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RESEARCH PAPER

# The second extracellular loop of $\alpha_{2A}$-adrenoceptors contributes to the binding of yohimbine analogues

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**Background and purpose:** Rodent $\alpha_{2A}$-adrenoceptors bind the classical $\alpha_2$-antagonists yohimbine and rauwolscine with lower affinity than the human $\alpha_{2A}$-adrenoceptor. A serine-cysteine difference in the fifth transmembrane helix (TM; position 5.43) partially explains this, but all determinants of the interspecies binding selectivity are not known. Molecular models of $\alpha_{2A}$-adrenoceptors suggest that the second extracellular loop (XL2) folds above the binding cavity and may participate in antagonist binding.
**Experimental approach:** Amino acids facing the binding cavity were identified using molecular models: side chains of residues 5.43 in TM5 and xl2.49 and xl2.51 in XL2 differ between the mouse and human receptors. Reciprocal mutations were made in mouse and human $\alpha_{2A}$-adrenoceptors at positions 5.43, xl2.49 and xl2.51, and tested with a set of thirteen chemically diverse ligands in competition binding assays.
**Key results:** Reciprocal effects on the binding of yohimbine and rauwolscine in human and mouse $\alpha_{2A}$-adrenoceptors were observed for mutations at 5.43, xl2.49 and xl2.51. The binding profile of RS-79948-197 was reversed only by the XL2 substitutions.
**Conclusions and implications:** Positions 5.43, xl2.49 and xl2.51 are major determinants of the species preference for yohimbine and rauwolscine of the human *versus* mouse $\alpha_{2A}$-adrenoceptors. Residues at positions xl2.49 and xl2.51 determine the binding preference of RS-79948-197 for the human $\alpha_{2A}$-adrenoceptor. Thus, XL2 is involved in determining the species preferences of $\alpha_{2A}$-adrenoceptors of human and mouse for some antagonists.
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**Keywords:** $\alpha_2$-adrenoceptor; GPCR; yohimbine; ligand binding; rauwolscine; RS-79948-197; second extracellular loop; receptor models

**Abbreviations:** $B_{max}$, receptor density; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; $K_d$, equilibrium dissociation constant; TM, transmembrane (domain); XL, extracellular loop

## Introduction

The $\alpha_2$-adrenoceptors, members of the rhodopsin-like family of G protein-coupled receptors (GPCRs), are involved in many physiological processes through their activation by the neurotransmitters/hormones noradrenaline and adrenaline. For example, $\alpha_2$-adrenoceptors mediate feedback inhibition of catecholamine release in sympathetic nerve endings, vascular smooth muscle contraction and central regulation of blood pressure (Link *et al*., 1996; MacMillan *et al*., 1996; Altman *et al*., 1999). The $\alpha_2$-adrenoceptors are currently targeted by several clinically important drugs, including clonidine and dexmedetomidine, but a better understanding of the structural determinants of selectivity among the $\alpha_2$-adrenoceptor subtypes would have obvious therapeutic applications. For example, subtype-selective agonists and antagonists could possibly improve the treatment of hypertension, depression, pain and opioid withdrawal symptoms (Ruffolo and Hieble, 1994; MacDonald *et al*., 1997).

In mammals, three distinct genes encode three $\alpha_2$-adrenoceptor subtypes, named $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$. They differ in their patterns of tissue expression and mechanisms of regulation (Eason *et al*., 1994; MacDonald *et al*., 1997; Richman *et al*., 2001). Recently, in zebrafish, we have identified a fourth $\alpha_2$-adrenoceptor subtype present as two duplicates that has no ortholog in mammals; this new subtype is present in many other fish species as well as in some tetrapods (Ruuskanen *et al*., 2004). In accordance with IUPHAR rules,

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we have named the duplicates of this new receptor subtype $\alpha_{2Da}$- and $\alpha_{2Db}$-adrenoceptors (Ruuskanen *et al*., 2004; Bylund, 2005).

The three mammalian $\alpha_2$-adrenoceptor subtypes bind the endogenous catecholamines adrenaline and noradrenaline with similar affinities, while their binding affinities for non-natural ligands may differ (Uhlén *et al*., 1998; Molderings *et al*., 2000; Bylund, 2005). In addition, when orthologs of a given $\alpha_2$-adrenoceptor subtype are compared between species, the ligand binding affinities may also differ, for example, the murine and rat $\alpha_{2A}$-adrenoceptor subtypes bind the classical $\alpha_2$-antagonists yohimbine and rauwolscine with lower affinity in comparison to the orthologous $\alpha_{2A}$-adrenoceptor subtypes of several other species, for example, human, pig, rabbit and zebrafish (Link *et al*., 1992; Bylund, 2005; Ruuskanen *et al*., 2005). Indeed, based on these pharmacological differences, the rodent $\alpha_{2A}$-adrenoceptor was at one time called the $\alpha_{2D}$-adrenoceptor (Michel *et al*., 1989; Simonneaux *et al*., 1991).

Rodents are frequently used as animal models in pharmaceutical development. It is therefore important to understand, on the molecular level, the origin of their pharmacological differences from humans. Currently, a detailed experimental structure of an adrenoceptor is still lacking, hampering our understanding of the origin of specificity of different drug molecules for diverse receptors, receptor subtypes and species variants. Adrenoceptors, like other GPCRs, fold into a bundle of seven hydrophobic transmembrane $\alpha$-helices (TM1–TM7), as seen in the bovine rhodopsin structure (Palczewski *et al*., 2000), the only high-resolution GPCR structure known so far. In rhodopsin, the photoactivatable 11-*cis*-retinal ligand binds within a pocket embedded within the protein core and formed by amino acids from TM2–TM7. For the adrenoceptors, a similar location of the binding site was predicted long ago and is now commonly accepted (Wong *et al*., 1988; Trumpp-Kallmeyer *et al*., 1992). Earlier studies have focused on the amino-acid differences along the TMs in order to explain the distinct pharmacology of the murine $\alpha_{2A}$-adrenoceptor compared to its human ortholog. A serine-cysteine amino-acid substitution in TM5 (position 5.43) only partly explains the species-specific differences between the human and rodent $\alpha_{2A}$-adrenoceptors in the recognition of yohimbine and rauwolscine (Link *et al*., 1992; Bylund, 2005). In this paper, amino-acid residues are numbered according to the Ballesteros–Weinstein nomenclature (Ballesteros and Weinstein, 1995). In this indexing system, the first number refers to the transmembrane helix where the residue is located and the number after the decimal point refers to the residue position with respect to the most conserved residue in that helix, which has been arbitrarily assigned the number 50. The Ballesteros–Weinstein numbering scheme was extended to the second extracellular loop; thus, the conserved cysteine in XL2 that forms a putative disulphide bond is indicated as Cxl2.50 (Xhaard *et al*., 2005).

In rhodopsin, the second extracellular loop (XL2) that connects TM4 and TM5 folds down between the transmembrane domains and forms part of the binding site, in direct contact with 11-*cis*-retinal (Palczewski *et al*., 2000; Teller *et al*., 2001; Okada *et al*., 2002). A disulphide bond between cysteines at positions 3.25 and xl2.50 (see above for numbering convention extended to XL2) constrains the position of XL2 in rhodopsin. As these cysteines are conserved among most rhodopsin-like GPCRs, the disulphide bond would very likely be present and serve to constrain the XL2 domain to a similar location 'above' the binding cavity in many GPCRs, including the $\alpha_2$-adrenoceptors. The structure of rhodopsin and molecular models of the $\alpha_2$-adrenoceptors suggest that residues in XL2, especially in the vicinity of the cysteine at xl2.50, may contribute to the differences in ligand binding among $\alpha_2$-adrenoceptors from different subtypes and species (Ruuskanen *et al*., 2005; Xhaard *et al*., 2005). Experimental evidence has been reported for other types of GPCRs where determinants of the binding affinities for amine ligands were attributed to XL2 (Zhao *et al*., 1996; Wurch *et al*., 1998; Shi and Javitch, 2004).

In this study, we have investigated in more detail the interspecies differences between the human and mouse $\alpha_{2A}$-adrenoceptors. Reciprocal mutations were generated for arginine/serine (human/mouse) at xl2.49 (residue 187 in both human and mouse; located adjacent to the conserved cysteine, residue 188, at position xl2.50) and glutamate/lysine at xl2.51 (residue 189), as well as for cysteine/serine at position 5.43 (residue 201). A panel of 13 structurally diverse ligands was selected and tested with transfected recombinant cells expressing wild-type and mutated $\alpha_{2A}$-adrenoceptors. Ten of these ligands are antagonists, among which ARC239, atipamezole, WB4101, rauwolscine, RS-79948-197 and yohimbine discriminated between the human and mouse receptors with at least threefold differences in affinity. The four remaining antagonists, chlorpromazine, doxazosin, MK-912 and prazosin, served as controls to rule out any indiscriminate effects of the mutations, as they are known to bind to mouse and human $\alpha_{2A}$-adrenoceptors with similar affinities. An earlier modelling study (Xhaard *et al*., 2006) had suggested a potential role for XL2, also in agonist binding. Therefore, we tested the effects of the mutations within XL2 on the affinity of the $\alpha_2$-adrenoceptors for the endogenous catecholamines adrenaline, noradrenaline and dopamine. We show that reciprocal substitutions at positions 5.43, xl2.49 and xl2.51 reverse the binding profiles of human and mouse $\alpha_{2A}$-adrenoceptors for yohimbine, rauwolscine and RS-79948-197, indicating a role of XL2 in the determination of species-specific ligand binding profiles.

## Methods

*Molecular modelling of the binding cavities, human–mouse comparisons and construction of receptor–ligand complexes*

The amino acids facing the binding cavity were defined using modelled receptor–ligand complexes. Structural models of individual receptors were constructed as reported previously (Nyrönen *et al*., 2001; Xhaard *et al*., 2005, 2006). Briefly, bovine rhodopsin solved at 2.6 Å resolution in the inactive state was used as a structural template (Palczewski *et al*., 2000; Okada *et al*., 2002; Li *et al*., 2004; PDB codes 1F88, 1HZX, 1L9H). The inactive state of bovine rhodopsin should be well suited to model the site where antagonists

bind (Bissantz *et al*., 2003; Xhaard *et al*., 2005). In order to construct the models, pairwise sequence alignments obtained previously using Malign (Johnson and Overington, 1993) and corrected manually were used, and the models were constructed using Modeller v8.0 (Sali and Blundell, 1993) with the standard options.

The antagonists yohimbine, rauwolscine, RS-79948-197 and MK-912 (see Figure 1 for ligand structures) were manually docked into structural models of the human and mouse $\alpha_{2A}$-adrenoceptors in two different ways; first, based on the classical hypothesis of ion pairing between D3.32 and the protonated amine of the ligands, and secondly, using the alternative hypothesis proposed by Xhaard *et al*. (2005) based on cation–$\pi$ interactions. Before docking, the three-dimensional structures of yohimbine (CSD code: YOHIMB, YOHIMB10) were taken from the Cambridge Structure Database (CSD) and the three-dimensional structures of rauwolscine, RS-79948-197 and MK-912, which are not available in the CSD, were constructed using SYBYL (Tripos Corp., St Louis, MO, USA).

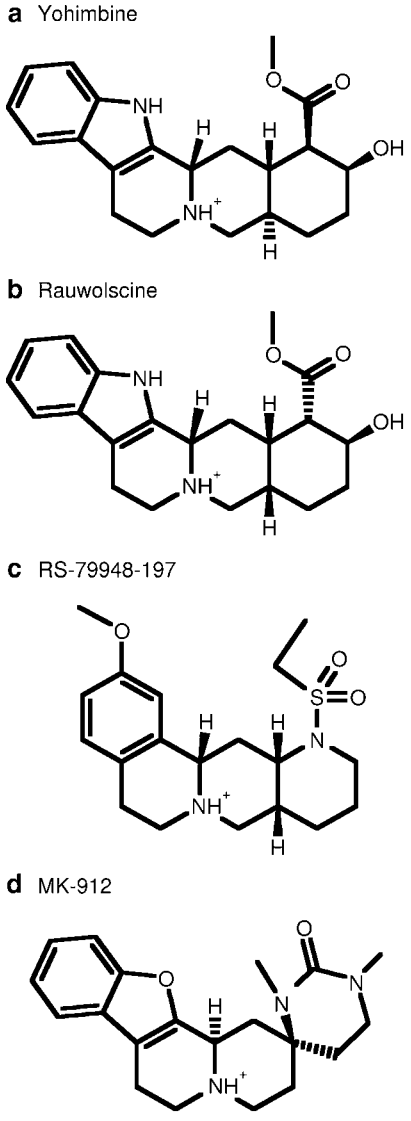


**Figure 1** Molecular structures of (**a**) yohimbine, (**b**) rauwolscine, (**c**) RS-79948-179 and (**d**) MK-912.

*Mutagenesis and expression vectors*

A human $\alpha_{2A}$-adrenoceptor cDNA clone was obtained from the UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO, USA). The murine $\alpha_{2A}$-adrenoceptor cDNA clone was originally provided by Dr BK Kobilka (Stanford University, Stanford, CA, USA; clone M$\alpha_2$-10H; Link *et al*., 1992).

The human $\alpha_{2A}$-adrenoceptor wild-type cDNA was cloned into the pcDNA3.1+ expression vector (Invitrogen, NV Leek, The Netherlands). The cDNAs encoding the murine wild-type and S5.43C-mutated $\alpha_{2A}$-adrenoceptors were subcloned into pcDNA3.1+ from expression constructs in pREP4 (Marjamäki *et al*., 1998). Mutagenesis primers were obtained from TAG Copenhagen A/S (Copenhagen, Denmark). Site-directed mutagenesis was performed utilizing the Gene Editor *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI, USA). The mutated expression vector constructs were sequenced with vector- and gene-specific primers to confirm the correctness of the sequence and the success of the desired XL2 mutations.

*Cell culture and transfections*

Adherent Chinese hamster ovary (CHO) cells (K1 strain) (American Type Culture Collection, Manassas, VA, USA) were cultured in $\alpha$-minimum essential medium supplemented with 2 mM glutamine, 20 mM $NaHCO_3$, 5% heat-inactivated fetal calf serum, penicillin (50 U ml$^{-1}$) and streptomycin (50 $\mu$g ml$^{-1}$). Cells were grown in 5% $CO_2$ at 37°C. CHO cells expressing human C5.43S-mutated $\alpha_{2A}$-adrenoceptors, reported previously (Peltonen *et al*., 2003), were revived from liquid nitrogen and cultured. The pcDNA3.1+-based expression constructs were transfected into CHO cells using the Lipofectamine 2000 reagent kit (Invitrogen Life Technologies Inc., Rockville, MD, USA) with slight modifications to the manufacturer's instructions. Stable transfections were selected using 800 $\mu$g ml$^{-1}$ of the neomycin analogue G418 (Calbiochem, San Diego, CA, USA). After selection, transfected cell cultures were examined for their ability to bind the $\alpha_2$-adrenoceptor antagonist radioligand [$^3$H]RX821002 (see below). The transfected cell clones with the highest expression levels were chosen for further competition binding studies and were subsequently maintained in 200 $\mu$g ml$^{-1}$ G418. Confluent cells were harvested into chilled phosphate-buffered saline, pelleted and frozen at −70°C.

*Membrane preparation*

All procedures were performed on ice. The harvested recombinant CHO cell pellets were thawed and suspended in hypotonic lysis buffer (10 mM Tris-HCl, 0.1 mM EDTA, 0.32 mM sucrose, pH 7.4) and homogenized using an Ultra-Turrax homogeniser (3 × 10 s at 800 r.p.m.). The homogenate was centrifuged at 23 000 *g* for 30 min, and the pellet was re-homogenized and again centrifuged at 23 000 *g* for 30 min.

The membrane pellet was suspended in hypotonic lysis buffer and stored at −70°C until used. Protein concentrations were determined with the method of Bradford (1976) using bovine serum albumin as reference.

*Saturation binding assays*

The receptor expression levels of the transfected cell lines were determined in saturation binding experiments using the $\alpha_2$-adrenoceptor antagonist [$^3$H]RX821002 as radioligand. Saturation binding assays were performed as described previously (Halme *et al*., 1995). Briefly, whole-cell homogenates (40–120 $\mu$g of protein per sample) or membrane preparations (4–20 $\mu$g of protein per sample) were incubated (in 50 mM potassium phosphate buffer, pH 7.4) with final concentrations of [$^3$H]RX821002 ranging from 0.125 to 8 nM. After 30 min incubation at 25°C, reactions were terminated using rapid filtration through glass fibre filters (Whatman GF/B). The filters were washed (50 mM Tris-HCl, 10 mM EDTA, pH 7.4), placed into scintillation vials with Optiphase 'HiSafe' III (Wallac Oy, Turku, Finland) and bound radioactivity was measured in a scintillation counter (Wallac 1410). Nonspecific binding was defined in parallel tubes with phentolamine (10 $\mu$M). Specific binding was defined as the difference between total and nonspecific binding. For each studied cell line, saturation experiments were performed at least three times. The $B_{max}$ values were different between the cell lines, but no relationship between the observed expression levels and $K_d$ values for [$^3$H]RX821002 was seen. Equilibrium dissociation constants ($K_d$) and receptor expression levels ($B_{max}$) were calculated from saturation binding experiments (GraphPad Prism Software, San Diego, CA, USA).

*Competition binding assays*

Competition binding assays were carried out using two systems: either a Beckman Biomek 2000 Laboratory Automation Workstation (Beckman Instruments Inc., Palo Alto, CA, USA) with 96-well plates, or a MultiScreen Vacuum Manifold system (Millipore Corporation, Bedford, MA, USA) with Millipore MultiScreen FB 96-well filtration plates. The affinities of adrenaline, prazosin, yohimbine and rauwolscine at C5.43S-substituted human $\alpha_{2A}$-adrenoceptors were determined using both systems, with an excellent correlation ($r = 0.99$) between the results obtained, indicating that the type of system had no significant influence on the outcome. Competition binding experiments were performed in 50 mM potassium phosphate buffer (pH 7.4) using [$^3$H]RX821002 at concentrations close to its affinity constant ($K_d$) for each receptor variant, 6–8 serial dilutions of the competitor ligands, and cell membrane preparations with 2–10 $\mu$g of protein per sample. After 30 min incubation at room temperature, reactions were terminated by rapid vacuum filtration. Filters were washed three times with ice-cold buffer, dried and impregnated with Meltilex B/HS scintillation wax (1205-422, Wallac, Turku, Finland) or Super Mix cocktail (Wallac), depending on the system used. The incorporated radioactivity was determined in a Wallac 1205 or Wallac 1450 Betaplate liquid scintillation counter.

Ligands included in the competition binding assays were chosen on the basis of their chemical structure: some 'bulky antagonists' (ARC239, doxazosin, prazosin, WB4101), the imidazole atipamezole, the tricyclic antipsychotic drug chlorpromazine, yohimbine and its structural analogues rauwolscine, MK-912 and RS-79948-197. In addition, endogenous catecholamines were tested (adrenaline, noradrenaline and dopamine). The apparent affinity (apparent $K_i$) of each ligand was determined using nonlinear regression analysis (GraphPad Prism), assuming one-site binding. For conversion of $IC_{50}$ into $K_i$ values, the Cheng–Prusoff equation was applied (Cheng and Prusoff, 1973). The statistical significance of affinity differences between receptor variants was evaluated with unpaired *t*-tests.

*Materials*

[$^3$H]RX821002 was purchased from Amersham Pharmacia Biotech (Bucks, UK). ARC239 and RS-79948-197 were obtained from Tocris (Bristol, UK). MK-912 (L-657.743) was a gift from Merck & Co. (Whitehouse Station, NJ, USA), and atipamezole was a gift from Orion Pharma (Turku, Finland). Other ligands were purchased from Sigma-Aldrich (St Louis, MO, USA). Cell culture reagents were supplied by Life Technologies Inc. (Rockville, MD, USA). Other reagents were of analytical or reagent grade and were purchased from commercial suppliers.

## Results

*Identification of amino acids likely to face the ligand-binding cavity: TM regions and XL2*

Adrenoceptors bind their endogenous ligands adrenaline and noradrenaline inside a binding cavity that has been defined over the last 20 years (Dixon *et al*., 1987; Strader *et al*., 1987; Schwartz, 1994). There is compelling evidence that this cavity is located within the TM region, surrounded by TM2–TM7. This notion is based on several lines of evidence, including site-directed mutagenesis studies combined with modified ligands, studies based on cysteine-reactive probes (Nyrönen *et al*., 2001; Peltonen *et al*., 2003), and consideration of the three-dimensional structure of the distant but recognizably similar rhodopsin (Wong *et al*., 1988), whose ligand-bound complex is now known. The location of the site of antagonist binding is less well defined than that of agonists, and some bound antagonists may only partially overlap with the location of bound agonists.

Most of the amino acids forming the agonist binding site are located in the TMs (Salminen *et al*., 1999; Nyrönen *et al*., 2001; Xhaard *et al*., 2005, 2006). For endogenous agonists, the amino acids predicted to face the ligand in the human $\alpha_{2A}$-adrenoceptor are from TM2 (V2.53 and V2.57); from TM3 (C3.25, Y3.28, D3.32, V3.33, C3.36, T3.37 and I3.40); from TM4 (I4.52, I4.56 and P4.60); from TM5 (Y5.38, V5.39, S5.42, C5.43, S5.46 and F5.47); from TM6 (F6.44, W6.48, F6.51, F6.52 and Y6.55); and from TM7 (F7.39, G7.42, Y7.43 and N7.45). For antagonists, the most common way to place (dock) them in the binding cavity is to form an ion pair between a positively charged nitrogen of the ligand and the

side chain of D3.32 in the receptor (Strader *et al*., 1987), based on analogy with agonist binding. Such a binding mode shifts the location of the antagonist ligands away from TM5 and positions them close to TM1 (Xhaard *et al*., 2005); thus, GPCRs modelled on the basis of the rhodopsin structure would require expansion of the agonist binding site in order to accommodate antagonists. For example, Surgand *et al*. (2006) suggest that L1.35, A1.39, L1.42, I2.58, S2.61 and E2.65 in the monoamine receptors face antagonist ligands in this expanded binding site. Alternatively, other binding modes for antagonists have been proposed that do not require an expanded binding site: we have suggested (Xhaard *et al*., 2005), based on automatic docking and supported by the covalent binding of the antagonist phenoxybenzamine to C3.36 (Frang *et al*., 2001), that antagonists also could fully occupy the 'agonist' binding pocket and take advantage of cation–$\pi$ interactions and carboxylate–ring interactions that would effectively replace the classical ion pair seen for agonist–receptor interactions.

The second extracellular loop, XL2, folds as a $\beta$-hairpin and covers the binding cavity in the X-ray structure of bovine rhodopsin, where it interacts directly with the endogenously bound ligand 11-*cis*-retinal. In rhodopsin, cysteine Cxl2.50 in XL2 is attached to cysteine C3.25 in TM3 with a disulphide bridge; cysteine xl2.50 and its neighbouring residues are thus constrained to form the extracellular surface – the 'top' – of the binding cavity. As cysteines C3.25 and Cxl2.50 are conserved among the GPCRs, it is very likely that XL2 is similarly constrained and forms the 'top' of the binding cavity in the $\alpha_2$-adrenoceptors, too (Figure 2). In the $D_2$ dopamine receptor, a close relative of the $\alpha_2$-adrenoceptors (Xhaard *et al*., 2006), XL2 has been shown by a site-directed cysteine accessibility study to be exposed to the binding cavity (Shi and Javitch, 2004). Thus, in the modelled structures of the human $\alpha_{2A}$-adrenoceptor, the side chains of the amino acids neighbouring xl2.50, that is, Rxl2.49, Exl2.51 and Ixl2.52, as well as some main-chain atoms from xl2.49–xl2.52, are likely to form the extracellular surface of the binding site where they could participate in important interactions with ligands.

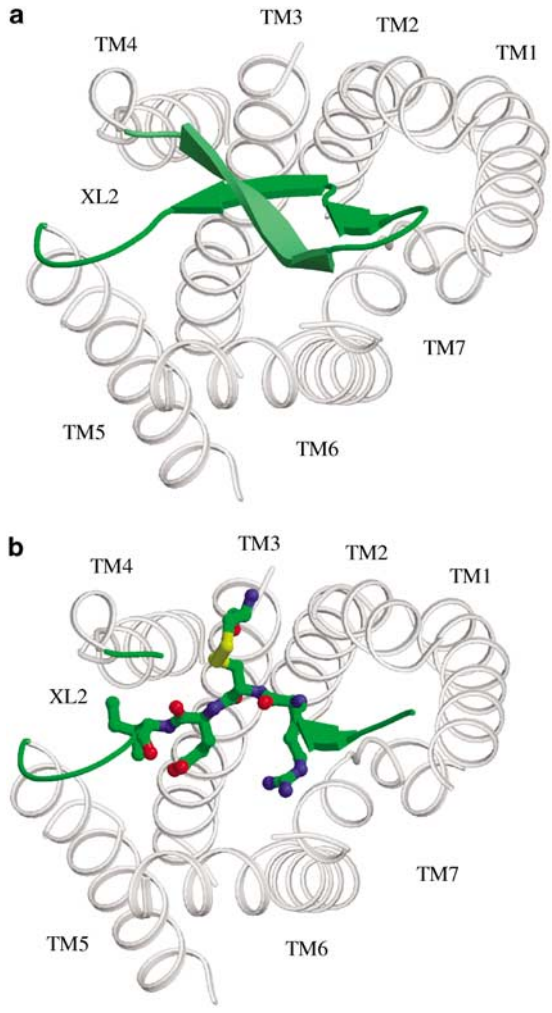


**Figure 2** Molecular model of the human $\alpha_{2A}$-adrenoceptor, viewed from the extracellular surface. For clarity, only the TM helices are shown (indicated as ribbons). (**a**) The XL2 domain that forms a $\beta$-hairpin (individual strands indicated by green ribbons). (**b**) The side chains forming the extracellular surface of the binding cavity, xl2.49, xl2.50, xl2.51 and xl2.52 are indicated, and are constrained on top of the cavity by the disulphide bridge connecting xl2.50 (XL2) to 3.25 (TM3).

*Comparisons of the binding cavities of the modelled human and mouse $\alpha_{2A}$-adrenoceptors*

The predicted binding cavities of the $\alpha_{2A}$-adrenoceptors of human and mouse (or rat; mouse and rat $\alpha_{2A}$-adrenoceptors have identical amino acids facing the binding cavity) are very similar: the amino acids facing the binding cavities are identical except for three, that is, C5.43, Rxl2.49 and Exl2.51 in the human receptor and S5.43, Sxl2.49 and Kxl2.51 in the mouse and rat receptors (Figure 3). In the extended binding cavity model of Surgand *et al*. (2006), the number of differences in the binding site does not increase, since all amino acids closest to the binding cavity in TM1, TM2 and TM7 are identical in the human and mouse $\alpha_{2A}$-adrenoceptors. Based on the comparison of the mouse and human $\alpha_{2A}$-adrenoceptors, we have focused on the three amino acids that are different within the binding cavity. We have introduced replacements in the human and mouse receptors by site-directed mutagenesis and tested their effects on ligand binding. Five mutants were constructed: a human $\alpha_{2A}$-adrenoceptor with two mutations in XL2 (Rxl2.49S and Exl2.51K) and the reciprocal mouse $\alpha_{2A}$-adrenoceptor double mutant (Sxl2.49R and Kxl2.51E); a human TM5 mutant (C5.43S) and the reciprocal mouse TM5 mutant (S5.43C). In order to validate the results of the double mutants, an additional mouse receptor construct was mutated at all three positions (S5.43C, Sxl2.49R and Kxl2.51E).

*Binding preferences of wild-type human and mouse $\alpha_{2A}$-adrenoceptors for antagonists*

The $K_d$ values for [$^3$H]RX821002 determined in saturation binding assays and the receptor densities of the CHO cell lines are summarized in Table 1. We determined the binding affinities (apparent $K_i$ values; Table 2) for a set of 10 structurally diverse antagonist ligands, using competition

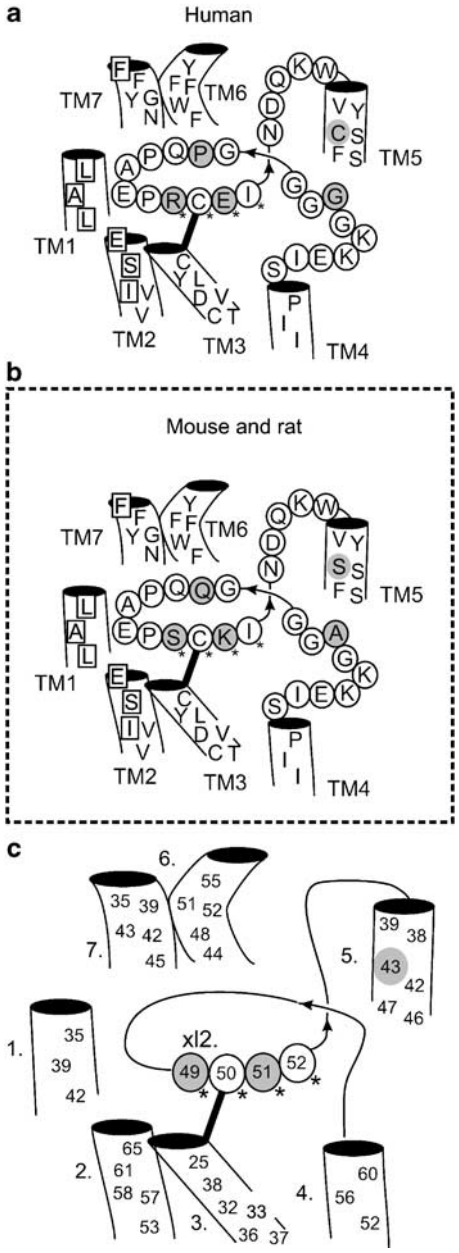


**Figure 3** Schematic comparison of the $\alpha_{2A}$-adrenoceptors from (**a**) human, (**b**) mouse and rat, and corresponding (**c**) amino-acid codes according to the Ballesteros and Weinstein (1995) numbering scheme. Amino acids facing the ligand binding cavity in the transmembrane domains and in XL2 are indicated with one-letter codes. Residues differing between the human and mouse receptors are indicated with grey. Only four residues from XL2 (*) are suggested to face the ligand binding cavity. Amino acids from the expanded binding site close to TM1 suggested by Surgand *et al.* (2006) are boxed. Cysteines at 3.25 and xl2.50 are connected by a disulphide bridge.

binding assays for the wild-type human and mouse $\alpha_{2A}$-adrenoceptors, as well as for five mutants of these receptors. Yohimbine and its chiral analogue rauwolscine showed significant discrimination between the wild-type mouse and human $\alpha_{2A}$-adrenoceptors, as expected (Uhlén *et al.*, 1998): rauwolscine preferred the human wild-type

**Table 1** Binding affinities of [$^3$H]RX821002 and receptor densities in recombinant cell lines (means±s.e.m.)

| *Receptor* | $K_d$ *(nM)* | $B_{max}$ *(pmol mg$^{-1}$ of protein)* |
|---|---|---|
| h$\alpha_{2A}$ C/R/E (wild type) | 1.10±0.17 | 29.5±2.26 |
| h$\alpha_{2A}$ C/S/K (XL2) | 1.20±0.12 | 14.5±1.62 |
| h$\alpha_{2A}$ S/R/E (TM5) | 0.50±0.02 | 43.3±1.51 |
| m$\alpha_{2A}$ S/S/K (wild type) | 0.54±0.02 | 8.49±0.03 |
| m$\alpha_{2A}$ S/R/E (XL2) | 0.93±0.10 | 28.0±5.28 |
| m$\alpha_{2A}$ C/S/K (TM5) | 1.88±0.13 | 5.33±0.10 |
| m$\alpha_{2A}$ C/R/E (TM5 and XL2) | 1.59±0.28 | 2.57±0.25 |

Abbreviations: h, human; m, mouse; TM, transmembrane (domain); XL2, second extracellular loop.
Here, in Table 2 and Figure 3, receptor variants are named based on the amino acids at positions 5.43/xl2.49/xl2.51, for example, C/R/E for the human wild-type $\alpha_{2A}$-adrenoceptor.

$\alpha_{2A}$-adrenoceptor by about 20-fold over the mouse ortholog, while for yohimbine this ratio was 15-fold. Another four antagonist compounds discriminated moderately between the human and mouse $\alpha_{2A}$-adrenoceptors: WB4101 (sevenfold) and RS-79948-197 (fourfold) preferred the human $\alpha_{2A}$-adrenoceptor, while ARC239 (<4-fold) and atipamezole (<4-fold) preferred the mouse ortholog.

The apparent affinities of another four antagonists, doxazosin, chlorpromazine, MK-912 and prazosin, were similar with at most twofold differences (chlorpromazine, doxazosin) between the human and mouse $\alpha_{2A}$-adrenoceptors. Since their affinities remained similar in all mutant receptors, it is likely that the properties of the mutated binding pockets remained intact, and that the receptor mutants provided a valid framework for studying the selectivity of antagonists that discriminate between human and mouse $\alpha_{2A}$-adrenoceptors.

### *Mutations in TM5 and XL2 reciprocally reverse the binding profiles of human and mouse $\alpha_{2A}$-adrenoceptors for yohimbine, rauwolscine and RS-79948-197*

For the reciprocal mutations in XL2 and TM5, reciprocal changes in the binding affinities were seen for the four ligands, rauwolscine, yohimbine, RS-79948-197 and WB4101, which preferred the human $\alpha_{2A}$-adrenoceptor over the mouse receptor (Figure 4). The human double mutant substituted at positions Rxl2.49S and Exl2.51K changed the ligand binding profile so that the $K_i$ increased for rauwolscine (fourfold), yohimbine (sixfold) and RS-79948-197 (sevenfold). The reciprocal double mutation (Sxl2.49R and Kxl2.51E) in the mouse receptor led to opposite effects on the binding of these ligands: decreases in the $K_i$ for rauwolscine (<2-fold), yohimbine (fourfold) and RS-79948-197 (fourfold). A corresponding reciprocal effect for rauwolscine and yohimbine was seen with substitutions at position 5.43, and the mutation C5.43S in the human receptor led to an increase in the $K_i$ for rauwolscine (eightfold) and yohimbine (sixfold), whereas the reciprocal S5.43C mutation in the mouse receptor led to a reduced $K_i$ for rauwolscine (threefold) and yohimbine (sixfold). The mutations at 5.43 did not, however, alter the affinity for RS-79948-197. When all three positions were simultaneously mutated in the

**Table 2** Competition binding affinities of different ligands obtained with [$^3$H]RX821002 at human and mouse wild-type and mutant $\alpha_{2A}$-adrenoceptors expressed in CHO cells

| *Ligand* | *Human* $\alpha_{2A}$ | | | *Mouse* $\alpha_{2A}$ | | | |
|---|---|---|---|---|---|---|---|
| | *WT (C/R/E)* | *xl2 (C/S/K)* | *TM5 (S/R/E)* | *WT (S/S/K)* | *xl2 (S/R/E)* | *TM5 (C/S/K)* | *TM5 and xl2 (C/R/E)* |
| Rauwolscine | 1.1 (0.48–2.5)●● | 4.7 (3.4–6.5)** | 9.0 (6.8–12)*** | 22 (15–32) | 15 (12–20) | 6.9 (4.6–10)* | 3.9 (2.6–5.7)** |
| RS-79948-197 | 0.16 (0.054–0.53) | 1.1 (0.72–1.7)*** | 0.19 (0.15–0.24) | 0.64 (0.33–1.2) | 0.15 (0.087–0.28)* | 0.63 (0.51–0.78) | 0.11 (0.046–0.28)* |
| WB4101 | 4.5 (2.0–7.4)●● | 5.9 (3.2–11) | 2.7 (0.46–17) | 32 (9.0–100) | 44 (7.8–170) | 11 (1.7–85) | 8.1 (2.3–32) |
| Yohimbine | 2.1 (1.6–4.6)●● | 13 (6.3–23)*** | 12 (8.8–16)*** | 31 (21–46) | 7.0 (3.4–15)** | 4.9 (2.2–11)** | 2.0 (1.2–4.1)*** |
| ARC239 | 1800 (1400–2400)●●● | 3000 (1300–9200) | 2400 (1000–5600) | 500 (200–1300) | 550 (240–1700) | 650 (210–2100) | 280 (120–710) |
| Atipamezole | 2.3 (1.3–3.9)●●● | 3.4 (1.5–8.4) | 1.4 (0.47–5.3) | 0.63 (0.52–0.76) | 1.9 (0.84–5.6)** | 3.6 (1.2–11)** | 4.3 (1.4–13)*** |
| Chlorpromazine | 200 (67–630) | 280 (95–840) | 210 (82–570) | 100 (38–280) | 100 (43–260) | 200 (75–540) | 150 (66–370) |
| Doxazosin | 710 (330–1600) | 1600 (670–5100) | 1300 (270–6500) | 1600 (340–8100) | 1600 (470–8900) | 2300 (630–8800) | 720 (330–2700) |
| MK-912 | 2.5 (1.2–5.6) | 1.5 (0.64–3.4) | 1.2 (0.87–1.6) | 2.7 (1.7–4.2) | 2.6 (1.4–4.7) | 2.5 (1.1–5.8) | 2.8 (1.8–4.3) |
| Prazosin | 880 (250–3400) | 1600 (770–3700) | 650 (200–2100) | 510 (290–1400) | 780 (180–2400) | 390 (130–1300) | 190 (68–510) |
| Adrenaline | 880 (260–2100) | 1900 (510–7200) | 1900 (1400–2700) | 910 (200–4300) | 5300 (530–61 000) | 570 (240–2000) | 500 (140–2200) |
| Noradrenaline | 2400 (940–6800) | 2200 (1000–5200) | 1000 (340–4100) | 1900 (400–12 000) | 6200 (900–48 000) | 1400 (510–3900) | 590 (180–2000) |
| Dopamine | 4200 (1500–13 000) | 5900 (3300–11 000) | 3200 (1100–9500) | 8000 (1500–72 000) | 21 000 (4900–110 000) | 3400 (760–17 000) | 5000 (1000–18 000) |
| Chlorpromazine/yohimbine | 95 | 22 | 18 | 3 | 14 | 41 | 75 |

Abbreviation: CHO, Chinese hamster ovary.
For other abbreviations see Table 1.
Results are expressed as apparent $K_i$ (nM) and 95% confidence intervals of 3–5 independent experiments. Ligands are grouped as (1) antagonists with more than fourfold different affinity between the human and mouse wild-type receptors, (2) antagonists with less than fourfold difference in affinity between the human and mouse wild-type receptors, (3) endogenous agonists. The pharmacological comparison of receptors is illustrated by the ratios of the $K_i$ values for chlorpromazine and yohimbine. *$P<0.05$; **$P<0.01$; ***$P<0.001$ mutant versus wild type; ●$P<0.05$; ●●$P<0.01$; ●●●$P<0.001$ human versus mouse.

mouse $\alpha_{2A}$-adrenoceptor (S5.43C, Sxl2.49R and Kxl2.51E), the largest affinity shifts were observed, with significant increases seen for yohimbine (16-fold; $P=0.0007$), rauwolscine (sixfold; $P=0.0084$) and RS-79948-197 (sixfold; $P=0.026$). The affinities were now similar to those of the human wild-type $\alpha_{2A}$-adrenoceptor.

Of the other ligands, WB4101 preferred the human $\alpha_{2A}$-adrenoceptor by about sevenfold over the mouse receptor (apparent $K_i$, 4.5 versus 32 nM; $P=0.0051$), with a tendency for improved affinity at the TM5 and triple mutant of the mouse receptor; however, reciprocal effects were not seen in the human receptor. These results were characterized by large overlapping confidence intervals, and thus remain inconclusive. Two ligands, ARC239 and atipamezole, preferred the mouse ortholog by less than fourfold over the human ortholog, but none of the mutations significantly affected the binding affinity of ARC239 compared to the wild-type receptors. For atipamezole, mutations in both XL2 and TM5 in the mouse receptor significantly decreased its binding affinity (XL2, threefold; TM5, sixfold) to resemble the human receptor, and correspondingly in the human receptor, the mutation of 5.43 in TM5 (but not those in XL2) tended to increase the affinity.

In summary, positions xl2.49 (S/R) and xl2.51 (E/K) in XL2, and position 5.43 (C/S) in TM5 are important for determining the binding preference of yohimbine and rauwolscine for the human $\alpha_{2A}$-adrenoceptor versus its mouse ortholog. In contrast, xl2.49 (S/R) and xl2.51 (E/K), but not the residue at 5.43, are important for determining the binding preference of RS-79948-197 for the human $\alpha_{2A}$-adrenoceptor. For WB4101 and atipamezole, for which the initial difference between the mouse and human receptor affinities was small, no definitive conclusions can be reached. For ARC239 and prazosin, which share rather poor affinity for the human (and zebrafish, Ruuskanen *et al*., 2005) $\alpha_{2A}$-adrenoceptor, the mutations did not explain the small preference for the mouse receptor observed here.

### *Binding preferences of wild-type human and mouse $\alpha_{2A}$-adrenoceptors for endogenous agonists*

Three endogenous agonists of $\alpha_{2A}$-adrenoceptors, adrenaline, noradrenaline and dopamine, were also tested. The agonist affinities did not differ significantly between the wild-type mouse and human $\alpha_{2A}$-adrenoceptors (Table 2). In most cases, the agonist affinity values exhibited greater experimental variability – larger confidence intervals – than those of the antagonists. The agonist results are more likely to be affected by the variation in the expression levels of the receptors, as a high level of expression leads to a predominance of the low-affinity conformation of the receptor, thus shifting the agonist binding curve to the right. The order of preference ($K_i$) for the catecholamines was conserved between the receptor variants: adrenaline <noradrenaline <dopamine.

## Discussion and conclusions

In the present study, we have investigated the effects of mutations on ligand selectivity towards the mouse and

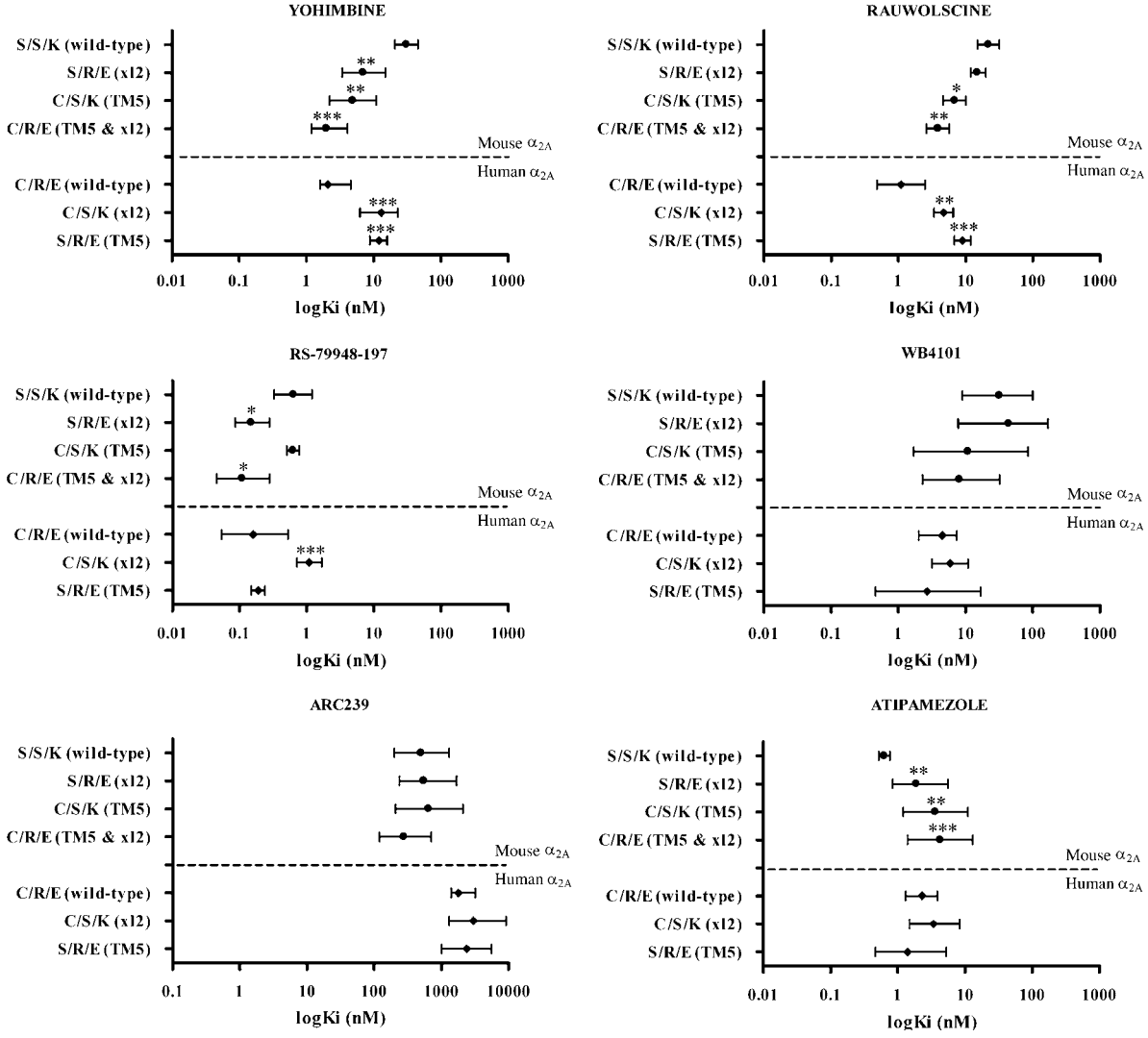


**Figure 4** Comparison of the binding affinities of six selected antagonists towards the mouse and human $\alpha_{2A}$-adrenoceptors and their mutants. Error bars represent the 95% confidence intervals of 3–5 separate experiments. Statistical significance compared to the wild-type receptor (unpaired *t*-test): *$P<0.05$, **$P<0.01$, ***$P<0.001$.

human $\alpha_{2A}$-adrenoceptors. Only three positions that are accessible in the binding cavity are predicted to differ. We confirmed that position 5.43 in TM5 affects reciprocally the binding of two classical $\alpha_2$-adrenoceptor antagonists, yohimbine and rauwolscine (Link *et al*., 1992; Cockcroft *et al*., 2000). In addition, we show that two residues in the XL2 domain of the $\alpha_{2A}$-adrenoceptors, xl2.49 and xl2.51, affect the binding affinities of yohimbine and rauwolscine, contributing to the discrimination between the mouse and human receptors to the same extent as the residue at 5.43. The binding of RS-79948-197 was significantly influenced by the XL2 mutations but not by changes at 5.43.

*XL2 and 5.43 may provide anchoring points for antagonists*

Molecular modelling was used to predict the amino acids facing the ligand-binding cavity, and identified three positions that differ within the cavity (Figures 2 and 3). Our results (Table 2 and Figure 4) suggest that two positions in XL2, combined with 5.43, contribute to the species-dependent discrimination by the receptors of some yohimbine-like antagonists. The presence of XL2 as a $\beta$-hairpin, forming the extracellular surface of the binding cavity, is supported by the proposed disulphide bridge connecting C3.25 and Cxl2.50, which is found in the rhodopsin structure and is undoubtedly present in these receptors, too. The disulphide bridge provides an extremely valuable spatial restraint since the sequence similarity between rhodopsin and the $\alpha_2$-adrenoceptors is low within the loop regions. Since the mutated positions in XL2 are located adjacent to Cxl2.50, the position of xl2.49 and xl2.51 relative to the binding cavity should be very well modelled.

We manually docked yohimbine, rauwolscine, RS-79948-197 and MK-912 into the binding site of the $\alpha_{2A}$-adrenoceptor model structures built for the human (Figure 5) and

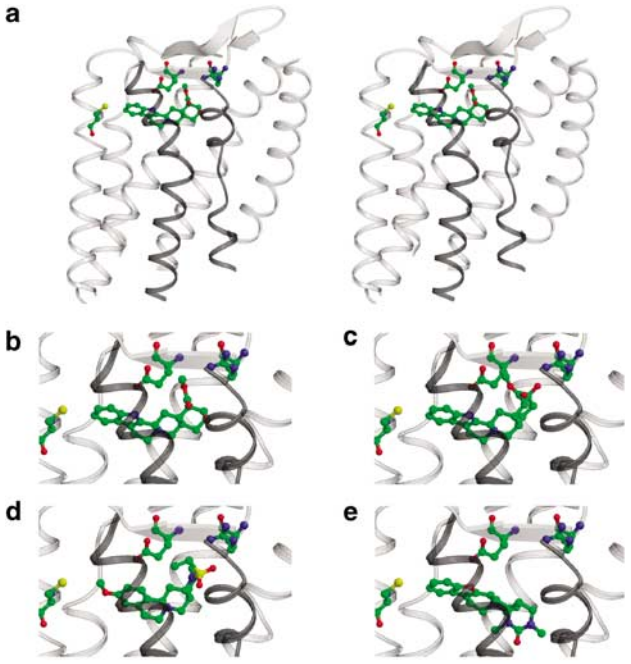


**Figure 5** Docking of yohimbine into the molecular model of the human $\alpha_{2A}$-adrenoceptor. The view is from the plane of the membrane through TM6 and TM7 (dark helices); the 7TM bundle is oriented so that TM5 is on the left, TM1 is on the right and XL2 forms the upper surface of the receptor. (**a**) Overall structure with yohimbine docked within the agonist binding site (in stereo); a close-up view is shown in (**b**). The left-most ring of (**c**) rauwolscine, (**d**) RS-79948-197 and (**e**) MK-912 was superposed on the ring from yohimbine docked to the human $\alpha_{2A}$-adrenoceptor. MK-912, in contrast to yohimbine, rauwolscine and RS-79948-197, clashes with the receptor (especially with W6.48 in TM6) and thus another mode of binding likely takes place. The three side chains mutated in this study are shown: cysteine 5.43 (left of the ligand), glutamate xl2.51 (above the ligand, left) and arginine xl2.49 (above the ligand, right). Carbon atoms are shown in green.

mouse receptors. Our assumption, based on the experimental results, was that these ligands would be bound in direct contact with the side chains at 5.43, xl2.49 and xl2.51. In order to fulfil these requirements, the antagonist ligands should be placed close to TM5 (Xhaard *et al*., 2005), where they fully occupy the agonist-binding pocket. Note that our proposed modes of binding differ from those where the ligand must be shifted away from TM5 in order to form a direct ion-pair interaction between D3.32 and the ligand's protonated amine (Surgand *et al*., 2006).

The agonist binding cavity is elongated and oriented horizontally with respect to the cell membrane. Yohimbine, rauwolscine and RS-79948-197 are also elongated, nearly planar, 'L'-shaped molecules formed from four or five ring systems. Both yohimbine and rauwolscine have an aromatic, hydrophobic end, as well as a polar group (carboxymethyl and hydroxyl groups) at the opposite end, which in the models would match well the location of the residues at 5.43 and xl2.49 and xl2.51 (Figures 5b and c). In line with this view, the human $\alpha_{2A}$-adrenoceptor with a non-polar cysteine at 5.43 binds yohimbine and rauwolscine with higher affinity than the mouse/rat receptor having a serine. When the $SO_2$ group of RS-79948-197 is superimposed on the polar carboxymethyl group of yohimbine and rauwolscine (Figure 5d), the methoxy group at the other end of RS-79948-197 is located near TM5 and could form favourable interactions with either a cysteine or a serine at 5.43; accordingly, the binding of RS-79948-197 was not affected by C/S substitutions at 5.43. Like the yohimbine analogues, MK-912 contains a ring system. When MK-912 was docked onto yohimbine with their aromatic ends similarly placed closest to TM5 (Figure 5e), clashes with the receptor structure, especially with W6.48 in TM6, occurred as a result of the molecules being bent in opposite directions. Thus, it is probable that MK-912 on one hand and yohimbine, rauwolscine and RS-79948-197 on the other hand adopt different modes of binding. Binding of MK-912 was affected neither by mutations at 5.43 nor at xl2.49/xl2.51.

We have previously suggested (Xhaard *et al*., 2006) that the $\beta$-hydroxyl group of the adrenoceptor agonists noradrenaline and adrenaline could hydrogen-bond to the amide backbone of XL2 at xl2.52 and possibly to the functional groups of long side chains at xl2.49 or xl2.51, too. However, although long side chains are often found at these positions in the adrenoceptors, the residue types are generally not conserved. Here, the preference order for the agonists, adrenaline > noradrenaline > dopamine, was conserved, and the introduced mutations did not significantly influence the binding of adrenaline and noradrenaline relative to dopamine. This result is consistent with the proposal that the $\beta$-hydroxyl of adrenaline and noradrenaline interacts with the amide backbone of XL2, possibly from xl2.52, rather than with the side chains of xl2.49 or xl2.51.

### *Relative contributions of XL2 and 5.43 to the binding affinity of $\alpha_{2A}$-adrenoceptors for yohimbine in different species*

Yohimbine binds with relatively low affinity to guinea-pig and bovine $\alpha_{2A}$-adrenoceptors that have pharmacological profiles similar to mouse and rat $\alpha_{2A}$-adrenoceptors, while chicken, zebrafish, rabbit and pig $\alpha_{2A}$-adrenoceptors have profiles more similar to the human receptor ortholog (Bylund *et al*., 1988; O'Rourke *et al*., 1994; Svensson *et al*., 1996; Uhlén *et al*., 1998; Bylund, 2005). The binding cavity of the $\alpha_{2A}$-adrenoceptor was modelled for each of these species (Figure 6). In seven out of the eight animal species, the presence of a cysteine or serine at position 5.43 correlated well with the receptor's affinity for yohimbine, that is, serine is linked to the lower affinity binding (except in chicken), whereas cysteine is present in receptors that bind yohimbine with higher affinity. Indeed, the pharmacological profiles of four species, human, rabbit, cow and guinea-pig, which are otherwise identical at xl2.49 and xl2.51, are segregated according to the residue present at position 5.43, that is, cow and guinea-pig have here a serine and mouse-like pharmacology, while human and rabbit have a cysteine and human-like pharmacology (Svensson *et al*., 1996; Uhlén *et al*., 1998). Therefore, the amino acid at position 5.43 alone is a major determinant of the binding profile for these species, consistent with the findings of this study. The chicken receptor is an exception to this pattern, suggesting that also other regions of the binding site may have significant effects on ligand binding (Bylund, 2005).

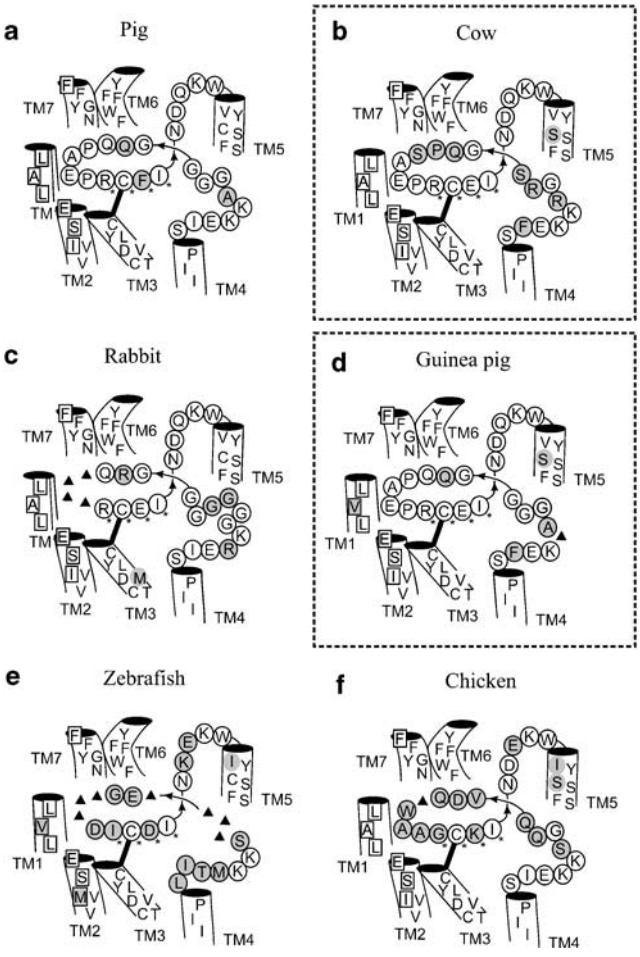


**Figure 6** Schematic comparison of the binding cavities of $\alpha_{2A}$-adrenoceptors from (**a**) pig, (**b**) cow, (**c**) rabbit, (**d**) guinea-pig, (**e**) zebrafish and (**f**) chicken. The codes used for comparison to the human receptor are as in Figure 2, but amino-acid deletions are shown as triangles. The receptors of species with 'mouse-like' pharmacology are boxed – with lower affinity for yohimbine.

This study provides the first evidence that substitutions at positions xl2.49 and xl2.51 influence the pharmacological profiles of human and mouse $\alpha_{2A}$-adrenoceptors for yohimbine, rauwolscine and RS-79948-179, showing that these positions have an effect on their own. Indeed, the triple mutant with substitutions at xl2.49, xl2.51 and 5.43 produces a fully reversed pharmacological profile for yohimbine binding, showing that the effect of xl2.49/xl2.51 is additive to that of 5.43. For yohimbine, rauwolscine and RS-79948-197, the observed differences for mutants at xl2.49 and xl2.51 were two- to sevenfold with respect to the wild-type receptors, that is, in the same order as was observed for mutants at 5.43 (0- to 8-fold). Thus, the impact of residues from the XL2 domain on species selectivity for these yohimbine analogues appears to be equivalent to that of 5.43. Since amino acids at xl2.49 and xl2.51 are identical among human, rabbit, cow and guinea-pig $\alpha_{2A}$-adrenoceptors, these two positions would not explain any differences between these species in the binding of a ligand, but this does not mean that they do not mediate important interactions with ligands. Indeed, for other species comparisons, for example, human versus mouse, differences at these positions reveal their importance for ligand binding.

Curiously, the chicken $\alpha_{2A}$-adrenoceptor, with a serine at position 5.43 as in the mouse receptor, has a pharmacological profile closer to that of the human $\alpha_{2A}$-adrenoceptor (Bylund *et al*., 1988; Blaxall *et al*., 1993). The $\alpha_{2A}$-adrenoceptors of mouse and chicken differ at several positions within XL2, for example, one amino acid downstream of xl2.49 is deleted in the chicken receptor, and glycine is found at xl2.49 in the chicken receptor whereas serine is present in the mouse receptor. This suggests that XL2 also contributes to the binding preferences of the chicken $\alpha_{2A}$-adrenoceptor. In addition to XL2, for several species, amino acids facing the binding cavity in the TM regions also differ (Figure 6). For example, chicken and zebrafish $\alpha_{2A}$-adrenoceptors have isoleucine at position 5.39 (valine in human and mouse), the rabbit receptor has methionine at position 3.33 (valine) and guinea-pig and zebrafish receptors have valine at position 1.39 (alanine). These differences may contribute to some extent to the binding profiles of the receptors towards different ligands.

The present study provides further evidence that antagonist affinities at monoamine GPCRs may be significantly influenced by interactions with XL2. In the $\alpha_1$-adrenoceptor subtypes A and B, amino-acid substitutions in XL2 at xl2.51, xl2.52 and xl2.53 have altered the subtype-selectivity of phentolamine and WB4101 (Zhao *et al*., 1996). Positions xl2.52 and xl2.53 are conserved between the human and mouse $\alpha_{2A}$-adrenoceptors, and therefore cannot contribute to the species preferences observed here. For another monoamine GPCR, the type-1D serotonin receptor, an 8-amino-acid replacement in XL2, positions xl2.44 to xl2.51, affected the binding of ketanserin (Wurch *et al*., 1998). XL2 also has a role in antagonist binding to the $D_1$ and $D_2$ dopamine receptors (Lan *et al*., 2006). In conclusion, this is the first study to show that XL2 has a direct role in the binding of some $\alpha_{2A}$-adrenoceptor ligands, that is, yohimbine, rauwolscine and RS-79948-197.

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## Conflict of interest

Mika Scheinin has patents related to $\alpha_2$-adrenoceptor pharmacology, owns stock in Juvantia Pharma Ltd., a drug discovery company with an interest in $\alpha_2$-adrenoceptor pharmacology, and conducts contract research for Orion Pharma Ltd, the manufacturer of atipamezole. Mark S Johnson is CEO of and owns stock in FBD Ltd, a computational chemistry consultancy enterprise.

## References

Altman JD, Trendelenberg AU, MacMillan L, Bernstein D, Limbird L, Starke K *et al.* (1999). Abnormal regulation of the sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. *Mol Pharmacol* **56**: 154–161.

Ballesteros JA, Weinstein H (1995). Integrated methods for the construction of three-dimensional models and computational probing of structure–function relations in G protein-coupled receptors. In: Sealfon SC (ed). *Receptor Molecular Biology*. Academic Press Inc.: San Diego, pp 366–427.

Bissantz C, Bernard P, Hibert M, Rognan D (2003). Protein-based virtual screening of chemical databases. II. Are homology models of G-protein coupled receptors suitable targets? *Proteins* **50**: 5–25.

Blaxall HS, Heck DA, Bylund DB (1993). Molecular determinants of the alpha-2D adrenergic receptor subtype. *Life Sci* **53**: 255–259.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **7**: 248–254.

Bylund DB (2005). Alpha-2 adrenoceptor subtypes: are more better? *Br J Pharmacol* **144**: 159–160.

Bylund DB, Rudeen PK, Petterborg LJ, Ray-Prenger C (1988). Identification of alpha 2-adrenergic receptors in chicken pineal gland using [$^3$H]rauwolscine. *J Neurochem* **51**: 81–86.

Cockcroft V, Frang H, Pihlavisto M, Marjamäki A, Scheinin M (2000). Ligand recognition of serine-cysteine amino acid exchanges in transmembrane domain 5 of alpha2-adrenergic receptors by UK 14,304. *J Neurochem* **74**: 1705–1710.

Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108.

Dixon RA, Sigal IS, Candelore MR, Register RB, Scattergood W, Rands E *et al.* (1987). Structural features required for ligand binding to the beta-adrenergic receptor. *EMBO J* **11**: 3269–3275.

Eason MG, Jacinto MT, Theiss CT, Liget SB (1994). The palmitoylated cysteine of the cytoplasmic tail of alpha 2A-adrenergic receptors confers subtype-specific agonist-promoted downregulation. *Proc Natl Acad Sci USA* **91**: 11178–11182.

Frang H, Cockcroft V, Karskela T, Scheinin M, Marjamäki A (2001). Phenoxybenzamine binding reveals the helical orientation of the third transmembrane domain of adrenergic receptors. *J Biol Chem* **276**: 31279–31284.

Halme M, Sjöholm B, Savola JM, Scheinin M (1995). Recombinant human alpha 2-adrenoceptor subtypes: comparison of [$^3$H]rauwolscine, [$^3$H]atipamezole and [$^3$H]RX821002 as radioligands. *Biochim Biophys Acta* **1266**: 207–214.

Johnson MS, Overington JP (1993). A structural basis for sequence comparisons. An evaluation of scoring methodologies. *J Mol Biol* **233**: 716–738.

Lan H, Durand CJ, Teeter MM, Neve KA (2006). Structural determinants of pharmacological specificity between D(1) and D(2) dopamine receptors. *Mol Pharmacol* **69**: 185–194.

Li J, Edwards PC, Burghammer M, Villa C, Schertler GF (2004). Structure of bovine rhodopsin in a trigonal crystal form. *J Mol Biol* **343**: 1409–1438.

Link R, Daunt D, Brash G, Chruscinski A, Kobilka B (1992). Cloning of two mouse genes encoding alpha 2-adrenergic receptor subtypes and identification of a single amino acid in the mouse alpha 2-C10 homolog responsible for an interspecies variation in antagonist binding. *Mol Pharmacol* **42**: 16–27.

Link RE, Desai K, Hein L, Stevens ME, Chruscinski A, Bernstein D *et al.* (1996). Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtype b and c. *Science* **273**: 803–805.

MacDonald E, Kobilka BK, Scheinin M (1997). Gene targeting – homing in on alpha 2-adrenoceptor-subtype function. *Trends Pharmacol Sci* **18**: 211–219.

MacMillan LB, Hein L, Smith MS, Piascik MT, Limbird LE (1996). Central hypotensive effects of the alpha2a-adrenergic receptor subtype. *Science* **273**: 801–803.

Marjamäki A, Pihlavisto M, Cockcroft V, Heinonen P, Savola JM, Scheinin M (1998). Chloroethylclonidine binds irreversibly to exposed cysteines in the fifth membrane-spanning domain of the human alpha2A-adrenergic receptor. *Mol Pharmacol* **53**: 370–376.

Michel AD, Loury DN, Whiting RL (1989). Differences between the alpha 2-adrenoceptor in rat submaxillary gland and the alpha 2A-and alpha 2B-adrenoceptor subtypes. *Br J Pharmacol* **98**: 890–897.

Molderings GJ, Bonisch H, Bruss M, Likungu J, Gothert M (2000). Species-specific pharmacological properties of human alpha(2A)-adrenoceptors. *Hypertension* **36**: 405–410.

Nyrönen T, Pihlavisto M, Peltonen JM, Hoffrén AM, Varis M, Salminen T *et al.* (2001). Molecular mechanism for agonist-promoted alpha(2A)-adrenoceptor activation by norepinephrine and epinephrine. *Mol Pharmacol* **59**: 1343–1354.

Okada T, Fujiyoshi Y, Silow M, Navarro J, Landau EM, Shichida Y (2002). Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. *Proc Natl Acad Sci USA* **99**: 5982–5987.

O'Rourke MF, Iversen LJ, Lomasney JW, Bylund DB (1994). Species orthologs of the alpha-2A adrenergic receptor: the pharmacological properties of the bovine and rat receptors differ from the human and porcine receptors. *J Pharmacol Exp Ther* **271**: 735–740.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA *et al.* (2000). Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **289**: 739–745.

Peltonen JM, Nyrönen T, Wurster S, Pihlavisto M, Hoffrén AM, Xhaard H *et al.* (2003). Molecular mechanisms of ligand–receptor interactions in transmembrane domain V of the alpha2A-adrenoceptor. *Br J Pharmacol* **140**: 347–358.

Richman JG, Brady AE, Wang Q, Hensel JL, Colbran RJ, Limbird LE (2001). Agonist-regulated interaction between alpha2-adrenergic receptors and spinophilin. *J Biol Chem* **276**: 15003–15008.

Ruffolo Jr RR, Hieble JP (1994). Alpha-adrenoceptors. *Pharmacol Ther* **61**: 1–64.

Ruuskanen JO, Laurila J, Xhaard H, Rantanen VV, Vuoriluoto K, Wurster S *et al.* (2005). Conserved structural, pharmacological and functional properties among the three human and five zebrafish alpha 2-adrenoceptors. *Br J Pharmacol* **144**: 165–177.

Ruuskanen JO, Xhaard H, Marjamäki A, Salaneck E, Salminen T, Yan YL *et al.* (2004). Identification of duplicated fourth alpha2-adrenergic receptor subtype by cloning and mapping of five receptor genes in zebrafish. *Mol Biol Evol* **21**: 14–28.

Sali A, Blundell TL (1993). Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* **234**: 779–815.

Salminen T, Varis M, Nyrönen T, Pihlavisto M, Hoffrén AM, Lönnberg T *et al.* (1999). Three-dimensional models of alpha(2A)-adrenergic receptor complexes provide a structural explanation for ligand binding. *J Biol Chem* **274**: 23405–23413.

Schwartz TW (1994). Locating ligand-binding sites in 7TM receptors by protein engineering. *Curr Opin Biotechnol* **4**: 434–444.

Shi L, Javitch JA (2004). The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice. *Proc Natl Acad Sci USA* **101**: 440–445.

Simonneaux V, Ebadi M, Bylund DB (1991). Identification and characterization of alpha 2D-adrenergic receptors in bovine pineal gland. *Mol Pharmacol* **40**: 234–241.

Strader CD, Sigal IS, Register RB, Candelore MR, Rands E, Dixon RA (1987). Identification of residues required for ligand binding to the beta-adrenergic receptor. *Proc Natl Acad Sci USA* **84**: 4384–4388.

Surgand JS, Rodrigo J, Kellenberger E, Rognan D (2006). A chemogenomic analysis of the transmembrane binding cavity of human G-protein-coupled receptors. *Proteins* **62**: 509–538.

Svensson SP, Bailey TJ, Porter AC, Richman JG, Regan JW (1996). Heterologous expression of the cloned guinea pig alpha 2A, alpha 2B, and alpha 2C adrenoceptor subtypes. Radioligand binding and functional coupling to a CAMP-responsive reporter gene. *Biochem Pharmacol* **51**: 291–300.

Teller DC, Okada T, Behnke CA, Palczewski K, Stenkamp RE (2001). Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* **40**: 7761–7772.

Trumpp-Kallmeyer S, Hoflack J, Bruinvels A, Hibert M (1992). Modeling of G-protein-coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. *J Med Chem* **35**: 3448–3462.

Uhlén S, Dambrova M, Näsman J, Schiöth HB, Gu Y, Wikberg-Matsson A *et al*. (1998). [$^3$H]RS79948-197 binding to human, rat, guinea pig and pig alpha2A-, alpha2B- and alpha2C-adrenoceptors. Comparison with MK912, RX821002, rauwolscine and yohimbine. *Eur J Pharmacol* **343**: 93–101.

Wong SK, Slaughter C, Ruoho AE, Ross EM (1988). The catecholamine binding site of the beta-adrenergic receptor is formed by juxtaposed membrane-spanning domains. *J Biol Chem* **263**: 7925–7928.

Wurch T, Colpaert FC, Pauwels PJ (1998). Chimeric receptor analysis of the ketanserin binding site in the human 5-hydroxytryptamine$_{1D}$ receptor: importance of the second extracellular loop and fifth transmembrane domain in antagonist binding. *Mol Pharmacol* **54**: 1088–1096.

Xhaard H, Nyrönen T, Rantanen VV, Ruuskanen JO, Laurila J, Salminen T *et al*. (2005). Model structures of alpha-2 adrenoceptors in complex with automatically docked antagonist ligands raise the possibility of interactions dissimilar from agonist ligands. *J Struct Biol* **150**: 126–143.

Xhaard H, Rantanen VV, Nyrönen T, Johnson MS (2006). Molecular evolution of adrenoceptors and dopamine receptors: implications for the binding of catecholamines. *J Med Chem* **49**: 1706–1719.

Zhao MM, Hwa J, Perez DM (1996). Identification of critical extracellular loop residues involved in alpha 1-adrenergic receptor subtype-selective antagonist binding. *Mol Pharmacol* **50**: 1118–1126.