}-adrenoceptors might thus provide an explanation for the relatively high K_d value of [^{3}H]RX821002 in α_{2A}-KO cortex. For [methyl-^{3}H]rauwolscine, there were no significant differences in affinities neither between genotypes nor brain regions. At the radioligand concentrations used, there is little binding of [methyl-^{3}H]rauwolscine to α_{2A}-adrenoceptors, and $\alpha_{2B/C}$-adrenoceptors were thus the major determinants of the K_d values.

[Ethyl-^{3}H]RS-79948-197 labelled a larger number of specific binding sites compared to [^{3}H]RX821002 with the exception of α_{2A}-KO cortex, where the numbers were similar. [^{3}H]RX821002 could under the experimental conditions used have underestimated the true receptor number because of the fast dissociation rate of [^{3}H]RX821002 (Langin et al., 1989; Galitzky et al., 1990; Halme et al., 1995). It is hard to explain why the B_{max} values of [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 in α_{2A}-KO cortex do not differ from each other, but the low binding levels in α_{2A}-KO cortex might have made it difficult to accurately estimate the B_{max} values. The B_{max} values obtained with [ethyl-^{3}H]RS-79948-197 and [methyl-^{3}H]rauwolscine in α_{2A}-KO mice were in excellent agreement with each other, suggesting that both radioligands recognised the same $\alpha_{2B/C}$-adrenoceptor population.

Based on [ethyl-^{3}H]RS-79948-197 binding in WT and α_{2A}-KO mice, WT mouse cortex contained 83% α_{2A}-adrenoceptors and 17% $\alpha_{2B/C}$-adrenoceptors, and caudate putamen 28% α_{2A}-adrenoceptors and 72% $\alpha_{2B/C}$-adrenoceptors. With [^{3}H]RX821002 as reference, the corresponding numbers were 73% α_{2A}-adrenoceptors and 27% $\alpha_{2B/C}$-adrenoceptors in cortex and 29% α_{2A}-adrenoceptors and 71% $\alpha_{2B/C}$-adrenoceptors in caudate putamen. These numbers differ from those reported for membrane preparations of rat cortex (approximately 90% α_{2A}-adrenoceptors, 10% α_{2C}-adrenoceptors) and caudate putamen (72% α_{2A}-adrenoceptors, 28% α_{2C}-adrenoceptors), where the α_{2A}- and α_{2C}-adrenoceptor proportions were estimated using multicurve modelling of [methyl-^{3}H]MK-912 and guanfacine-blocked [methyl-^{3}H]MK-912 binding data (Uhlén et al., 1992, 1997). The observed differences may be due to both methodological and species differences. Significant alterations of $\alpha_{2B/C}$-adrenoceptor expression levels in these brain regions in the α_{2A}-KO mice as an adaptive consequence of, e.g., changes in noradrenaline turnover are unlikely, since such changes should have been revealed by our [methyl-^{3}H]rauwolscine saturation binding results.

4.4. Conclusions

In summary, the results demonstrate that [ethyl-^{3}H]RS-79948-197, as an alternative receptor autoradiography ligand to [^{3}H]RX821002, is well suited for localising and characterising α_2-adrenoceptor subtypes in mouse brain sections. Non-specific [ethyl-^{3}H]RS-79948-197 binding, although exceeding that of both [^{3}H]RX821002 and [methyl-^{3}H]rauwolscine at higher ligand concentrations, was low at concentrations around the apparent K_d, resulting in a high signal to noise ratio, which is an important determinant of the sensitivity of binding site detection. [Methyl-^{3}H]rauwolscine, at concentrations relevant for receptor autoradiography, does not label the α_{2A}-adrenoceptor. Compared to [^{3}H]RX821002, the advantages of [ethyl-^{3}H]RS-79948-197 include slower dissociation of binding, higher specific radioactivity, and higher affinity for α_{2B}- and α_{2C}-adrenoceptors. More generally, this study illustrates the usefulness of genetically engineered mouse lines for the pharmacological characterisation of receptor ligands.

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