TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 962

MEDICA - ODONTOLOGICA

α_2-ADRENOCEPTORS: STRUCTURE AND LIGAND BINDING PROPERTIES AT THE MOLECULAR LEVEL

by

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TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2011

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ISBN 978-951-29-4606-8 (PRINT)
ISBN 978-951-29-4607-5 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2011

In memory of my father

Tapani Laurila (1939-2006)

Jonne M.M. Laurila

α_2-Adrenoceptors: structure and ligand binding properties at the molecular level

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Annales Universitatis Turkuensis, Painosalama Oy, Turku, Finland 2011

ABSTRACT

The mouse is the most frequently used animal model in biomedical research, but the use of zebrafish as a model organism to mimic human diseases is on the increase. Therefore it is considered important to understand their pharmacological differences from humans also at the molecular level.

The zebrafish α_2-adrenoceptors were expressed in mammalian cells and the binding affinities of 20 diverse ligands were determined and compared to the corresponding human receptors. The pharmacological properties of the human and zebrafish α_2-adrenoceptors were found to be quite well conserved.

Receptor models based on the crystal structures of bovine rhodopsin and the human β_2-adrenoceptor revealed that most structural differences between the paralogous and orthologous α_2-adrenoceptors were located within the second extracellular loop (XL2). Reciprocal mutations were generated in the mouse and human α_{2A}-adrenoceptors. Ligand binding experiments revealed that substitutions in XL2 reversed the binding profiles of the human and mouse α_{2A}-adrenoceptors for yohimbine, rauwolscine and RS-79948-197, evidence for a role for XL2 in the determination of species-specific ligand binding.

Previous mutagenesis studies had not been able to explain the subtype preference of several large α_2-adrenoceptor antagonists. We prepared chimaeric α_2-adrenoceptors where the first transmembrane (TM1) domain was exchanged between the three human α_2-adrenoceptor subtypes. The binding affinities of spiperone, spiroxatrine and chlorpromazine were observed to be significantly improved by TM1 substitutions of the α_{2A}-adrenoceptor. Docking simulations indicated that indirect effects, such as allosteric modulation, are more likely to be involved in this phenomenon rather than specific side-chain interactions between ligands and receptors.

Key words: α_2-adrenoceptor, GPCR, ligand binding, XL2, receptor models

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α_2-Adrenoseptorit: rakenne ja lääkeaineiden sitoutumisominaisuudet molekyylitasolla
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Annales Universitatis Turkuensis, Painosalama Oy, Turku 2011

TIIVISTELMÄ

Biolääketieteen tutkimuksessa hiiri on yleisimmin käytetty koe-eläin, mutta seeprakalan käyttö ihmisen sairauksien mallinnuksessa on lisääntymässä. Siksi on tärkeää tutkia ja ymmärtää näiden eläinten ja ihmisten farmakologisten ominaisuuksien eroavaisuuksia myös molekyylitasolla.

Seeprakalan α_2-adrenoseptoreja tuotettiin nisäkässoluissa ja verrattiin 20 erilaisen lääkeyhdisteen sitoutumista niihin ja ihmisen vastaaviin reseptoreihin. Ihmisen ja seeprakalan α_2-adrenoseptorien farmakologiset ominaisuudet todettiin hyvin samanlaisiksi.

Naudan rodopsiinin ja ihmisen β_2-adrenoseptorin kiderakenteisiin perustuvat α_2-reseptorien rakennemallit osoittivat, että suurimmat rakenne-erot sekä reseptorialatyyppien että eri eläinlajien reseptorien välillä löytyvät reseptorien toisesta solunulkoisesta silmukkarakenteesta (XL2). Erilaiset aminohapot vaihdettiin hiiren ja ihmisen α_{2A}-adrenoseptorien välillä toistensa kaltaisiksi. Näillä muokatuilla reseptoreilla tehdyt sitoutumiskokeet osoittivat kolmen testatun lääkeaineen, johimbiinin, rauwolskiinin ja RS-79948-197:n, sitoutumishanakkuuden muuttuvan vastaavasti. Tulos osoitti, että XL2:n rakenne osaltaan määrittää eläinlajille tyypillisen lääkeaineiden sitoutumisprofiilin sen α_{2A}-reseptoreihin.

Aiemmat reseptorien muokkaamiseen perustuvat tutkimukset eivät olleet kyenneet selittämään isokokoisten α_2-reseptoreja salpaavien lääkeainemolekyylien valikoivaa sitoutumista α_2-reseptorialatyyppeihin. Sen vuoksi valmistettiin ns. kimeerisiä α_2-reseptoreja, joissa ensimmäistä solukalvon läpäisevää rakennejaksoa (TM1) vaihdeltiin systemaattisesti ihmisen kolmen α_2-reseptorialatyypin välillä. TM1-jakson vaihdon seurauksena kolmen reseptorinsalpaajan, spiperonin, spiroksatriinin ja klooripromatsiinin havaittiin sitoutuvan aiempaa selvästi hanakammin α_{2A}-reseptoreihin. Reseptorien tietokonemalleilla suoritetut lääkeaineiden sovittamissimulaatiot viittasivat siihen, että lisääntynyt sitoutumishanakkuus ei johdu spesifisistä vuorovaikutusten muutoksista reseptorien sitomiskohtien ja lääkeaineiden välillä vaan epäsuorista vaikutuksista reseptorien kolmiulotteiseen muotoon.

Avainsanat: α_2-adrenoseptori, GPCR, lääkeaineiden sitoutuminen, XL2, reseptorimallit

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ABBREVIATIONS

5-HT	5-Hydroxytryptamine, serotonin
B_{max}	Maximal binding capacity
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CNS	Central nervous system
C-terminus	Carboxyl terminus (of polypeptide)
DRY	Aspartic acid-arginine-tyrosine motif
EC_{50}	Drug concentration that elicits 50 % of the maximal response
EDTA	Ethylenediaminetetraacetic acid
G protein	Guanine nucleotide binding protein
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
GTPγS	Guanosine 5´-[gamma-thio]triphosphate
IC_{50}	Drug concentration that inhibits 50 % of specific binding
K_d	Equilibrium dissociation constant
K_i	Binding inhibition constant
MAO	Monoamine oxidase
NSB	Non-specific binding
N-terminus	Amino terminus (of polypeptide)
PCA	Principal component analysis
PCR	Polymerase chain reaction
PTX	Pertussis toxin
RT	Room temperature
RT-PCR	Reverse transcription PCR
SCAM	Substituted cysteine accessibility method
TM (TM1-TM7)	Transmembrane domain (1-7)
XL2	Second extracellular loop

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-III. In addition, some unpublished data are presented.

I Ruuskanen JO, **Laurila J**, Xhaard H, Rantanen V-V, Vuoriluoto K, Wurster S, Marjamäki A, Vainio M, Johnson MS, and Scheinin M (2005). Conserved structural, pharmacological and functional properties among the three human and five zebrafish α_2-adrenoceptors. *Br J Pharmacol* **144:** 165-177.

II **Laurila JM**, Xhaard H, Ruuskanen JO, Rantanen MJ, Karlsson HK, Johnson MS, and Scheinin M (2007). The second extracellular loop of α_{2A}-adrenoceptors contributes to the binding of yohimbine analogues. *Br J Pharmacol* **151:** 1293-1304.

III **Laurila JM**, Wissel G, Xhaard H, Ruuskanen JO, Johnson MS, and Scheinin M (2011). Involvement of the first transmembrane segment of human α_{2A}-adrenoceptors in the subtype-selective binding of chlorpromazine, spiperone and spiroxatrine. *Br J Pharmacol under review.*

1. INTRODUCTION

Proteins embedded in cell membranes represent a major proportion (~25 %) of all proteins encoded by mammalian genomes. G-protein coupled receptors (GPCRs) are a large and biologically very important superfamily of cell membrane proteins. They mediate extracellular signals to intracellular effector pathways by activating membrane-associated guanine nucleotide binding regulatory proteins, or G-proteins. GPCRs are important targets for pharmaceutical development as they regulate a wide range of physiological processes. It is estimated that almost 40 % of all currently used therapeutic drugs mediate their effects *via* GPCRs.

All GPCRs share a common basic structure, consisting of seven α-helical transmembrane (TM) domains connected by intracellular and extracellular loops. The adrenoceptors (α_1-, α_2- and β-adrenoceptors) are members of the rhodopsin-like GPCR subfamily. They mediate cellular responses to the adrenomedullary hormone adrenaline and the neurotransmitter noradrenaline. They posses a membrane-embedded water-accessible ligand binding cavity contained between the hydrophobic TM domains. The amino acids that form the surface of the ligand binding cavity are considered to be potential contact sites for selective ligand recognition. Other amino acids in the TM domains lack direct contacts with ligands but have at least a structural role, thereby having a possibility to indirectly influence ligand binding.

Humans and other mammalian species have three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}) that are encoded by three distinct intronless genes. The three α_2-adrenoceptors show high structural similarity, especially within their TM domains, where 70-80 % of the amino acids are identical between any two receptor subtypes. Their conserved structure is also reflected in their very similar ligand binding properties, especially for the endogenous catecholamines, adrenaline and noradrenaline, which bind with rather similar affinities to the receptor subtypes within an animal species (paralogues) and to homologous subtypes between species (orthologues). Nonetheless, differences have been found in the binding affinities of some synthetic ligands, especially for antagonist ligands, whose binding to α_2-adrenoceptors is less well understood than that of catecholamines and related agonists. In spite of the advent of modern drug discovery technologies, current clinically available α_2-adrenoceptor drugs have only marginal subtype-selectivity, which limits their therapeutic usefulness because of various side-effects. Subtype-selective drugs could have therapeutic applications *e.g.* in the treatment of elevated blood pressure, in relief of pain and of opioid and alcohol withdrawal symptoms, as neuropsychiatric therapeutic agents and in anaesthetic care.

A better understanding of the molecular basis of drug actions on α_2-adrenoceptors, in terms of receptor structure and function, may in the future allow the design and development of new subtype-selective drug molecules. In this thesis work, computer-based molecular modelling and sequence comparisons between paralogous and orthologous receptor subtypes along with experimental site-directed mutagenesis and pharmacological *in vitro* assays were used to characterise the structural determinants of human, mouse and zebrafish α_2-adrenoceptors with respect to their specific ligand binding properties.

2. REVIEW OF THE LITERATURE

2.1 α_2-Adrenoceptors, members of the G-protein coupled receptor (GPCR) superfamily

2.1.1 Concept of GPCRs

The concept of receptor molecules was first postulated by John N. Langley at the turn of the 20th century. According to his theory, the effects of curare and nicotine on skeletal muscle could be attributed to special "receptive substances" or "receptors" (Langley, 1905; reviewed by Maehle, 2004). Later, this concept was broadened and developed to successfully explain the phenomenon that membrane-bound cell surface receptors were the molecular entities responsible for the communication of signalling events between the extracellular and intracellular environments. It is now known that various endogenous signalling molecules such as hormones, neurotransmitters, neuromodulators and growth factors exert their cellular signals through these proteins (Bockaert and Pin, 1999).

G-protein coupled receptors (GPCRs) comprise one of the largest families of proteins in humans, and all cell types express some subset of GPCRs. GPCRs are integral membrane proteins that span the depth of the cell membrane. They transduce extracellular signals into intracellular messages by acting on membrane-associated guanine nucleotide binding regulatory proteins, or G-proteins. Genes encoding GPCRs comprise about 3 % of all genes in the human genome (Milligan and Kostenis, 2006). GPCRs are composed of a common "canonical" motif of seven α-helical transmembrane domains (TM1-TM7), connected to each other by three flexible intracellular and extracellular loops, an extracellular amino terminus and an intracellular carboxyl-terminal tail. The perpendicularly oriented TM domains are composed of 25-35 hydrophobic amino acids, and are embedded in the phospholipid-rich plasma membrane in an anticlockwise manner (as seen from the outside), forming a central receptor core that in many cases is involved in ligand recognition and binding (Figure 1). In order to conduct comparative structural biological comparisons in receptor characterization, different indexing systems for the amino acids have been established. In the Ballesteros-Weinstein convention (Ballesteros and Weinstein, 1995), the amino acid residues are numbered according to an indexing system where the first number refers to the TM 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. This indexing system was further extended to encompass also the extracellular loops.

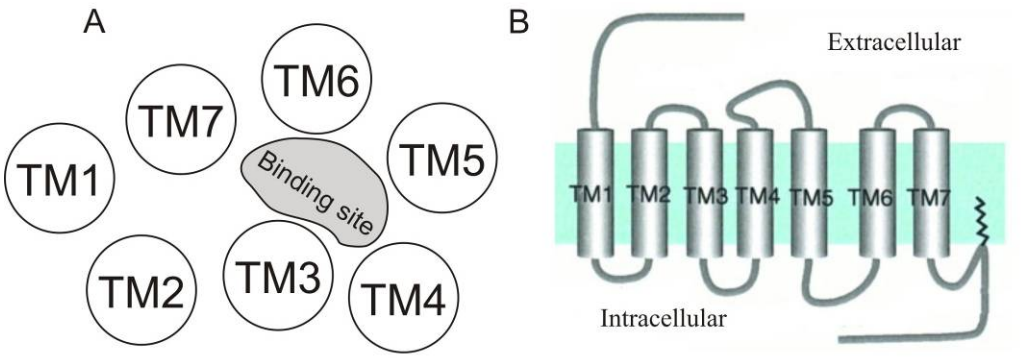


Figure 1. Schematic representation of TM helices organized in a counterclockwise fashion, viewed from the extracellular side of the cell membrane. The putative ligand binding site is shown between the TM helices (A). A conventional 2-dimensional "snake diagram" of the seven TM domains embedded in the plasma membrane. The intracellular carboxyl-terminal tail is attached to the plasma membrane via cysteine-linked palmitoylation (B).

The common fundamental structure of GPCRs was verified by X-ray crystallography of bovine rhodopsin about ten years ago (Palczewski *et al.*, 2000). The lack of stability and higher-order symmetry of membrane proteins in the native state effectively hindered the development of diffraction methods for other GPCRs for a long time. In the past few years, very significant progress in technology has made crystallization and high resolution X-ray diffraction analysis possible also for some other GPCRs, and the crystal structures of the human β_2-adrenoceptor, the turkey β_1-adrenoceptor, and the human A_{2A} adenosine, dopamine D_3 and CXCR4 chemokine receptors have now been characterized at the time of writing of this review (Cherezov *et al.*, 2007, Rasmussen *et al.*, 2007, Jaakola *et al.*, 2008, Warne *et al.*, 2008, Chien *et al.*, 2010, Wu *et al.*, 2010). This major advance has provided a wealth of new information on the structural biology of GPCRs and it is highly likely that more receptor structures will be reported in the near future.

GPCRs mediate effects of a wide array of endogenous and exogenous ligands, and regulate many important cellular functions. GPCRs are involved in almost all physiological processes and have been associated with the pathology or therapy of many common diseases such as impaired vision (involving rhodopsin mutations; Menon *et al.*, 2001), many cardiovascular diseases (where especially β_1-adrenoceptor antagonists are widely used; McNamara *et al.*, 2002), and asthma (where β_2-adrenoceptor agonists are one form of therapy; Johnson, 2001). Their location in the plasma membrane makes GPCRs readily accessible to endogenous signalling molecules but it also makes them very attractive drug targets. It has been estimated that about 25-40 % of the current clinically used drugs act on GPCRs, and many of them are found among the 100 best-selling pharmaceutical products (Lagerström and Schiöth, 2008). On the other hand, the human genome contains approximately 100 GPCRs whose ligands and biological functions are still unknown (Suwa and Ono, 2009). These "orphan" receptors are very interesting for pharmacologists and the pharmaceutical industry as they may be potential drug targets.

GPCRs have been highly conserved throughout evolution. These receptors are expressed in almost all living organisms, from prokaryotic bacteria to eukaryotic human cells. About 800 genes encode human GPCRs, of which more than 300 are non-olfactory and about 400 are olfactory or other chemosensory receptors (Fredriksson *et al.*, 2003, Niimura and Nei, 2006, Schiöth *et al.*, 2007, Lagerström and Schiöth, 2008, Nordström *et al.*, 2009). In addition, many of the GPCR genes can yield several transcripts or splice variants. GPCRs are involved in a broad range of physiological functions - in taste, olfaction, vision, immune responses as well as in higher functions of the nervous system, *e.g.* memory and other cognitive functions, emotions, attention and pain. The chemical diversity of endogenous GPCR ligands is large, encompassing molecules like small and large peptides, monoamine neurotransmitters, nucleotides, amino acids, lipids, even larger signalling hormones such as chemokines (Kristiansen, 2004, Lagerström and Schiöth, 2008).

GPCRs are classified into subfamilies based on several classification systems related to their sequence similarity and protein structure, ligand structure, ligand binding mode and receptor function. One of the most commonly used systems groups GPCRs into six classes, A-F, of which classes D, E and F contain receptors that are not expressed in humans (Attwood and Findlay, 1994, Kolakowski, 1994). In this system, group A, the rhodopsin-like receptors, is the most numerous. Its subfamily 1 contains GPCRs for small ligands including the adrenergic, adenosine and serotonin receptors. In another more recent classification system (also known as the GRAFS classification) that is based on a phylogenetic analysis of more than 800 human GPCRs, the receptors are clustered into five main families: glutamate (G, with 15 members), rhodopsin-like (R, n = 701), adhesion (A, n = 24), frizzled/taste2 (F, n = 24), and secretin (S, n = 15) (Fredriksson *et al.*, 2003). Of these, the large rhodopsin-like receptor family consists of four main groups (α, β, γ, δ) and 13 subfamilies. This overall mapping system of the entire GPCR superfamily suggests that all GPCRs share a common evolutionary origin traceable to a single ancestral gene.

2.1.2 Subtypes of α_2-adrenoceptors

The first classification of "adrenotropic" receptors was postulated by Raymond Ahlquist in 1948 and was based on the different pharmacological characteristics of adrenaline-like agonists in different tissues (Ahlquist, 1948). Ahlquist noted that there must be at least two types of adrenotropic receptors: those whose activation resulted in excitation and those whose activation evoked inhibition of the target cells. Ahlquist defined these receptor types as α- and β-receptors. Subsequently, the "adrenotropic" receptors have been divided into three different main classes and nine mammalian subtypes.

Today, receptors are mainly classified based on their primary amino acid sequences. The first cloned adrenoceptor was the hamster β_2-adrenoceptor (Dixon *et al.*, 1986). Soon thereafter, the genes of the other members of the adrenoceptor family were cloned. In the current scheme, adrenoceptors are classified into three main classes, α_1-, α_2- and β-adrenoceptors, based on their amino acid sequences and biological and pharmacological properties, and each of these classes has been further divided into three subtypes (Bylund *et al.*, 1992) (Figure 2). These nine adrenoceptor subtypes, encoded by distinct genes, have been identified in many mammalian species. All

adrenoceptors share a relatively high degree of amino acid identity, especially within their TM domains that form the membrane-embedded core of the proteins and contain the ligand binding pocket (66-75 % identity within TM regions between subtypes for α_1-, 77-79 % for α_2-, and 62-70 % for β-adrenoceptors) (Xhaard *et al.*, 2006). The three α_2-adrenoceptor subtypes, initially designated as α_2-C10 (Kobilka *et al.*, 1987), α_2-C2 (Lomasney *et al.*, 1990) and α_2-C4 (Regan *et al.*, 1988) based on the localization of their genes on human chromosomes 10, 2 and 4, are better known as the subtypes α_{2A}, α_{2B} and α_{2C}. The current molecular classification agrees with the earlier receptor subtype classification that was based on their pharmacological properties. The three α_2-adrenoceptor subtypes have many similarities but also show variations in protein sequence, ligand binding, regulation, as well as in patterns of tissue and cell expression (Eason *et al.*, 1994, MacDonald *et al.*, 1997, Richman and Regan, 1998).

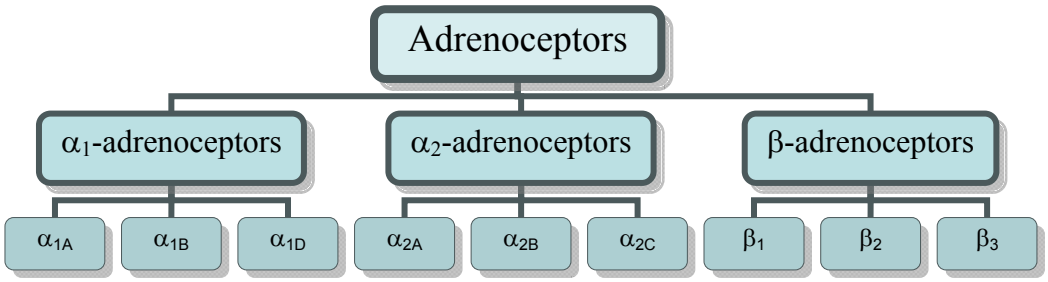


Figure 2. Classification of mammalian adrenoceptors

The rodent (rat and mouse) α_{2A}-adrenoceptor was initially "misleadingly" identified as a fourth α_2-adrenoceptor subtype, α_{2D}, because of some differences in its pharmacological properties compared to the human α_{2A}-adrenoceptor with regard to the classical α_2-adrenoceptor antagonists, yohimbine and rauwolscine (Michel *et al.*, 1989, Lanier *et al.*, 1991, Simonneaux *et al.*, 1991, Bylund *et al.*, 1992). Subsequently, cloning of the mouse α_{2A}-adrenoceptor gene demonstrated that its lower affinity for yohimbine in comparison to the human α_{2A}-adrenoceptor was to be ascribed at least in part to a single cysteine/serine difference in TM5 (position 5.43) (Link *et al.*, 1992, Cockcroft *et al.*, 2000).

A real fourth α_2-adrenoceptor subtype was cloned from zebrafish (*Danio rerio*), in which it is present as two duplicates of a gene that has been lost during mammalian evolution. It appears to be present also in many other fish species and in some non-mammalian tetrapods (Ruuskanen *et al.*, 2004). This receptor was named α_{2D} according to the nomenclature recommendations of IUPHAR (International Union of Basic and Clinical Pharmacology), in spite of the potential for confusion with regard to the erroneously named mouse/rat α_{2A}-adrenoceptor. Based on the conserved syntenies and identified molecular fingerprints of the gene sequences from several vertebrate species, the set of four α_2-adrenoceptor subtype genes was postulated to have arisen by two rounds of duplication of the whole genome in early vertebrate evolution (Vernier *et al.*, 1995, Ruuskanen *et al.*, 2004). The presence of the fourth α_2-adrenoceptor subtype gene in zebrafish as two duplicates (*adra2da* and *adra2db*) is in agreement with the concept that an additional third round of large-scale duplication (~100 million years ago) of at least part of the genome occurred in many teleost fish lineages

(Postlethwait *et al.*, 1998, Shin and Fishman, 2002, Ruuskanen *et al.*, 2004). Based on this hypothesis, *e.g.* the pufferfish genome was proposed to contain as many as eight α_2-adrenoceptor subtype genes, making the GPCR subfamily of α_2-adrenoceptor subtypes even more complex (Ruuskanen *et al.*, 2004, Bylund, 2005). So far, no evidence has been published about the expression patterns of these putative α_2-adrenoceptor subtypes of the pufferfish.

2.2 Structure-function relationships of α_2-adrenoceptors

2.2.1 α_2-Adrenoceptor ligands and subtype selectivity

The naturally occurring catecholamines, noradrenaline and adrenaline and their chemical precursor dopamine, are sympathomimetic "fight-or-flight" hormones (adrenaline) and neurotransmitters (dopamine and noradrenaline) that are derived from the amino acid, tyrosine. They are rapidly degraded either by methylation by catechol-*O*-methyltransferases (COMT) or by deamination by monoamine oxidases (MAO). Structurally, catecholamines belong to the chemical class of phenylethylamines, consisting of a catechol moiety, *i.e.* a benzene ring with two adjacent hydroxyl groups, and an aliphatic amine side chain (Figure 3). The biosynthesis of endogenous catecholamines is driven by successive enzymatic reactions; in the case of adrenaline, a total of four enzymes is involved (Figure 4). Catecholamine synthesis can be inhibited by α-methyl-*p*-tyrosine (AMPT), which inhibits tyrosine hydroxylase, and has been in clinical use in the treatment of pheochromocytomas, catecholamine-producing tumours of the adrenal medulla (Hengstmann *et al.*, 1979).

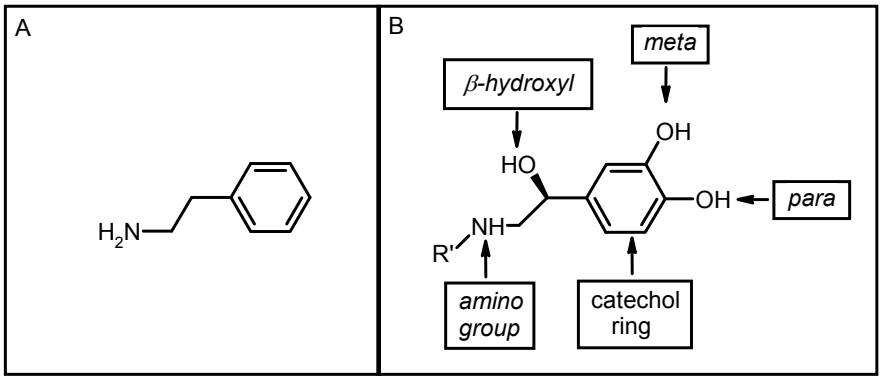


Figure 3. Chemical structures of the phenylethylamine scaffold (A) and (-)-noradrenaline (B). The meta- and para-hydroxyls, the aromatic catechol ring, the protonated amine and the chiral β-hydroxyl are indicated. R' = H in noradrenaline, CH_3 in adrenaline.

Adrenaline and noradrenaline are able to activate all adrenoceptor subtypes, which limits their usefulness for experimental purposes. Moreover, they share rather similar binding affinities and functional properties at all three α_2-adrenoceptor subtypes as well as binding to other adrenoceptors. Therefore, synthetic ligands are more commonly used when studying α_2-adrenoceptors and their subtypes. Several synthetic

agonists and antagonists have been developed, with different pharmacological properties at α_2-adrenoceptors. Structurally, there is a large diversity of compounds, but they can be grouped into some main classes based on their chemical scaffolds: synthetic phenylethylamines, imidazol(in)es, *i.e.* compounds that contain an imidazole or imidazoline moiety (*e.g.* atipamezole, brimonidine, clonidine, medetomidine, oxymetazoline, idazoxan and RX821002), guanidines (guanabenz and guanfacine), yohimbine derivatives (yohimbine, MK-912 (L657.743), rauwolscine and RS-79948-197), tricyclic antipsychotics (chlorpromazine and clozapine), and other "bulky" compounds with various larger molecular structures (ARC239, prazosin, spiperone, spiroxatrine and WB-4101).

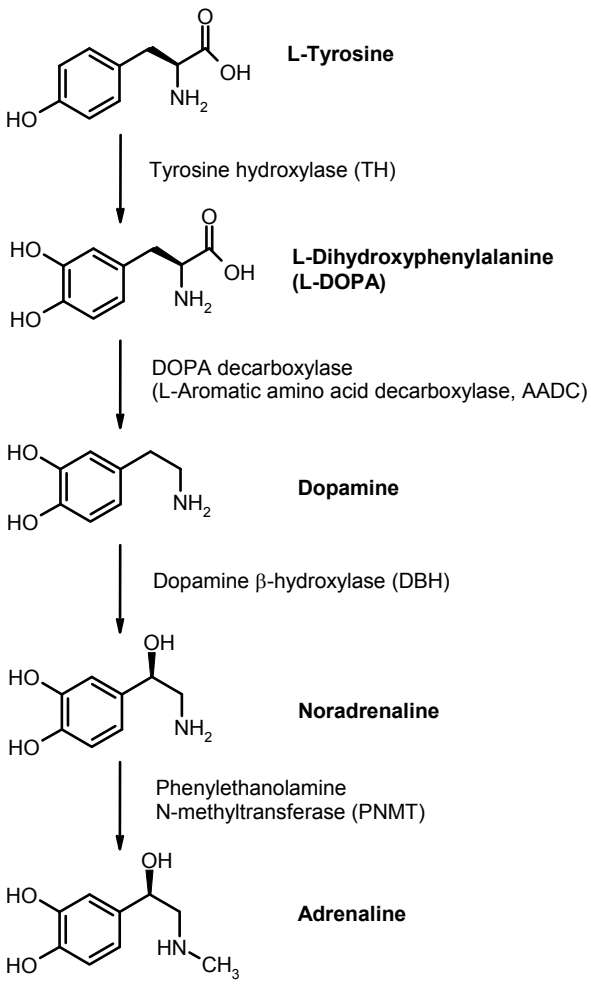


Figure 4. Biosynthetic pathway of catecholamines from the amino acid tyrosine to adrenaline.

Although some synthetic ligands show preference for certain α_2-adrenoceptor subtypes (Table 1), none of the current agents is truly α_2-adrenoceptor subtype-selective. Furthermore, many of them have pharmacological effects at some other GPCRs in addition to α_2-adrenoceptors, *e.g.* clozapine and chlorpromazine bind to dopamine

receptors and many other GPCRs, oxymetazoline, rauwolscine, spiroxatrine and yohimbine bind to serotonin receptors, and prazosin, WB-4101, clonidine and oxymetazoline bind to α_1-adrenoceptors (http://www.iuphar-db.org/). With respect to the imidazoline-based ligands, some of their effects were suggested to be mediated by distinct imidazoline-binding sites, instead of α_2-adrenoceptors (see *e.g.* Bousquet *et al.*, 1984). Three types of non-adrenergic imidazoline-binding sites (I_1, I_2 and I_3) have been proposed, with different tissue distributions, ligand binding profiles and functions, but molecular characterisation of these binding proteins as distinct gene products is still lacking (Dardonville and Rozas, 2004, Gongadze *et al.*, 2008). They may not be receptors at all in the biological sense of the term; indeed, the I_2-imidazoline site was actually shown to be identical with the enzyme MAO-B (Sastre and Garcia-Sevilla, 1993).

Table 1. Binding preferences, functional properties and chemical classification of selected α_2-adrenoceptor ligands.

Ligand	*Binding preference*	*Agonist efficacy*	*Chemical classification*
Endogenous ligands			
Adrenaline	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	Full	Phenylethylamine
Dopamine*	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	Full: α_{2A}; (*n.d.* α_{2B}, α_{2C})	Phenylethylamine
Noradrenaline	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	Full	Phenylethylamine
Synthetic agonists			
2-Amino-1-phenylethanol*	$\alpha_{2A} > \alpha_{2C} \approx \alpha_{2B}$	Partial	Phenylethylamine
Brimonidine (UK14,304)	$\alpha_{2A} \approx \alpha_{2B} > \alpha_{2C}$	Full: α_{2A}; Partial: α_{2B}, α_{2C}	Imidazoline
Biphenyline	$\alpha_{2A} > \alpha_{2B} \approx \alpha_{2C}$	Partial	Imidazoline
Clonidine	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	Partial	Imidazoline
Dexmedetomidine	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	Full: α_{2B}; Partial α_{2A}, α_{2C}	Imidazole
Guanfacine	$\alpha_{2A} >> \alpha_{2B} \approx \alpha_{2C}$	Partial	Guanidine
Oxymetazoline	$\alpha_{2A} >>> \alpha_{2C} > \alpha_{2B}$	Partial	Imidazoline
Synthetic antagonists			
ARC239	$\alpha_{2B} \approx \alpha_{2C} >> \alpha_{2A}$	-	Piperazine
Atipamezole	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	-	Imidazole
BRL44408	$\alpha_{2A} >>> \alpha_{2B} \approx \alpha_{2C}$	-	Imidazole
Chlorpromazine	$\alpha_{2B} > \alpha_{2C} >> \alpha_{2C}$	-	Phenothiazine
Clozapine*	$\alpha_{2C} >> \alpha_{2A} \approx \alpha_{2B}$	-	Dibenzazepine
Idazoxan	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	-	Imidazoline
L657.743 (MK912)	$\alpha_{2C} >> \alpha_{2A} \approx \alpha_{2B}$	-	Quinazoline
Prazosin	$\alpha_{2B} \approx \alpha_{2C} > \alpha_{2A}$	-	Quinazoline
Phentolamine	$\alpha_{2A} \approx \alpha_{2B} > \alpha_{2C}$	-	Imidazoline
Rauwolscine	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	-	Yohimban
RS-79948-197	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	-	Decahydronaphthyridine Tetrahydroisoquinoline
RX821002	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	-	Imidazoline
Spiperone*	$\alpha_{2B} \approx \alpha_{2C} >>> \alpha_{2A}$	-	Butyrophenone
Spiroxatrine	$\alpha_{2B} \approx \alpha_{2C} >>> \alpha_{2A}$	-	Benzodioxane
WB4101	$\alpha_{2B} > \alpha_{2A} \approx \alpha_{2C}$	-	Benzodioxane
Yohimbine	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$	-	Yohimban

Derived from Lomasney *et al.*, 1991, Bylund *et al.*, 1992, Devedjian *et al.*, 1994, Uhlèn *et al.*, 1994, Jasper *et al.*, 1998, Peltonen *et al.,* 1998, Peltonen *et al.,* 2003. *Based on the binding affinities shown in papers I and III.

2.2.2 Experimental probing of receptor structure and ligand interactions

The binding sites of monoamine-activated GPCRs are accessible to small cationic ligands such as adrenaline and noradrenaline, and are located within the core of the receptor proteins formed of the seven α-helical TM domains. The general structural architecture of GPCRs, the orientation of the TM helices and the location of the binding site were confirmed already some years before the bovine rhodopsin 3D structure was resolved by X-ray crystallography, by using molecular imaging techniques such as cryo-electron microscopy of bacteriorhodopsin (Henderson *et al.*, 1990) and frog rhodopsin (Schertler and Hargrave, 1995) and electron paramagnetic resonance spectroscopy of rhodopsin (Altenbach *et al.*, 1994). Even though the bacterial light-sensing protein bacteriorhodopsin is not a GPCR, as it does not signal through G-proteins and shares no sequence identity with GPCRs, it is considered to have structural similarities with rhodopsin and other GPCRs. Based on the structures of bacteriorhodopsin and rhodopsin, the ligand binding site of GPCRs was proposed to be contained between the TM domains, in a fold or cavity extending from the extracellular surface of the proteins into their transmembrane core, where the surface of the binding cavity is formed by residues that constitute contact sites for the recognition and binding of specific ligands. This has been demonstrated for retinal in the case of rhodopsin and for other chromophores in the case of bacteriorhodopsin (Henderson and Schertler, 1990). However, in contrast to other GPCRs which provide an open access for entrance of diffusible ligands, the retinal binding pocket is closed in rhodopsin to protect retinal from solvent molecules (Hildebrand *et al.*, 2009). The present understanding of rhodopsin-like GPCR structures is illustrated below in a comparison of the model structures of the human α_{2A}-adrenoceptor based on the crystal structures of bovine rhodopsin and the human β_2-adrenoceptor (Figure 5).

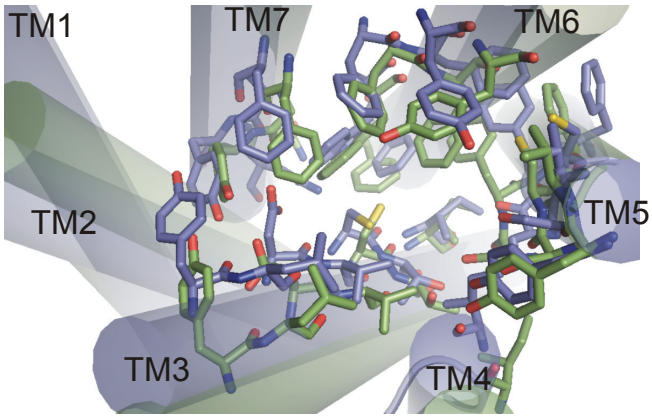


Figure 5. Molecular model and the binding cavity of the human α_{2A}-adrenoceptor viewed from the extracellular side of the cell membrane, based on both the bovine rhodopsin (green) and human β_2-adrenoceptor structures (blue). The surface of the binding cavity is formed by amino acids of the TM helices. Here, only some key amino acids are shown. Modified from paper III, by courtesy of Henri Xhaard.

Over the past 25 years, several experimental approaches have been developed and applied to generate the current knowledge of the structural determinants that contribute to the specific ligand binding of various GPCRs. Site-directed mutagenesis combined with ligand binding experiments has been an important method for mapping the molecular interactions between ligands and receptors. For example, receptor binding assays performed with asymmetric ligand enantiomers or analogues with different functional groups combined with amino acid substitutions in the receptor proteins have been useful in probing the catecholamine binding site of α_2-adrenoceptors at the atomic level (Salminen *et al.*, 1999, Nyrönen *et al.*, 2001, Peltonen *et al.*, 2003). Another mutagenesis-based method, termed the Substituted Cysteine Accessibility Method (SCAM), has yielded information on the amino acids that contribute to the surface of the binding cavity. In this approach, candidate residues are mutated to cysteines, one by one, and residues facing the water-accessible binding cavity are observed to react much faster with alkylating sulphydryl-specific reagents than other residues facing the interior of the receptor protein or the phospholipid bilayer of the cell membrane (Liapakis *et al.*, 2001). Several irreversible sulphydryl-reactive probes with different pharmacological properties at α_2-adrenoceptors have been employed for this purpose. These include several ligands whose covalent binding properties were initially identified in other contexts, *e.g.* N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinole (EEDQ) (Garvey *et al.*, 1972), benextramine (Melchiorre and Gallucci, 1983), phenoxybenzamine (Regan *et al.*, 1984, Frang *et al.*, 2001), phenylmercuric chloride (Regan *et al.*, 1986), pyrextramine (Brasili *et al.*, 1986) and chloroethylclonidine (Bultmann and Starke, 1993, Marjamäki *et al.*, 1998). Some of these compounds were used in the initial characterization of α_2-adrenoceptor structures. For example, chloroethylclonidine and phenoxybenzamine were used in SCAM experiments to elucidate the critical structural determinants of TM3 and TM5 that are exposed in the α_2-adrenoceptor ligand binding pocket and contribute to receptor activation (Marjamäki *et al.*, 1998, Marjamäki *et al.*, 1999, Frang *et al.*, 2001).

Although advances in gene manipulation technologies have allowed the mapping of structural determinants important for ligand binding, caution is warranted when interpreting results obtained with mutated receptors, as the observed alteration of a receptor's pharmacological profile is not always a direct consequence of changes in the contact between ligand and receptor. Mutations can also disrupt the overall structure of the receptors, cause conformational rearrangements, posttranslational modifications or disturb cell surface expression, which all may have indirect effects on ligand binding. In addition, differences in experimental conditions may have important effects *e.g.* on the apparent binding affinity (Deupree *et al.*, 1996). As a consequence, it may be very difficult to differentiate direct from indirect effects of experimental mutations on ligand binding.

2.2.3 Structural determinants of α_2-adrenoceptor ligand recognition and binding

Cell membrane receptors recognize ligand molecules present in the extracellular environment and transmit messages from the extracellular space to the intracellular components of the cellular signal transduction pathways. Receptor-ligand interactions may be highly specific, because each receptor type has unique structural elements for

ligand recognition and binding, which have developed during the course of evolution. For α_2-adrenoceptors and other monoamine receptors, the ligand binding cavity is thought to be mainly formed by residues in TM3, TM5, TM6, TM7 and the second extracellular loop (XL2). Experimental studies, for example on alkylating ligand derivatives targeted to specific residues in the proposed binding site (Marjamäki *et al.*, 1999, Salminen *et al.*, 1999, Frang *et al.*, 2001), site-directed mutagenesis studies (Wang *et al.*, 1991), and competitive binding experiments where labelled ligands are displaced by other non-labelled ligands, also in terms of asymmetric (chiral) compounds (Nyrönen *et al.*, 2001, Peltonen *et al.*, 2003), have revealed several amino acids that appear to play important roles in ligand binding or recognition in α_2-adrenoceptors (Figure 6). A highly conserved aspartate present in all rhodopsin-like GPCRs in the top portion of TM3 at position 3.32 and its negatively charged side-chain carboxyl group have in several studies been shown to provide an important anchoring point for ligands, providing direct polar ion-pair interactions for compounds that contain a positively charged amine or imine moiety (Wang *et al.*, 1991, Nyrönen *et al.*, 2001, Xhaard *et al.*, 2006). Additionally, one of the two side-chain oxygens of the aspartate at position 3.32 has been hypothesized to provide a hydrogen bonding site for the β-hydroxyl group of the *R*-enantiomers of catecholamine ligands, whereas the *S*-enantiomers of catecholamines cannot undergo a similar interaction. Thus, the affinities of *S*-enantiomers are much weaker than those of the *R*-enantiomers. Furthermore, the clearly lower affinity of dopamine at α_2-adrenoceptors in comparison to noradrenaline was interpreted to be a consequence of dopamine lacking the β-hydroxyl moiety (Nyrönen *et al.*, 2001). Other residues believed to interact with the positively charged *N*-methyl group of the catecholamine ligands *via* hydrophobic contacts are two phenylalanines at positions 7.38 and 7.39 in TM7 (Nyrönen *et al.*, 2001). Two serine residues in TM5 were shown to provide hydrogen bonding sites for the two catecholic hydroxyl groups of the catecholamines with the *meta*-OH probably interacting with the serine in position 5.42 and the *para*-OH with the serine in position 5.46 of the human α_{2A}-adrenoceptor (Marjamäki *et al.*, 1998, Rudling *et al.*, 1999, Salminen *et al.*, 1999). These interactions have also been postulated to be influenced by the conformational changes that occur upon receptor activation, *e.g.* rotation of TM5, and in that way to play a significant role in agonist-dependent receptor activation (Nyrönen *et al.*, 2001, Peltonen *et al.*, 2003). In addition, they are important for the orientation of ligands within the binding pocket. The side chains of a threonine at position 3.37 in TM3 and a cysteine at position 5.43 in TM5 of the human α_{2A}-adrenoceptor have also been shown to interact with the catecholic OH groups, but in contrast to the serines at 5.42 and 5.46, they were not observed to play a role in receptor activation (Nyrönen *et al.*, 2001, Peltonen *et al.*, 2003). Other residues of the α_{2A}-adrenoceptor that are likely to interact through a π-π stacking interaction with the aromatic ring of phenylethylamine-type ligands include a conserved valine at position 3.33 in TM3, the conserved aromatic side chain of the phenylalanine at position 5.47 in TM5, the conserved aromatic side chains of a tryptophan at position 6.48, phenylalanines at positions 6.51 and 6.52, and a tyrosine at position 6.55 in TM6 (Nyrönen *et al.*, 2001, Peltonen *et al.*, 2003, Gentili *et al.*, 2004). In docking simulations, the *N*-methyl group of adrenaline-like phenylethylamines was proposed to become packed against aromatic side chains of phenylalanines at positions 7.38 and

7.39 in TM7, but experimental support for this proposal is still lacking (Nyrönen *et al.*, 2001). Previously, also serines at positions 2.61 in TM2 and 7.46 in TM7 were claimed to be involved in the stereoselectivity of the α_{2A}-adrenoceptor for the (-)- or *R*-enantiomers of catecholamine agonists, and in addition to play a role in the attachment of the β-hydroxyl groups of the catecholamines to α_{2A}-adrenoceptors (Hehr *et al.*, 1997, Hieble *et al.*, 1998). However, the current understanding of the molecular structure of α_{2A}-adrenoceptors points to some indirect effects being involved in these findings rather than specific side-chain interactions between catecholamines and the receptor.

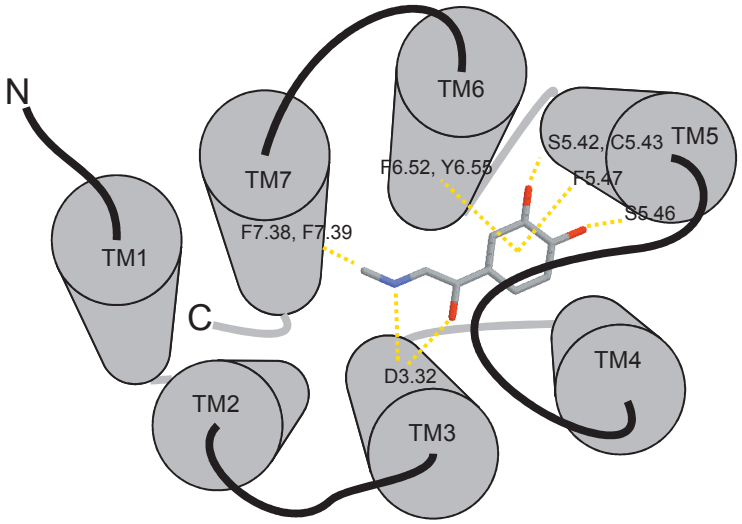


Figure 6. Major interactions between the agonist adrenaline and the human α_{2A}-adrenoceptor. Transmembrane domains are shown as cylinders, extracellular loops and the N-terminus as black lines, and intracellular loops as gray lines.

In contrast to agonists, the binding of antagonists to α_2-adrenoceptors is less well understood. The chemical diversity of antagonists that bind to α_2-adrenoceptors is much greater than that of agonists, indicating more divergent and complex modes of binding. No general "antagonist binding site" has so far been reported for adrenoceptors or any other types of GPCRs. However, in monoamine-binding GPCRs, including the adrenoceptors, antagonists are proposed to bind at least in part within the same orthosteric binding site as agonists. This proposal is based on the markedly reduced binding affinity of antagonists when the receptors have been mutated at the highly conserved aspartate at 3.32 in TM3 (reviewed by Shi and Javitch, 2002). For α_2-adrenoceptors, this was demonstrated by the failure of yohimbine to bind to a D3.32N-mutated α_{2A}-adrenoceptor (Wang *et al.*, 1991), although possible indirect mechanisms were not ruled out in that study. This observation was proposed to support a general concept where an ion-pair interaction with the aspartate at position 3.32 was a key feature also for antagonist binding. Nonetheless, some conflicting reports also exist, *e.g.* for a D3.32N mutant of the type 1A serotonin receptor (5-HT_{1A}), and D3.32A and D3.32K mutants of the α_{1B}-

adrenoceptor, where the binding of antagonists was shown to be similar to that of the corresponding wild-type receptors, while agonist binding was impaired (Ho *et al.*, 1992, Porter *et al.*, 1996). Other residues believed to be involved in antagonist binding are a cysteine at position 3.36 that was found to interact with covalently bound phenoxybenzamine, and which is also important for binding of imidazol(in)e based ligands at the human α_{2A}-adrenoceptor (Frang *et al.*, 2001). A phenylalanine at position 7.39 was also found to be critical for the binding of yohimbine to the α_{2A}-adrenoceptor (Suryanarayana *et al.*, 1991).

In addition to differences in the amino acids that line the binding cavity, the size of the binding pocket was also suggested to differ among the α_2-adrenoceptor subtypes as shown in a comparative molecular modelling study. The α_{2B}-adrenoceptor was found to contain the largest and the α_{2C}-adrenoceptor the smallest cavity (Balogh *et al.*, 2009). Differences in binding pocket volume may also have a role in the subtype selectivity of some ligands.

All adrenoceptors are proposed to have overall tertiary structures similar to rhodopsin, but rhodopsin shares only 20-23 % sequence identity in its TM regions with members of the monoamine GPCR subfamily. Recently, the structures of three much closer homologues of the α_2-adrenoceptors, namely the human β_2-adrenoceptor (Cherezov *et al.*, 2007), the turkey β_1-adrenoceptor (Warne *et al.*, 2008) and the human dopamine D3 receptor (Chien *et al.*, 2010), were published. Of these, the β-adrenoceptors share, on the average, 37-43 % identical aligned amino acids in their TM regions with the human α_2-adrenoceptor subtypes and may thus serve as more accurate templates for α_2-adrenoceptor models than rhodopsin. A common feature of all rhodopsin-like GPCR crystal structures is the involvement of XL2 in ligand binding. In the crystal structure of bovine rhodopsin, XL2 folds as a β-hairpin, composed of two four-residue β-strands, and it dives down into the pocket between the TM helices, forming an aromatic "lid" over the binding cavity, and it is also in direct contact with covalently bound 11-*cis*-retinal (Palczewski *et al.*, 2000, Teller *et al.*, 2001, Okada *et al.*, 2002). A single 7 Å disulphide bond connects XL2 (Cxl2.50) to TM3 (C3.25) and constrains the position of XL2 in rhodopsin. This feature is thought to be highly conserved across all rhodopsin-like GPCRs, and is also seen in the crystal structures of the β-adrenoceptors as well as in the human dopamine D3 receptor and A_{2A}-adenosine receptor structures (Cherezov *et al.*, 2007, Rasmussen *et al.*, 2007, Jaakola *et al.*, 2008, Warne *et al.*, 2008, Chien *et al.*, 2010). In spite of this conserved disulphide bridge, many significant structural differences are also apparent in the extracellular loops of the solved X-ray structures, as in contrast to rhodopsin, in the human β_2-adrenoceptor structure (and also in the turkey β_1-adrenoceptor structure) XL2 is partly folded as an α-helix, and is further stabilised by an additional internal loop formed between cysteines Cxl2.43 and Cxl2.49 (Cherezov *et al.*, 2007). Although the conformation of XL2 in the β_2-adrenoceptor is more rigid than in rhodopsin, it is believed to lie above the binding cavity and is in direct contact with the partial inverse agonist carazolol, and is thought to play an important role in ligand binding by stabilizing the core of the receptor (Bokoch *et al.*, 2010). Also in the dopamine D_2 receptor which is closely related to the α_2-adrenoceptors, XL2 has been shown to be accessible to the binding cavity, as evidenced with SCAM and sulphydryl-reactive probes (Shi and Javitch, 2004). In the α_{2A}-adrenoceptor, XL2

was first implicated in antagonist binding by using a chimaeric receptor approach. When the region from TM3 to TM5 of the mouse α_{2A}-adrenoceptor was replaced with the corresponding human sequence, the binding affinity of yohimbine became closer to that seen with the human α_{2A}-adrenoceptor (with a cysteine in position 5.43) than to the wild-type mouse receptor (with a serine in position 5.43) or to a S5.43C substituted mouse α_{2A}-adrenoceptor (Link *et al.*, 1992). In this chimaeric receptor, there were no other sequence differences facing the orthosteric binding cavity except for the differences within XL2 and in position 5.43, suggesting that XL2 is participating in antagonist binding. In the α_{1A}- and α_{1B}-adrenoceptors and the type-1D serotonin receptor (5-HT_{1D}) that are closely related to the α_2-adrenoceptors, site-directed mutagenesis has also revealed XL2 to affect the binding properties of antagonists (Zhao *et al.*, 1996, Wurch *et al.*, 1998). The predicted position of XL2 above the binding cavity in the two model structures of the human α_{2A}-adrenoceptor is shown in Figure 7.

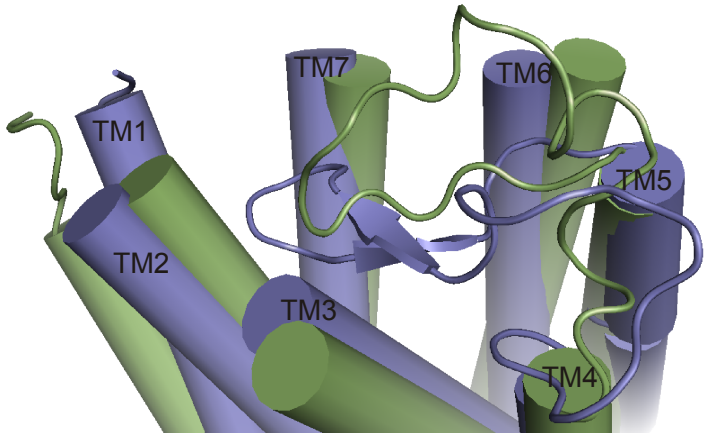


Figure 7. Molecular models (viewed in the plane of the cell membrane from the extracellular side) of the human α_{2A}-adrenoceptor and the position of XL2, based on either the bovine rhodopsin (blue) or the human β_2-adrenoceptor (green) structure (Palczewski et al., 2000, Cherezov et al., 2007). The folding of XL2 is the most marked difference in the two model structures. The rhodopsin-based receptor model contains two parallel β-sheets folded similarly as in the bovine rhodopsin X-ray structure. The additional a-helix seen in the β_2-adrenoceptor X-ray structure is not probable in the α_{2A}-adrenoceptor structure. Modified from paper III, courtesy of Henri Xhaard.

The greatest extent of diversity in the amino acid compositions of the α_2-adrenoceptor subtypes is present in the amino terminus, the third intracellular loop, and the carboxyl terminus (Lomasney *et al.*, 1990). The long third intracellular loop, together with other cytoplasmic domains, has been shown to be involved in G-protein coupling and in receptor desensitisation (Eason and Liggett, 1995, Small *et al.*, 2000b, Small *et al.*, 2001, Jaakola *et al.*, 2005). Truncation of the third intracellular loop in the human α_{2B}-adrenoceptor altered the binding affinity of some agonist ligands, indicating that the conformation of the third intracellular loop may also affect agonist binding to the receptor (Jaakola *et al.*, 2005). The extracellular amino-terminal segment

of the α_{2A}- and α_{2C}-adrenoceptors, in contrast to the α_{2B}-adrenoceptor, contains two consensus sites for asparagine-linked glycosylation (Lomasney *et al.*, 1991). The exact functional role of amino-terminal glycosylation is still poorly understood but it is likely to improve overall receptor expression rather than affecting the intracellular trafficking characteristics of the receptors (Keefer *et al.*, 1994, Deslauriers *et al.*, 1999, Angelotti *et al.*, 2010). In addition, the size of the amino terminal segment differs significantly between the α_2-adrenoceptor subtypes, consisting of 28, 7 and 46 amino acids in the human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors, respectively. The hydrophobic repeat sequence "ALAAALAAAAA" near the beginning of the amino terminal segment of the α_{2C}-adrenoceptor was found by a chimaeric α_{2A}/α_{2C}-adrenoceptor strategy to be an endoplasmic reticulum retention signal responsible for the intracellular retention of the α_{2C}-adrenoceptor subtype (Angelotti *et al.*, 2010).

The conserved structure and the high extent of amino acid identity of the α_2-adrenoceptors (~75 % in the TM region; 175 out of all 450/450/461 amino acids in the human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors, respectively) would be expected to result in relatively similar ligand binding properties and possibly complicate the design of subtype-selective drugs. Therefore, any type of information on the structural determinants of ligand binding and recognition at α_2-adrenoceptors may be considered to be potentially important for subtype-selective drug design and development.

2.2.4 Molecular dynamics of α_2-adrenoceptor activation

The theory of receptor activation has advanced over the past century from the first "lock-and-key" concept, formulated by Emil Fisher (reviewed by Vernier *et al.*, 1995), to much more sophisticated and dynamic models that include also allosteric regulation of the receptors. In one generally used model based on the ternary complex concept (Figure 8), receptor activation requires the recognition of an extracellular ligand to cause conformational changes in the receptor structure, and transforms the receptor from an inactive state (R) to an active state (R*), followed by G-protein activation and the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the cognate G-protein α-subunit (see *e.g.* Weiss *et al.*, 1996, Kenakin, 2004). The heterotrimeric G-protein complex then dissociates to a GTP-bound α-subunit (39-46 kDa) and a βγ-dimer (β-subunit, 36–37 kDa and γ-subunit, 5–10 kDa), both of which can regulate specific downstream effector systems such as enzymes or ion channels (Pfeuffer and Helmreich, 1988, Birnbaumer, 1990). As a result of the intrinsic GTPase activity of the α-subunit, bound GTP is rapidly hydrolysed to GDP, followed by re-association of the α-subunit with the βγ-dimer, thereby completing the G-protein activity cycle. This cascade of G-protein and GPCR interactions represents a fundamental mechanism of cell signalling for all GPCRs.

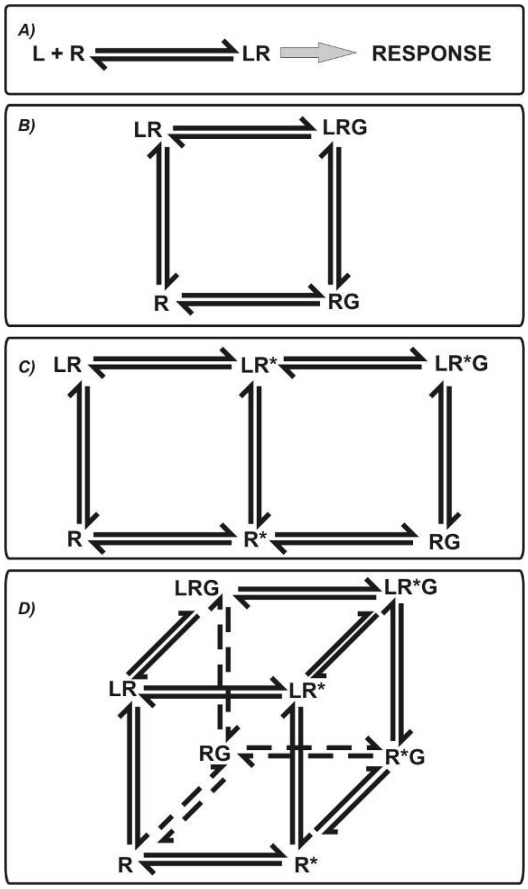


Figure 8. Evolution of receptor occupancy and activation models (Weiss et al., 1996). A) In the first simple model, binding of a ligand (L) to the receptor (R) leads to the generation of a response. B) In the ternary complex model, the receptor is coupled to a G-protein (G). C) In the extended ternary complex model, the receptor may exhibit constitutive signalling, which permits spontaneous isomerisation between inactive (R) and active (R*) receptor conformations. D) The cubic ternary complex model provides an additional interaction between the inactive receptor and the G-protein.

In humans, 17 genes encode G-protein α-subunits, 6 encode β-subunits and 12 encode γ-subunits (Jones and Assmann, 2004, Sprang *et al.*, 2007), and some of these genes are further expressed as splice variants. Based on their sequence, functional properties and toxin sensitivities, the α-subunits of G-proteins are classified into four main families, G_s, $G_{i/o}$, G_q and G_{12} (Birnbaumer, 1990, Sprang *et al.*, 2007). The specificity of a given receptor for the α-isoforms generally determines the specific intracellular messenger pathways of each receptor but also the βγ-subunits regulate certain intracellular effects. All three α_2-adrenoceptor subtypes are preferentially coupled to pertussis toxin-sensitive $G_{i/o}$-type G-proteins (Cerione *et al.*, 1986, Cotecchia *et al.*, 1990, Eason *et al.*, 1992). The activation of $G_{i/o}$ proteins leads primarily to reduced formation of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase enzymes and increased K^+ efflux *via* ligand-regulated K^+-channels,

but also to stimulation of Ca^{2+} release from intracellular stores (Limbird, 1988, Cotecchia *et al.*, 1990, Dorn *et al.*, 1997, Arima *et al.*, 1998, Kukkonen *et al.*, 1998) and to inhibition of N-, L- and P/Q-type voltage-activated Ca^{2+} channels (Soini *et al.*, 1998, Delmas *et al.*, 1999, Kolaj and Renaud, 2001, Timmons *et al.*, 2004). These signalling pathways have been demonstrated for all α_2-adrenoceptor subtypes in many different types of tissue- and cell-based experiments. Some additional coupling mechanisms have also been reported, *e.g.* coupling to activation of mitogen-activated protein (MAP) kinases (Richman and Regan, 1998), activation of phospholipase A_2 (Jones *et al.*, 1991), phospholipase C (Seuwen *et al.*, 1990, Koch *et al.*, 1994, Dorn *et al.*, 1997) and phospholipase D (MacNulty *et al.*, 1992).

In contrast to the original ternary complex model, it is now appreciated that receptors may exhibit spontaneous signalling activity also in the absence of activating agonist ligands, referred to as the phenomenon of constitutive activity or basal activity (Lefkowitz *et al.*, 1993). Mutations of the conserved DRY motif at the cytoplamic end of TM3 have clearly increased the level of constitutive activity of certain GPCRs including some adrenoceptors (Wurch *et al.*, 1999, Ge *et al.*, 2003). On the basis of constitutive activity, it is currently considered that receptors must exist in an equilibrium between a resting state and an active state, and that agonist ligands stabilise the receptor in its active conformation whereas inverse agonists suppress basal activity by stabilising the receptor in its inactive, resting conformation. Neutral antagonists that prevent other ligands, agonists and inverse agonists, from binding are capable of binding to both receptor conformations with (nearly) equal affinity and thus block their functional effects (Samama *et al.*, 1994).

This established view of receptor-G-protein-effector coupling presents a linear signalling cascade that has recently been challenged by suggestions of more complex signalling networks. Activated receptors are known to recruit not only G-proteins but also arrestins, and arrestin-mediated signalling may contribute to the net effects of receptor activation. Arrestins not only modulate G-protein signalling (Lohse *et al.,* 1990), a classical effect demonstrated already 25 years ago in the light-sensing cells of the retina (Wilden *et al.,* 1986), but they can also mediate G-protein-independent signalling in cells *via* pathways largely related to pro-survival and anti-apoptosis signals (Lefkowitz and Shenoy, 2005). Indeed, "biased ligands" may selectively activate G-protein- or arrestin-mediated signalling, resulting in a situation where the same receptor can mediate different cellular effects, depending on the activating agonist. This may provide novel opportunities for drug development (Violin and Lefkowitz, 2007).

In spite of the availability of crystal structures of seven different GPCRs, little is known at the time of writing this review about the structural basis of GPCR function: how does binding of an agonist lead to the conformational changes that are propagated from the extracellular portion of the receptor molecule to the cytoplasmic surface involved in G-protein binding? Each of the crystal structures of the GPCRs is just a "snapshot" of one receptor conformation, and they probably represent inactive conformations of the receptors, and therefore do not necessarily improve our understanding of the conformation changes occurring upon receptor activation. Receptor activation has, however, been extensively studied with other methods. Different biochemical and biophysical approaches have also been applied for some

adrenoceptors, including site-directed spin labelling techniques, site-directed mutagenesis and various fluorescent technologies (Javitch *et al.*, 1997, Jensen and Gether, 2000, Jaakola *et al.*, 2005, Zurn *et al.*, 2009). One general assumption is that the overall conserved receptor structures support a common molecular activation mechanism for all GPCRs (Oliveira *et al.*, 2003, Schwartz *et al.*, 2006). Nonetheless, the exact mechanisms are not yet fully understood and there are at least three proposed models for this: (1) in the "pivot" model, activation is driven by the rotation of TM3 and TM6, wherein the proline (P6.50) -induced kink in TM6 acts as a pivot, leading to subsequent rearrangements and conformational interchanges at the intracellular portion of the receptor (Chen *et al.*, 2002); (2) in the "see-saw" model, the mechanism is similar but is based on vertical see-saw movements around the proline-induced bend in TM6 (Schwartz *et al.*, 2006); (3) in the "toggle switch" model, receptor activation alters the configuration of the proline-induced kink in TM6 and causes the subsequent rotameric movement of the cytoplasmic end of TM6, *e.g.* allowing the straightening of the proline kink (Shi and Javitch, 2002). One common feature of all of these models is that the intracellular segments of the TMs, especially TM6, move apart during receptor activation and thereby expose receptor epitopes, such as the DRY motif at the end of TM3, allowing recognition of/interactions with intracellular signalling molecules, such as G-proteins.

If the mechanisms of receptor activation are not well known, the molecular mechanisms of basal (constitutive) activity and inhibition of basal activity by inverse agonists are even more poorly understood and difficult to study, because the constitutively active state is short-lived and represents a minor fraction of possible receptor conformations. Nonetheless, basal activity is thought to be physiologically relevant, and the ability of a drug to inhibit constitutive receptor activity may influence its therapeutic properties.

2.2.5 Physiological functions and patterns of tissue expression of α_2-adrenoceptors

Three basic types of techniques have been used to characterise the tissue distributions of the mammalian α_2-adrenoceptor subtypes, especially in humans and in rodents, *i.e.* techniques to detect ligand binding (receptor autoradiography and radioligand binding assays with tissue samples) (Boyajian *et al.*, 1987), techniques to monitor receptor gene expression on the messenger RNA (mRNA) level (*i.e. in situ* hybridisation and other methods to detect and quantitate mRNA species) (Nicholas *et al.*, 1993), and antibody-based methods (immunohistochemistry, Western blotting and related methods) (Kurose *et al.*, 1993). The three α_2-adrenoceptor subtypes were observed to have unique patterns of tissue distribution both in the central nervous system (CNS) and in peripheral tissues (MacDonald *et al.*, 1997). The α_{2A}-adrenoceptor is expressed widely throughout the CNS, abundantly in the *locus coeruleus* and in other noradrenergic cell body regions, but also in peripheral tissues. The α_{2B}-adrenoceptor appears to be expressed primarily in peripheral tissues and only low levels of expression are found in the CNS, especially in the thalamus. The α_{2C}-adrenoceptor appears to be expressed mainly in the CNS, but with a different expression pattern from the α_{2A}-adrenoceptor, being most abundant in the striatum (Nicholas *et al.*, 1993,

Scheinin *et al.*, 1994). Patterns of the tissue expression of the three α_2-adrenoceptor subtypes are summarized in Table 2.

Table 2. Tissue distributions of α_2-adrenoceptor subtypes in the central nervous system (CNS) and peripheral tissues assessed by receptor autoradiography, in situ hybridisation, radioligand binding assays, immunohistochemistry and reverse transcriptase-PCR (RT-PCR).

Subtype	*CNS*	*Peripheral tissues*
α_{2A}	Amygdala Locus coeruleus Lateral septum Brain stem Cerebral cortex Thalamus Hypothalamus Hippocampus Spinal cord Olfactory nucleus Retina	Kidney Vasculature Urethra Heart Blood platelets Spleen Salivary glands Pancreas Fat cells
α_{2B}	Thalamus	Kidney Placenta Liver Vasculature
α_{2C}	Striatum Olfactory tubercle Locus coeruleus Hippocampus Cerebral cortex Amygdala Substantia nigra	Kidney Adrenal gland Vasculature Pancreas

Derived from: Perälä *et al.*, 1992, McCune *et al.*, 1993, Nicholas *et al.*, 1993, Ruffolo and Hieble, 1994, Scheinin *et al.*, 1994, MacDonald and Scheinin, 1995, Rosin *et al.*, 1996, Tavares *et al.*, 1996, Trendelenburg *et al.*, 1997, Winzer-Serhan *et al.*, 1997, Lee *et al.*, 1998, Saunders and Limbird, 1999, Dossin *et al.*, 2000, Holmberg *et al.*, 2003, Peterhoff *et al.*, 2003, Chotani *et al.*, 2004, Happe *et al.*, 2004, Schambra *et al.*, 2005.

The first and most classical physiological function described for α_2-adrenoceptors is to mediate presynaptic feedback inhibition of neurotransmitter release from noradrenergic nerve endings (Langer, 1976). Today, based on molecular cloning, ligand binding and anatomical and functional studies of the different α_2-adrenoceptor subtypes, it is evident that α_2-adrenoceptors are also involved in many postsynaptic and extrasynaptic actions. However, the precise physiological functions of each of the three α_2-adrenoceptor subtypes have been difficult to elucidate, largely because of the lack of subtype-selective pharmacological probes. Anatomical data on receptor expression have not always been possible to definitively link to specific physiological functions of a particular receptor subtype. More recently, mice with altered α_2-adrenoceptor gene expression have become important tools to elucidate the distinct physiological functions

of the α_2-adrenoceptor subtypes. Several genetically engineered mouse lines have been generated, *e.g.* α_{2A}-, α_{2B}-, α_{2C}- and double α_{2AC}-deficient (knockout) mouse lines with disrupted gene expression of one or several of the α_2-adrenoceptor subtypes (Link *et al.*, 1996, Altman *et al.*, 1999, Hein *et al.*, 1999), a mouse line with increased α_{2C}-adrenoceptor expression (overexpressing mouse line) (Link *et al.*, 1995, Sallinen *et al.*, 1997) and a mouse line containing an inactivating point mutation of the α_{2A}-adrenoceptor (D2.50N substitution in the D79N mouse line) (MacMillan *et al.*, 1996, Lakhlani *et al.*, 1997). Studies on these gene-manipulated mice have emphasized the crucial role for α_{2A}-adrenoceptors in sedation, analgesia, hypotension, sympathetic inhibition, pupil control, alleviation of opioid and alcohol withdrawal symptoms, regulation of body temperature and blood glucose homeostasis and modulation of seizure susceptibility, all previously identified as important pharmacological effects mediated by α_2-adrenoceptors (Ruffolo *et al.*, 1993). Nonetheless, few of these effects can be totally and unequivocally attributed to a single receptor subtype. The α_{2A}-adrenoceptor has generally been observed to have a much more prominent role in CNS regulation than the α_{2C}-adrenoceptor (Trendelenburg *et al.*, 2001). Peripherally expressed α_{2B}-adrenoceptors appear to be involved in the regulation of blood pressure by controlling contraction and relaxation of vascular smooth muscle cells (Link *et al.*, 1996), but the evidence is still somewhat uncertain. Examples of some α_2-adrenoceptor-mediated physiological functions identified by experiments performed in transgenic mice with altered gene expression are summarised in Table 3.

Table 3. Overview of physiological functions and pharmacological responses mediated by α_2-adrenoceptor subtypes, as proposed from experiments with genetically engineered mice.

Receptor	*Physiological functions and responses*	*References*
α_{2A}	Analgesia	Hunter *et al.*, 1997
	Bradycardia and hypotension	MacMillan *et al.*, 1996
	Hypothermia	Lähdesmäki *et al.*, 2003
	Inhibition of epileptic seizures	Janumpalli *et al.*, 1998, Szot *et al.*, 2004
	Presynaptic inhibition of neurotransmitter release	Altman *et al.*, 1999, Hein *et al.*, 1999, Kable *et al.*, 2000
	Anxiety-like behaviour	Schramm *et al.*, 2001
	Sedation and anaesthesia	Lakhlani *et al.*, 1997
	Regulation of blood glucose and insulin homeostasis	Fagerholm *et al.*, 2004
	Decrease of intraocular pressure	Wikberg-Matsson and Simonsen, 2001
	Inhibition of gastrointestinal motility	Scheibner *et al.*, 2002
α_{2B}	Placental angiogenesis	Philipp *et al.*, 2002
	Salt-induced hypertension	Makaritsis *et al.*, 1999
	Vascular smooth muscle contraction	Hein *et al.*, 1999
α_{2C}	Presynaptic inhibition of catecholamine release	Hein *et al.*, 1999, Brede *et al.*, 2003
	Modulation of motor behaviour	Sallinen *et al.*, 1999
	Regulation of dopamine and serotonin balance in the brain	Sallinen *et al.*, 1998
	Vascular smooth muscle contraction	Hein *et al.*, 1999

On the cellular level, many GPCRs have been found to form homodimers and heterodimers, possibly implying increased biological and pharmacological complexity in receptor functions. The formation of dimers and higher-order oligomers may occur during the purification of receptor proteins with apparent molecular weights larger than expected. In living cells, α_{2A}-adrenoceptors and muscarinic M4 receptors as well as α_{2A}-adrenoceptors and μ- and δ-type opioid receptors have been proposed to form functional dimers (Jordan *et al.*, 2003, Rios *et al.*, 2004, Zhang and Limbird, 2004, Nobles *et al.*, 2005). These findings point to more complicated characteristics of α_2-adrenoceptors than initially believed; perhaps very few functions of α_2-adrenoceptors can ultimately be ascribed to a single α_2-adrenoceptor subtype acting alone. On the other hand, functional complexity based on receptor oligomerisation and other protein interactions may yield increased possibilities for drug discovery (Ferrè *et al.,* 2010).

The localisation of GPCRs in the plasma membrane is dynamic rather than static, with several highly regulated processes determining the density and spatial distribution of the receptors in different domains of the plasma membrane and in intracellular compartments. GPCRs are synthesised in the endoplasmic reticulum and then further processed in the Golgi apparatus before their transport to the plasma membrane. Agonist binding to the receptor not only leads to the generation of second messengers and intracellular responses, but may also trigger a series of adaptive changes that decrease the subsequent responsiveness of the receptor. This phenomenon is referred to as *desensitisation*, which is often accompanied by *internalisation* of the receptors. Desensitised receptors may become resensitised and recycled back into the plasma membrane ready to function in ligand recognition and signal transduction. Alternatively, internalised receptors may be transported to other intracellular compartments for further processing, leading to receptor "down-regulation", and subsequently to decreased receptor density.

Agonist-induced trafficking has also been reported for α_2-adrenoceptors. The human α_{2A}- and α_{2B}-adrenoceptors were observed to internalise into distinct intracellular vesicles in transfected Madin-Darby canine kidney (MDCK) cells, rat pheochromocytoma (PC12) cells and in human embryonic kidney (HEK-293) cells after agonist exposure (Keefer and Limbird, 1993, Daunt *et al.*, 1997, Olli-Lähdesmäki *et al.*, 1999, Taraviras *et al.*, 2002, Olli-Lähdesmäki *et al.*, 2003). Dynamic regulation of receptor density and signaling after exposure to an agonist has significant consequences for the pharmacological responsiveness to many drugs, and is therefore an important cellular phenomenon.

2.2.6 Therapeutic applications of α_2-adrenoceptors

Current clinically available α_2-adrenoceptor drugs show only marginal subtype selectivity, and partly as a consequence of this, several unwanted side-effects limit their therapeutic usefulness. This lack of agents that are at the same time subtype-selective and specific for α_2-adrenoceptors has also precluded the detailed elucidation of the physiological roles of the α_2-adrenoceptor subtypes and the clinical contexts where drugs acting on α_2-adrenoceptors could be applicable. Mice with genetically modified receptor gene expression are now available and are used extensively to study the physiological and pharmacological functions of the α_2-adrenoceptor subtypes. Gene-targeted mice are

expected to predict the pharmacological and therapeutic properties of subtype-selective drugs, but they are not expected to totally display the same phenotypic effects as could be seen by administering pharmacological agents to human subjects (MacDonald *et al.*, 1997). In addition to species differences between mice and humans and possible adaptations taking place in transgenic mice because of the life-long absence of a receptor type, genetic variation in the human population could also play a role. For example, several common polymorphisms have been identified within the coding regions of the human α_2-adrenoceptor genes (Heinonen *et al.*, 1999, Small *et al.*, 2000a, Small *et al.*, 2000b). Some of these sequence variants, *e.g.* substitutions or deletions of amino acids in the third intracellular loop, have been demonstrated to affect receptor function at least in cell models *in vitro* (Small *et al.*, 2000b, Small and Liggett, 2001). Clinical genetic studies have associated α_2-adrenoceptor sequence variants with certain clinical phenotypes (Snapir *et al.*, 2001, Snapir *et al.*, 2003), and genetic variation may at least in part explain the observed substantial inter-individual variability in responses to α_2-adrenoceptor agonists and antagonists (Kohli *et al.*, 2010). From a different viewpoint, genetic receptor variants may also be considered as potential drug targets in the future, in an era of individualized medicine.

Several subtype non-selective α_2-adrenoceptor agonists are in clinical use with well-established therapeutic indications, *e.g.* clonidine, α-methyldopa, guanfacine, guanabenz, moxonidine and rilmenidine in the treatment of hypertension (Ruffolo and Hieble, 1994). *Para*-aminoclonidine (apraclonidine) and brimonidine (bromoxidine) are dispensed as eyedrops to treat glaucoma (Serle, 1994). A structural analogue of clonidine, tizanidine, is an effective muscle relaxant and useful in the treatment of spasticity in several neurological diseases such as multiple sclerosis and spinal cord injury (Bes *et al.*, 1988, Eyssette *et al.*, 1988, Mathias *et al.*, 1989). Oxymetazoline, an imidazoline derivative, is used as a nasal spray formulation as a decongestant to provide rapid relief of nasal obstruction (Fox *et al.*, 1979).

In veterinary surgery, α_2-adrenoceptor agonists such as xylazine, detomidine, medetomidine and dexmedetomidine are widely used to achieve sedation, analgesia, anxiolysis, hypnosis and immobilization of animals, while the α_2-adrenoceptor antagonist atipamezole is available for reversal of sedation and immobilization (Savola *et al.*, 1986, Virtanen, 1989, Schwartz and Clark, 1998, Paddleford and Harvey, 1999). Dexmedetomidine is also used clinically in human medicine in the intensive care setting in the United States and in about 40 other non-EU countries. It has sympatholytic and antinociceptive effects that provide hemodynamic stability during surgery and other painful and stressful procedures, and has some beneficial therapeutic effects in comparison with other clinically used anaesthetics (Aantaa and Jalonen, 2006, Arcangeli *et al.*, 2009).

Even though many functional effects have been described for α_2-adrenoceptors, there are currently almost no human therapeutic applications for α_2-adrenoceptor antagonists. Yohimbine and phentolamine were used by some in the treatment of male sexual impotence before modern phosphodiesterase type 5 (PDE5) inhibitors became available (Andersson and Stief, 2001). Mianserin and mirtazapine are commonly used as antidepressants, but these drugs possess many other pharmacological effects in addition to antagonism of α_2-adrenoceptors (Stimmel *et al.*, 1997). Some other conditions have been hypothesized to be targeted by α_2-adrenoceptor antagonists, *e.g.* non-insulin-dependent diabetes and obesity (Ruffolo and Hieble, 1994).

3. AIMS OF THE STUDY

The three mammalian α_2-adrenoceptor subtypes bind the endogenous catecholamines adrenaline and noradrenaline with similar affinities, while their binding affinities for many synthetic ligands differ. The present clinically available α_2-adrenoceptor drugs, agonists and antagonists, are not subtype-selective, which limits their therapeutic usefulness. Understanding of the molecular basis of drug actions on α_2-adrenoceptors, in terms of receptor structure and function, is expected to provide valuable information for subtype-selective drug development. Site-directed mutagenesis, ligand binding experiments, computer-based molecular modelling and sequence comparisons between paralogous and orthologous receptors are strategies used to reveal the key domains and amino acids that are important for specific ligand recognition and binding.

Improved understanding of differences between the α_2-adrenoceptor subtypes on the molecular level is expected to facilitate the design and development of new truly subtype-selective α_2-adrenoceptor drugs and to improve the accuracy of molecular models of the α_2-adrenoceptor proteins. From this point of view, the aims of the present studies were as follows:

1. Characterisation of the pharmacological properties of the recently cloned zebrafish α_2-adrenoceptor subtypes and their comparison with the corresponding human orthologues. This information was also expected to suggest amino acid domains and positions for subsequent mutagenesis experiments (study I).

2. Elucidation of the structural determinants that contribute to the different pharmacological profiles of the human and mouse (rodent) α_{2A}-adrenoceptors (study II).

3. Elucidation of the possible roles of the extracellular amino-terminal region and the first transmembrane domain of the receptors in the subtype-selective binding of antagonist ligands to human α_2-adrenoceptors (study III).

4. MATERIALS AND METHODS

4.1 Mutagenesis and expression vectors

Mutagenesis is a process where DNA, the molecular basis of genetic information, is permanently changed, either in nature or experimentally by the use of chemicals or radiation. For geneticists, the study of mutagenesis is important since mutations may reveal the genetic mechanisms underlying heredity and gene expression. In the life sciences and in molecular biology, mutagenesis is utilized as a tool to study protein function and structure. In site-directed mutagenesis, a pre-planned mutation is created at a defined site in a DNA molecule. This requires that the wild-type gene sequence is known.

Mutagenesis based on polymerase chain reaction (PCR) technology is a relatively simple modern method for generating site-directed mutations. PCR can be used to produce mutations such as nucleotide substitutions, insertions and deletions in double-stranded DNA without the need of subcloning into a bacteriophage plasmid. The procedure involves a PCR reaction using template DNA and synthetic oligonucleotide primers containing the desired mutation, *i.e.* the "mismatch", which are complementary to the opposite strands of the template DNA.

The wild-type cDNAs encoding human, mouse and zebrafish α_2-adrenoceptors used in the present studies were inserted either into the vector pREP4, pcDNA3 or pcDNA3.1(+) (Invitrogen Life Technologies Inc., Rockville, MD, USA). In study I, fragments containing the coding sequences of the zebrafish α_{2A}-, α_{2B}-, α_{2C}-, α_{2Da}- and α_{2Db}-adrenoceptors were generated either from genomic DNA or phage or cosmid clones with primers flanking the coding regions and containing artificial restriction enzyme recognition sites for ligation into the pREP4 vector (see Ruuskanen *et al.*, 2004). In study II, the cDNA encoding the human α_{2A}-adrenoceptor in the vector pcDNA3(+) was obtained from the UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO, USA) and the cDNA encoding the mouse α_{2A}-adrenoceptor (clone Mα_2-10H; Link *et al.*, 1992) in the vector pcDNA3 was originally provided by Dr. Brian Kobilka (Stanford University, Stanford, CA, USA). In study III, the cDNAs encoding human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors were cloned into the pREP4 (α_{2A} and α_{2B}) and pcDNA3 (α_{2C}) vectors, and were originally provided by Dr. Brian Kobilka.

Site-directed mutagenesis (in studies II and III) was performed utilising the GeneEditor™ *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI, USA). The system is designed for use with plasmid vectors conferring ampicillin resistance to the *E. coli* bacterial hosts, encoded by the TEM-1 β-lactamase (ampicillin resistance) gene. Synthetic mutagenic oligonucleotides complementary to the target templates but containing the desired mutations were annealed to denatured single-stranded DNA together with a selection oligonucleotide complementary to the β-lactamase gene, except for a 7-nucleotide sequence that encodes resistance to the GeneEditor™ Antibiotic Selection Mix. The hybridised oligonucleotide was extended

with DNA polymerase to form double-stranded DNA. Subsequently, the constructed cDNA was transformed into *E. coli* host cells (BMH71-18*mutS* and JM109 competent cells) and grown with the GeneEditor™ Antibiotic Selection Mix for selection of plasmids derived from the mutant strand.

Chimaeric receptor constructs based on the α_{2A}- and α_{2B}-adrenoceptors (in study III) were constructed using PCR-based mutagenesis and two pairs of primers designed for each subtype. Exchange of the TM1 domain and the preceding N-terminal segment between receptor subtypes was performed utilizing conserved threonine-serine sites at the distal end of TM1. A reverse primer to the distal part of TM1 and a forward primer to the first intracellular loop was designed to contain an artificial SpeI-site; recognition sequence ACTAGT, coding for threonine-serine. In addition, the N- and C-terminal primers contained restriction sites for subcloning into the pREP4 expression vector. A list of the primers used in studies II and III is shown in Table 4.

Table 4. A list of primers used for mutagenesis in studies II and III. Nucleotides that encode an artificial restriction enzyme recognition site or mutation are highlighted in colour: KpnI (blue), SpeI (red), HindIII (green) and mutation (bold black).

	GeneEditor™ *in vitro* Site-Directed Mutagenesis System primers	
Primer name	***Primer sequence***	***Study***
Human ADRA2A (RCExl2.49-xl2.51SCK)	3'-TCG GCC GGC TCG GCT CAA CGC CTT AGT TGC TGG TCT TCA CCA T-5'	II
Mouse ADRA2A (SCKxl2.49-xl2.51RCE):	5'-GGT CGT TGA TCT CGC ATC TTG GCT CGG CC-3'	II
ADRA2C-SpeI	5'-GCG CAG CGC CCG ACT AGT CAG CAC GGC GAT-3'	III
ADRA2C (ADRA2A Nter-TM1)-NheI	5´-TGC CAC CGC GCC CGC **GCT AGC** CTG GCC GCG CGG CGG-3´	III
ADRA2C (ADRA2A Nter-TM1) (restore)	5´-CAC CTG CAG GCT A**TA** CTG GCC GCG CG-3´	III
ADRA2A (ADCA2C Nter-TM1)-NheI	5´-CAG CGT CAC CTG CAG **GCT AGC** AGG GGT GGC CCG GGC-3´	III
ADRA2A (ADCA2C Nter-TM1) (restore)	5´-CCG CGC CCG CGC TA**T A**AG GGG TGG CCC GGG C-3´	III
	PCR-based mutagenesis primers	III
Primer name	***Primer sequence***	III
ADRA2A-TM1-KpnI (forward)	5'-TCG GTA CCA TGG GCT CCC TGC AGC CGG ACG CGG GCA ACG CGA GCT GGA A-3'	III
ADRA2A-TM1-SpeI (reverse)	5'-GAC TAG TGA ACA CGG CGA TGA TGA CGA GCA CGT T-3'	III
ADRA2A-cterm-SpeI (forward)	5'-AAC TAG TCG CGC GCT CAA GGC GCC CCA AAA CCT CTT-3'	III
ADRA2A-cterm-HindIII (reverse)	5'-TCG AG**A AGC TT**T CAC ACG ATC CGC TTC CTG T-3'	III
ADRA2B-TM1-KpnI (forward)	5'-TCG GTA CCA TGG ACC ACC AGG ACC CCT ACT CCG TGC AGG CCG CAG CGG CCA TA-3'	III
ADRA2B-TM1-SpeI (reverse)	5'-TAC TAG TCA ACA CAG CCA GGA TGA CCA GCG CGT T-3'	III
ADRA2B-cterm-SpeI (forward)	5'-AAC TAG TCG CTC GCT GCG CGC CCC TCG GAA-3'	III
ADRA2B-cterm-HindIII (reverse)	5'-TTA GG**A AGC TT**T CAC CAG GCC GTC TGG GTC CAC GGG CGG CAC AGG AT-3'	III
ADRA2C-TM1-KpnI (forward)	5'-TCG GTA CCA TGG CGT CCC CGG CGC TGG CGG CGG CGC TGG CGG TGG CGG CAG CGG CGG GCC CCA AT-3'	III
ADRA2C-TM1-SpeI (reverse)	5'-TAC TAG TCA GCA CGG CGA TCA CCA CCA GCA CGT T-3'	III

4.2 Cell culture and transfections

To express the constructed receptor proteins for subsequent ligand binding assays, the vector-based constructs were transfected into mammalian cells. Adherent Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection, Rockville, MD, USA) were cultured in α-MEM medium (α-Minimum Essential Medium, GIBCO^{TM}, Invitrogen) supplemented with 26 mM $NaHCO_3$, 50 IU/ml penicillin, 50 μg/ml streptomycin and 5 % heat-inactivated fetal bovine serum (FBS). Cells were grown at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2. The pcDNA3 and pcDNA3.1(+) -based expression constructs were linearised prior to transfection to increase genomic incorporation and to obtain stable expression. The construct cDNAs were transfected into the cells using either the Lipofectin or the Lipofectamine 2000 reagent kit (Invitrogen Life Technologies Inc., Rockville, MD, USA). Selection of transfected cells was performed using either the neomycin analogue G418 (Geneticin®) (Sigma-Aldrich, St. Louis, MO, USA) or Hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany), depending on the expression vector used. The production of stable CHO cell lines with expression vectors based on pMAMneo for expression of wild-type α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors has been described previously (Marjamäki *et al.*, 1992, Marjamäki *et al.*, 1993, Pohjanoksa *et al.*, 1997). Also the production of Shionogi 115 mouse mammary tumour cell lines (S115) stably expressing wild-type α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors (used in study I) has been described previously (Marjamäki et al., 1992).

4.3 Reverse transcription-PCR

Total RNA was isolated from cultured CHO cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesised with random hexamer primers using the DyNAmo^{TM} cDNA Synthesis Kit (Finnzymes, Espoo, Finland). Reverse transcription PCR (RT-PCR) was performed with gene-specific primers designed for the chimaeric human α_{2C}-adrenoceptor-based construct containing TM1 and the N-terminus from the α_{2B}-adrenoceptor. Primers were designed before and after the TM1 cut-off using the program PrimerSelect (DNASTAR Inc., Madison, WI, USA). Control reactions for RT-PCR were run with RNA from untransfected CHO cells.

4.4 Membrane preparation

Confluent CHO cells (>90 %) were detached from culture flasks with 0.25 % trypsin/0.02 % ethylenediamine tetraacetic acid (EDTA), centrifuged at 1500 rpm for 5 min at 4 ^{o}C, washed twice with chilled phosphate-buffered saline (PBS), and stored frozen at -20 ^{o}C until used. The harvested cell pellets were suspended in ice-cold homogenization buffer (10 mM Tris, 0.1 mM EDTA, 0.32 mM sucrose, pH 7.5), followed by homogenization with an Ultra-Turrax homogenizer (model T25, Janke & Kunkel, Staufen, Germany) (3 x 10 s at 800 rpm.). Cell homogenates were centrifuged at 108 *g* for 15 min at 4 ^{o}C, and supernatants were collected. The pooled supernatants

were further centrifuged at 50 227 *g* for 30 min at 4 °C. Membrane pellets were washed with sucrose-free Tris-EDTA buffer, aliquoted in homogenization buffer, and stored at -70 °C. Protein concentrations were determined with the method of Bradford (1976) using bovine serum albumin as reference.

4.5 Ligand binding assays

The radioligand binding assay is a relatively simple but powerful tool to study receptor-ligand interactions of GPCRs. There are three basic types of experiments based on this technique: (1) saturation experiments from which the affinity of the radioligand for the receptor and the binding site density can be determined; (2) competition experiments where the affinity of a competing, unlabeled compound for the receptor can be determined; and (3) kinetic experiments from which the association and dissociation rate constants for radioligand binding can be determined (reviewed extensively in Bylund and Toews, 1993).

4.5.1 Saturation experiments

In a saturation experiment, a receptor preparation (R) is incubated together with a radioligand (L) for a period of time until equilibrium is reached with regard to formation of a receptor-radioligand complex (RL). This can be represented by:

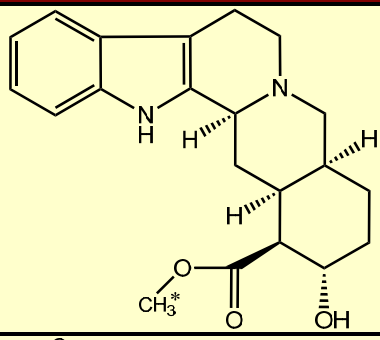
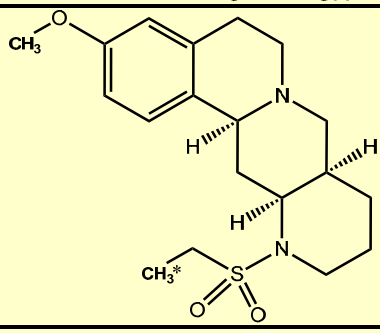
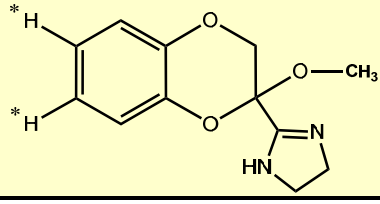
$$L + R \rightleftharpoons RL$$

The amount of RL is measured as a function of the free radioligand concentration [L]. The parameters obtained from a saturation experiment are the affinity, expressed as the dissociation constant, K_d (receptor affinity), and the number of binding sites present in the receptor preparation, B_{max} (maximal binding capacity).

In the present studies, transfected cell lines were screened for binding of three α_2-adrenoceptor specific antagonist radioligands [methyl-^{3}H]rauwolscine (NEN Life Science Products, Inc., Boston, MA, USA), [ethyl-^{3}H]RS79948-197 and [^{3}H]RX821002 (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Table 5). Saturation binding assays were performed in 50 mM potassium phosphate buffer (pH 7.4) as reported previously (Halme *et al.*, 1995, Marjamäki *et al.*, 1998). Either whole-cell homogenates (20-120 μg of protein per sample) or membrane preparations (4-20 μg of protein per sample) were incubated in 250 μl of buffer with serial dilutions of radioligand at 25 °C. Non-specific binding (NSB) was defined in parallel assay tubes in the presence of 10 μM phentolamine, an α_2-adrenoceptor antagonist. After 30 min incubation, reactions were terminated and bound radioactivity was separated by rapid filtration through pre-soaked glass fibre filters (Whatman GF/B) using a Brandel M-48R cell harvester (Medical Research and Development Laboratories, Gaithersburg, MD, USA) in a cold room (6 °C). Filters were washed twice with 5 ml of ice-cold buffer (50 mM Tris-HCl, 10 mM EDTA, pH 7.4) and placed into the bottom of scintillation vials with OptiPhase 'HiSafe' III (Wallac, Turku, Finland). The bound radioactivity on the filters was quantitated by

liquid scintillation counting (Wallac 1410) after 8 h incubation at room temperature (RT). Specific binding was defined as the difference between total binding and NSB. For each of the investigated cell lines, saturation experiments were performed in duplicate or triplicate and repeated at least three times. The K_d and B_{max} values were calculated from the results of saturation binding experiments using the GraphPad Prism software package (GrapPad Prism Software, San Diego, CA, USA).

Table 5. Details of the employed radiolabelled compounds. * indicates the position of the radioactive isotope.

Radioligand	*Chemical structure*	*Specific radioactivity*	*Binding preference*
[Methyl-^{3}H]Rauwolscine		77.5-71 Ci/mmol	$\alpha_{2A} \approx \alpha_{2B} < \alpha_{2C}$
[Ethyl-^{3}H]RS79948-197		88-81 Ci/mmol	$\alpha_{2A} \approx \alpha_{2B} \approx \alpha_{2C}$
[^{3}H]RX821002		60-41 Ci/mmol	$\alpha_{2A} \approx \alpha_{2C} > \alpha_{2B}$

4.5.2 Competition experiments

In a competition experiment, the amount of receptors and the radioligand concentration are kept constant, while the concentration of the unlabeled competing drug is varied. When the concentration of the unlabeled compound is zero, all receptors in the sample are free to be occupied by the radioligand. When the concentration of the unlabeled compound is increased, the unlabeled compound will compete with the radioligand for the receptor binding sites, decreasing the amount of receptor-radioligand complex being present. The parameters obtained from competition experiments are IC_{50} values

(the concentration of the competing drug that inhibits 50 % of the specific binding in a given experiment) and inhibition constants, K_i (the affinity of the competitor for the receptor). An IC_{50} is converted into a K_i-value using the Cheng-Prusoff equation, where [L] represents the free radioligand concentration and K_d represents the dissociation constant of the radioligand (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1+([L]/K_d)}$$

Ligands tested in the competition binding assays were chosen mainly on the basis of their chemical structure and known preference for different α_2-adrenoceptor subtypes (Table 6). The competition binding assays were implemented using either a Beckman Biomek 2000 Laboratory Automation Workstation (Beckman Instruments Inc., Palo Alto, CA, USA) with 96-well plate format, or a MultiScreen Vacuum Manifold system (Millipore Corporation, Bedford, MA, USA) with Millipore MultiScreen MSFBN 96-well glass fibre filtration plates. The experiments were performed in 50 mM potassium phosphate buffer (pH 7.4) using radioligands at concentrations close to their affinity constants (K_d) for each particular receptor, and 6-8 serial dilutions of the competing ligands, which were incubated with 2-10 μg of cell membrane protein per sample. After 30 min incubation at RT, reactions were terminated by rapid vacuum filtration. The filter plates were washed three times with ice-cold potassium phosphate buffer, dried and impregnated with Meltilex B/HS scintillation wax (1205-422, Wallac, Turku, Finland) or 50 μl SuperMix scintillation cocktail (Wallac), depending on the system used. The incorporated radioactivity was determined in a Wallac 1205 or Wallac 1450 Betaplate liquid scintillation counter. The apparent affinities (K_i) of the competing ligands at each receptor were determined *via* non-linear regression analysis (GraphPad Prism), assuming homogeneous one-site binding. The statistical significance of differences between the apparent affinities of two receptors was evaluated with unpaired *t*-tests.

Table 6. Ligands used in competition binding assays

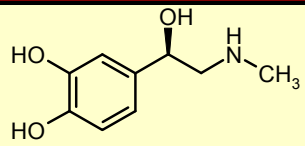
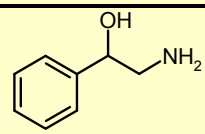
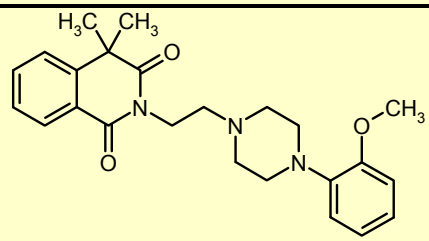
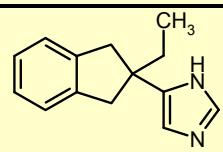
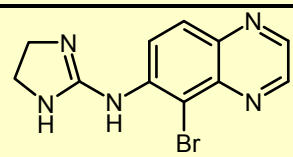
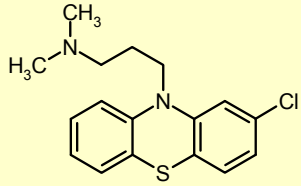
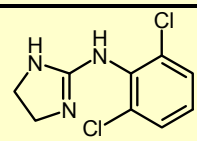
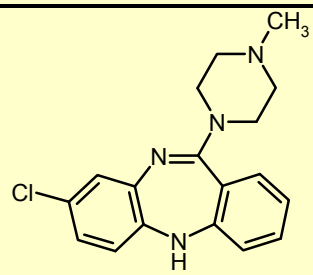
Ligand	*Chemical structure*	*Function*	*Study*
(-)-Adrenaline		Agonist	I, II
2-Amino-1-phenylethanol		Agonist	I
ARC239		Antagonist	I, II, III
Atipamezole		Antagonist	I, II, III
Brimonidine (UK-14.304)		Agonist	I
Chlorpromazine		Antagonist	I, II, III
Clonidine		Agonist	I
Clozapine		Antagonist	I

Table continued on next page.

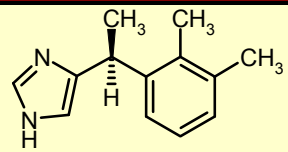
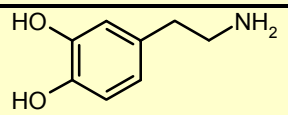
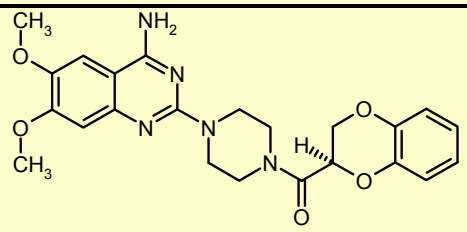
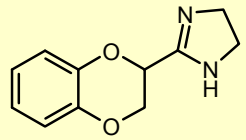
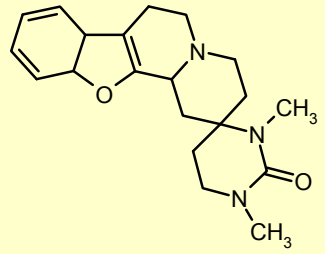
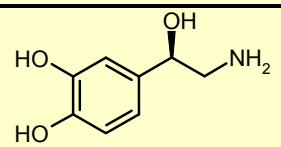
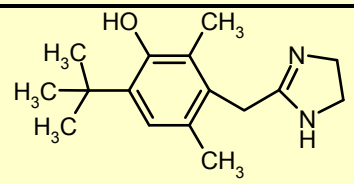
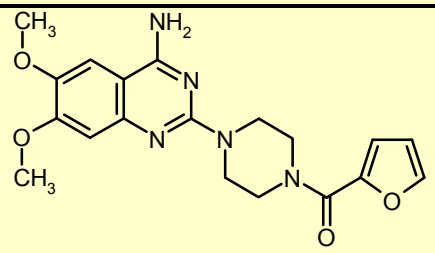
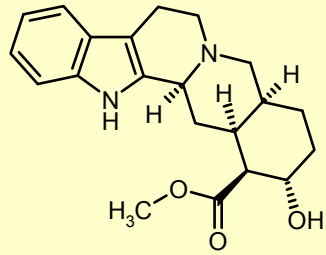
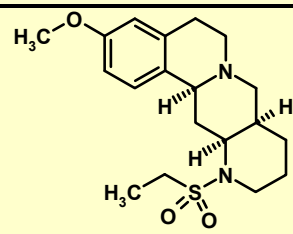
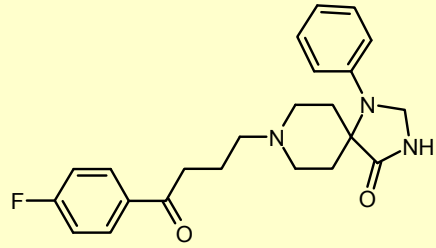
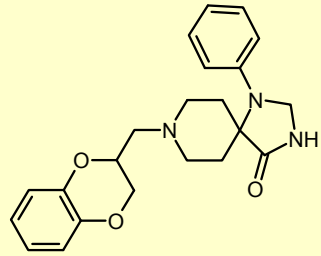
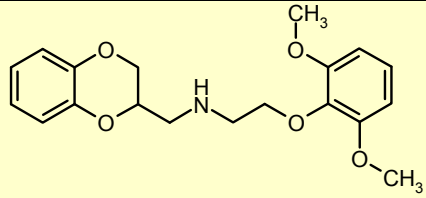
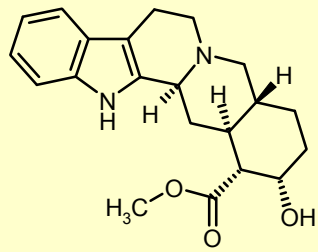
Ligand	*Chemical structure*	*Function*	*Study*
Dexmedetomidine		Agonist	I
Dopamine		Agonist	I, II
Doxazosin		Antagonist	II
Idazoxan		Antagonist	I, III
MK-912 (L657.743)		Antagonist	I, II
(-)-Noradrenaline		Agonist	I, II
Oxymetazoline		Agonist	I
Prazosin		Antagonist	I, II, III

Table continued on next page.

Ligand	*Chemical structure*	*Function*	*Study*
Rauwolscine		Antagonist	I, II, III
RS79948-197		Antagonist	II
Spiperone		Antagonist	I, III
Spiroxatrine		Antagonist	I, III
WB-4101		Antagonist	I, II
Yohimbine		Antagonist	I, II

4.5.3 Functional [^{35}S]GTPγS binding assay

The [^{35}S]GTPγS binding assay is a simple method to monitor the first step of the intracellular signalling cascade after receptor activation by exposure to an agonist ligand. Radiolabelled [^{35}S]GTPγS is a poorly hydrolysable GTP analogue, which binds almost irreversibly to G-protein α-subunits upon receptor activation.

Functional [^{35}S]GTPγS binding assays were conducted with membrane suspensions expressing zebrafish and human α_2-adrenoceptor subtypes (study I). The experiments were carried out in a reaction mixture based on 50 mM Tris-HCl buffer and containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 150 mM NaCl, 1 μM GDP and 30 μM ascorbic acid (pH 7.4) as final concentrations. An aliquot of 5-10 μg of the thawed membrane protein suspension per sample was added to the reaction buffer in a total volume of 0.3 ml. The samples were preincubated for 30 min at RT with serial dilutions of the agonists before 0.1 nM [^{35}S]GTPγS (NEN Life Science Products, Inc., Boston, MA, USA) was added for the 60 min incubation period. The incubations were terminated by rapid filtration through Whatman GF/B filters, the filters were rinsed (with cold 20 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4) and the bound radioactivity was counted in a liquid scintillation counter. Agonist-induced stimulation of [^{35}S]GTPγS binding was determined for the natural ligands (-)-adrenaline and (-)-noradrenaline and the synthetic agonists brimonidine, oxymetazoline and dexmedetomidine, displaying different efficacies and potencies at the three human α_2-adrenoceptor subtypes.

4.6 Molecular modelling and ligand docking

Molecular modelling is a computational technique to mimic the atomic-level description of the investigated molecules. In structural biology and chemistry, molecular modelling is used to visualize the three-dimensional shapes of the proteins of interest and interacting ligands. If the structure of the target protein is not known, homology modelling may be applied by using sequence alignment comparisons and a homologous protein with a known structure as template. For validation of homology models, it is important that experimental findings correlate with the structural features of the modelled protein.

Structural models of various orthologous and paralogous α_2-adrenoceptors (studies I and II) were constructed using the X-ray structure of bovine rhodopsin as template (Palczewski *et al.*, 2000). The structure of an inactive state conformation of bovine rhodopsin, bound with 11-*cis*-retinal, solved at 2.6 Å resolution, was obtained from the Protein Data Bank (PDB) (PDBcodes 1F88, 1HZX, 1LPH) (Palczewski *et al.*, 2000, Teller *et al.*, 2001, Okada *et al.*, 2002, Li *et al.*, 2004). A modified human α_{2A}-adrenoceptor structural model was reconstructed in study III based on the new X-ray structure of the human β_2-adrenoceptor (PDB code 2RH1) (Cherezov *et al.*, 2007, Rasmussen *et al.*, 2007). The human β_2-adrenoceptor structure was also solved in an inactive state in complex with the inverse agonist, carazolol. The human β_2-adrenoceptor structure was believed to provide a more accurate molecular model of the α_2-adrenoceptors than the bovine rhodopsin structure, as it belongs to the same subfamily of amine GPCRs and shares with the α_2-adrenoceptors both a higher level of

sequence identity – 37-43 % in the TM regions – and higher structural similarities *e.g.* with regard to the amino acids involved in the recognition of catecholamines.

In order to construct homology models of the α_2-adrenoceptor subtypes, multiple pairwise sequence alignments of the α_2-adrenoceptor sequences with the template sequence were first generated with the program MALIGN (Johnson and Overington, 1993) in the Bodil modelling environment (http://users.abo.fi/bodil/about.php) (Lehtonen *et al.*, 2004). Regions that were too dissimilar to be aligned, *i.e.* the N-terminal segment and the third intracellular loop, were deleted from the alignments and were not included in the models. The modelling procedures of α_2-adrenoceptors were performed using the program MODELLER (versions 6.0, 8.0 and 8.2) (Sali and Blundell, 1993) based on the template structures. Here, the deleted sequence segments were corrected manually in order to ensure that the modelled conformations of the α_2-adrenoceptors were relaxed and free of any undesirable atomic contacts. Both in the bovine rhodopsin and the human β_2-adrenoceptor structures, two cysteines, C3.25 in TM3 and Cxl2.50 in XL2, form a disulphide bridge, which constrains XL2 to fold above the binding cavity. In the rhodopsin structure, XL2 directly interacts with bound 11-*cis*-retinal, and in the β_2-adrenoceptor structure, it interacts with carazolol (Palczewski *et al.*, 2000, Cherezov *et al.*, 2007). In all α_2-adrenoceptors, cysteines are present at positions 3.25 and xl2.50, which suggests a similar position of XL2 as well as direct interactions of XL2 with bound ligands in the model structures of the α_2-adrenoceptors.

In the modelling procedures, 10-20 models for each of the receptor subtypes were constructed that varied in the side-chain conformations of the amino acids present in the putative ligand binding site. For those amino acids that are conserved among the α_2-adrenoceptors and the employed template, the conformations of the side-chains were used mainly as found in the template. For non-conserved residues, variable side-chain conformations were produced by MODELLER (for details, see Xhaard *et al.,* 2005).

Ligands docked into the structural models of the α_2-adrenoceptors varied in their size, structure, function and in the level of preference for individual α_2-adrenoceptor subtypes. Docking simulations were performed using either manual (I and II) or automated docking (III), where the program GOLD (version 4.0) (Jones *et al.*, 1997) was allowed to perform automated docking. The manual docking simulations for antagonists were performed in two different ways; first, based on the classical hypothesis of ion pair formation between the side-chain oxygen of D3.32 and the protonated nitrogen atom of the ligands, and secondly, using an alternative binding mode hypothesis based on cation-π interactions with aromatic residues, whereby ligands interact with D3.32 *via* carboxylate-aromatic interactions (Xhaard *et al.,* 2005).

5 RESULTS

5.1 Structural and pharmacological properties of the zebrafish α_2-adrenoceptors in comparison with the human orthologues (I)

5.1.1 Characterization of receptor expression in CHO cells

In saturation binding assays, the binding affinities of all three radioligands, [methyl-^{3}H]rauwolscine, [ethyl-^{3}H]RS79948-197 and [^{3}H]RX821002, were observed to be in the same low nanomolar range towards the five zebrafish α_2-adrenoceptors and their human orthologs (Table 7). Receptor densities of the studied cell lines are summarized in Table 8. In the recombinant CHO cell lines, the overall binding characteristics of [ethyl-^{3}H]RS79948-197 were observed to be comparable with values obtained in membrane preparations from zebrafish brain homogenate, where material collected from 21 pooled zebrafish brains yielded a K_d of 0.1 nM and a B_{max} of 475 fmol/mg protein.

Table 7. Binding affinities of three α_2-adrenoceptor preferring radioligands in CHO cell membranes expressing human and zebrafish α_2-adrenoceptors.

	Receptor (species, subtype) K_d (nM)							
Radioligand	**h α_{2A}**	**z α_{2A}**	**h α_{2B}**	**z α_{2B}**	**h α_{2C}**	**z α_{2C}**	**z α_{2Da}**	**z α_{2Db}**
[methyl-^{3}H]rauwolscine	0.9	0.2	1.3	6.3	0.3	1.5	0.3	0.05
[^{3}H]RX821002	1.1	5.0	4.6	3.3	0.9	1.7	6.0	6.8
[ethyl-^{3}H]RS79948-197	0.2	0.6	0.3	0.3	0.2	0.1	0.3	0.3

Results are expressed as mean values from at least three independent saturation binding experiments performed in duplicate or triplicate. h = human, z = zebrafish

Table 8. Receptor densities in recombinant CHO cell lines obtained from saturation binding experiments using [ethyl-^{3}H]RS79948-197 as radioligand.

Receptor (species, subtype) B_{max} (fmol/mg protein)							
h α_{2A}	**z α_{2A}**	**h α_{2B}**	**z α_{2B}**	**h α_{2C}**	**z α_{2C}**	**z α_{2Da}**	**z α_{2Db}**
5 500 ± 300	3 100 ± 100	1 300 ± 100	460 ± 10	1 700 ± 100	550 ± 10	2 300 ± 200	2 300 ± 300

Results are expressed as means ± s.e.m. of at least three independent experiments. h = human, z = zebrafish

5.1.2 Competition binding assays and cluster analysis of the receptors and ligands

Binding affinities of unlabelled compounds were determined in competition binding experiments performed mainly with the α_2-adrenoceptor antagonist radioligand [^{3}H]RX821002, but also [methyl-^{3}H]rauwolscine and [ethyl-^{3}H]RS79948-197 were used in some instances. In the conversion of IC_{50} into K_i values with the Cheng-Prusoff equation (Cheng and Prusoff, 1973), different K_d values for different radioligands and receptors were applied. The affinities of 20 structurally different ligands at three human and five zebrafish α_2-adrenoceptors are summarized in Table 9 as apparent K_i values. These radioligand binding assays were performed with receptors expressed in CHO-K1 cells, with the exception of clonidine, dexmedetomidine, oxymetazoline, WB-4101, dopamine and (-)-noradrenaline binding assays with the human α_{2A}-adrenoceptor, which were conducted with receptors expressed in the S115 host cell line. This difference in assay performance should not have any effect on the outcome, as 11 ligands were tested with α_{2A}-adrenoceptors in both CHO-K1 and S115 cell lines with an excellent correlation between the host cell types (Figure 9).

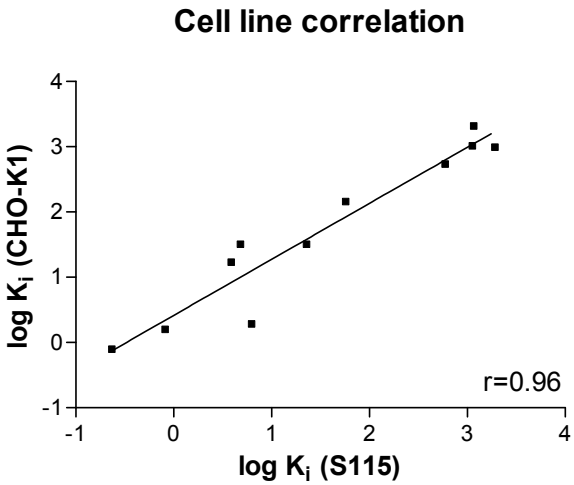


Figure 9. Correlation of ligand binding affinities of a set of 11 ligands obtained from CHO-K1 and S115 host cell lines stably expressing human α_{2A}-adrenoceptors.

The zebrafish and human α_2-adrenoceptors share generally similar ligand binding properties as seen in Table 9, *i.e.* the ligand binding affinities show greater similarities between receptor orthologues in comparison to the receptor paralogues. For receptor orthologues, the largest differences, up to 40-fold, are seen for chlorpromazine and spiroxatrine towards the human and zebrafish α_{2B}-adrenoceptors. Whereas for receptor paralogues, the largest differences, up to 100-fold, are seen for ARC239, oxymetazoline, spiperone, spiroxatrine and chlorpromazine, wherein the human and zebrafish α_{2A}-adrenoceptors bind these compounds with clearly lower affinity than the B and C subtypes. For the zebrafish α_{2Da}- and α_{2Db}-adrenoceptors, the pharmacological binding profiles were observed to be almost identical (r=0.98; Figure 10) as expected based on their close phylogenetic distance and high sequence similarity, with 75 % of the amino acids being identical within their entire sequences (Ruuskanen *et al.*, 2004).

For comparison of the binding affinity data of Table 9, two independent clustering methods were applied: the binary tree approach (see Figure 2A in I) and principal component analysis (PCA) (Figure 2B in I). From these visualizations, the close clustering of the zebrafish α_{2Da}- and α_{2Db}-adrenoceptors can be clearly seen, but for the human and zebrafish α_{2B}- and α_{2C}-adrenoceptors, the pairing is not resolved based on the binding affinity data. For the 20 investigated ligands, the binary tree approach and PCA clustering of the binding affinity data (Table 9) resulted in the segregation of the ligands based on their chemical structures and functional characteristics (see Figure 4 in I). In the binary tree-based clustering, all agonist ligands (clonidine, oxymetazoline, brimonidine, 2-amino-1-phenylethanol, dopamine, (-)-adrenaline and (-)-noradrenaline) except one (dexmedetomidine) clustered together and away from the inverse agonist/antagonist ligands.

Table 9. Competition binding affinities of 20 compounds at three human and five zebrafish α_2-adrenoceptors using [methyl-^{3}H]rauwolscine([a]), [ethyl-^{3}H]RS79948-197([b]) and [^{3}H]RX821002 as radioligands.

	Ligand	human α_{2A}	zebrafish α_{2A}		human α_{2B}	zebrafish α_{2B}		human α_{2C}	zebrafish α_{2C}		zebrafish α_{2Da}	zebrafish α_{2Db}
1	Atipamezole	1.6 (1.3-2.1)	13 (7.4-24)	**	1.5 (0.8-2.8)[a]	5.0 (3.2-7.9)[a]	**	4.3 (2.5-7.7)[a]	2.1 (1.9-2.3)[a]		5.1 (3.0-8.8)	6.90 (4.1-12)
2	Clonidine	10 (5-23)[a]	89 (62-130)	***	44 (21-94)[a]	250 (190-320)[a]	**	110 (51-240)[a]	55 (46-67)[a]		120 (65-210)	150 (98-240)
3	Dexmedetomidine	1.3 (0.5-3.4)[a]	2.2 (1.5-3.0)		4.7 (2.2-10)[a]	7.6 (5.0-12)[a]		6.5 (2.8-16)[a]	12 (9.9-15)[a]		4.1 (3.3-5.0)	3.7 (2.6-5.3)
4	Idazoxan	17 (13-21)	85 (48-150)	**	24 (18-34)[a]	40 (23-68)		17 (6.7-26)[a]	17 (13-22)		52 (31-85)	94 (42-210)
5	Oxymetazoline	2.1 (1.5-3.0)[a]	5.1 (3.2-8.0)	*	1100 (530-2300)[a]	1200 (1000-1400)[a]		130 (70-250)[a]	1300 (860-2000)[a]	**	1100 (540-2400)	440 (300-660)
6	Brimonidine	32 (28-37)	40 (23-69)		320 (200-510)[a]	1200 (940-1600)[a]	**	190 (140-270)[a]	700 (510-970)[a]	***	260 (200-320)	280 (210-370)
7	L657.743	0.8 (0.5-1.2)	6.9 (3.7-13)	*	0.7 (0.5-1.2)[a]	1.2 (1.0-1.6)[a]		0.09 (0.06-0.14)[a]	1.0 (0.9-1.1)[a]	***	1.6 (1.1-2.2)	1.3 (0.7-2.1)
8	Rauwolscine	1.9 (1.3-3.0)	1.0 (0.6-1.6)		1.1 (0.7-1.8)[a]	1.4 (0.6-3.2)		0.2 (0.1-0.5)[a]	0.5 (0.3-0.7)		2.3 (1.2-4.4)	2.3 (1.7-3.1)
9	Yohimbine	5.9 (4.9-7.1)[b]	5.2 (3.4-7.8)		7.5 (6.4-8.9)[b]	9.3 (7.0-12)[a]		4.6 (3.9-5.5)[b]	3.4 (3.0-3.7)[a]	*	6.4 (4.5-9.2)	4.0 (3.1-5.1)
10	Chlorpromazine	990 (500-1900)	110 (36-330)	**	43 (20-100)[a]	1.1 (0.8-1.6)[a]	*	330 (220-1900)[a]	83 (72-95)[a]	*	18 (13-26)	19 (10-36)
11	Clozapine	32 (15-66)	3.3 (2.6-4.3)	***	12 (5.0-28)[a]	9.3 (5.8-14)		2.1 (1.1-3.9)[a]	3.2 (1.8-5.7)		12 (9.5-16)	24 (15-38)
12	ARC239	2100 (860-5100)	1800 (870-3900)		9.6 (3.9-26)[a]	36 (31-42)[a]	*	66 (36-630)[a]	280 (210-370)[a]		55 (46.8-65)	44 (19-110)
13	Prazosin	1030 (540-2050)	330 (200-540)		66 (31-150)[a]	300 (260-360)[a]	***	31 (18-56)[a]	100 (78-130)[a]	*	68 (50-92)	64 (48-86)
14	Spiperone	540 (440-660)	45 (28-71)	***	12 (3.7-38)[a]	51 (38-67)[a]	**	11 (5.4-23)[a]	63 (44-91)[a]		15 (11-22)	18 (9.6-33)
15	Spiroxatrine	320 (240-430)[b]	150 (92-240)	*	2.4 (1.2-5.0)[a]	93 (62-140)[a]	***	3.1 (1.7-5.6)[a]	35 (22-56)	**	11 (3.7-33)	11 (4.7-86)
16	WB-4101	5.4 (2.0-15)[a]	11 (3.7-30)		60 (30-120)[a]	51 (45-59)[a]		1.9 (1.0-3.9)[a]	19 (16-24)[a]	***	31 (22-43)	16 (11-24)
17	2-amino-1-phenylethanol	1300 (940-1700)[b]	5400 (3100-9400)	**	4200 (2500-7100)[b]	9400 (7000-12600)[a]		8100 (6200-10600)[b]	5100 (3700-7000)[a]		3700 (2700-5100)	4000 (2200-7200)
18	Dopamine	2000 (1300-3000)[a]	790 (520-1200)	***	6300 (2800-14400)[a]	4400 (3800-5200)[a]		1200 (710-2300)[a]	3900(2100-7200)[a]		1300 (820-1900)	1700 (790-3700)
19	(-)-Adrenaline	150 (83-250)	140 (94-220)		710 (330-1600)[a]	910 (720-1200)[a]		130 (65-270)[a]	1080 (700-1700)[a]		500 (240-1100)	470 (300-740)
20	(-)-Noradrenaline	110 (32-400)[a]	260 (140-490)		680 (360-1300)[a]	647 (500-830)[a]		250 (120-550)[a]	580 (330-1000)[a]		380 (270-540)	510 (310-840)

Results are expressed as apparent K_i values (nM) and their 95 % confidence intervals (CÍ) from three to five separate experiments. Ligands are grouped into five clusters based on their chemical structures. Statistical significance of differences between the receptor orthologues A, B or C, *p<0.05, **p<0.01, ***p<0.001.

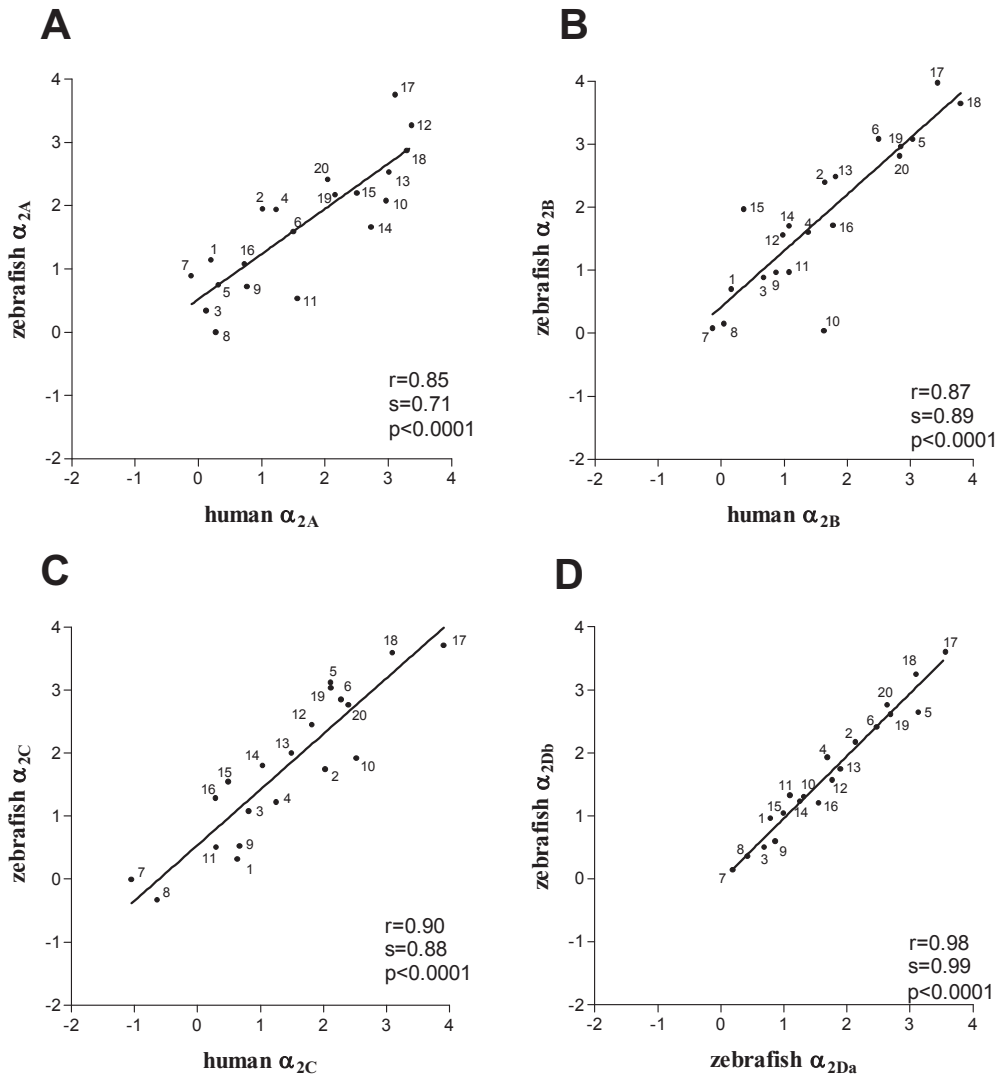


Figure 10. Linear regression analysis of the logarithmic K_i values (nM) for 20 competing ligands between the three orthologous human and zebrafish α_2-adrenoceptor subtypes (A-C) and the paralogous zebrafish α_{2Da}- and α_{2Db}-adrenoceptors (D). Regression lines, slopes (s), Spearman's correlation coefficients (r) and P-values for difference of the linear regression line from a line with zero slope are shown. Ligands are numbered in the order of Table 9. Modified from publication I.

5.1.3 Agonist-stimulated [^{35}S]GTPγS binding

The efficacies and potencies of (-)-adrenaline, (-)-noradrenaline, brimonidine, oxymetazoline and dexmedetomidine on the three human and the five zebrafish α_2-adrenoceptors were determined using the functional [^{35}S]GTPγS binding assay (Table 2 in paper I). For the human and zebrafish α_{2A}-adrenoceptors, the natural agonists (-)-adrenaline and (-)-noradrenaline exhibited high efficacy but rather low potency, while brimonidine, oxymetazoline and dexmedetomidine displayed only partial agonism but

higher potency. For the human α_{2B}-adrenoceptor, the maximal signal obtained with the natural ligands was smaller than for the human α_{2A}- and α_{2C}-adrenoceptors. On the other hand, they revealed even less efficacy at the zebrafish α_{2B}- and α_{2C}-adrenoceptors. Moreover, no clear stimulation was seen for brimonidine or oxymetazoline at the zebrafish α_{2B}- and α_{2C}-adrenoceptors. In addition, dexmedetomidine did not appreciably stimulate [^{35}S]GTPγS binding at the zebrafish α_{2B}-adrenoceptor. When α_2-adrenoceptor expressing CHO cells were treated with pertussis toxin (PTX) before being harvested, the responses to (-)-adrenaline were completely abolished for all adrenoceptor subtypes, indicating coupling to G_i proteins, which are inactivated by PTX (Freissmuth *et al.*, 1999).

5.1.4 Comparison of the structural models of the human and zebrafish α_2-adrenoceptors

Similarity in the pharmacological profiles of the human and zebrafish α_2-adrenoceptors can be thought to reflect similar requirements to bind endogenous catecholamines. In the structural models of the receptor proteins, the amino acids that were predicted to constitute the membrane-embedded ligand-accessible surface of the receptors are located in TM2-TM7 and in XL2, and most of them were found to be highly conserved (Figure 11). Only three residues (at positions 2.57, 5.39 and 5.43) of 27 ligand-accessible amino acids were found to differ within the TM domains in the structural models. Of these, the role of serine/cysteine variation at position 5.43 in the α_2-adrenoceptors has been studied extensively with regard to catecholamine binding (Rudling *et al.*, 1999, Nyrönen *et al.*, 2001, Peltonen *et al.*, 2003). Other amino acids known to be important for the binding of small catecholic ligands in TM3-TM6, D3.32, V3.33, C3.36, S5.42, S5.46, F5.47, W6.48, F6.51, F6.52 and Y6.55, are conserved among all of the human and zebrafish α_2-adrenoceptor subtypes (Marjamäki *et al.*, 1999, Nyrönen *et al.*, 2001, Peltonen *et al.*, 2003). Most of the amino acid variation within the proposed binding cavity of the human and zebrafish α_2-adrenoceptors was found to be concentrated in XL2. In particular, three amino acids, at positions xl2.49, xl2.51 and xl2.52, were predicted to be exposed to the binding cavity, and were found to differ both between paralogous and orthologous receptor subtypes.

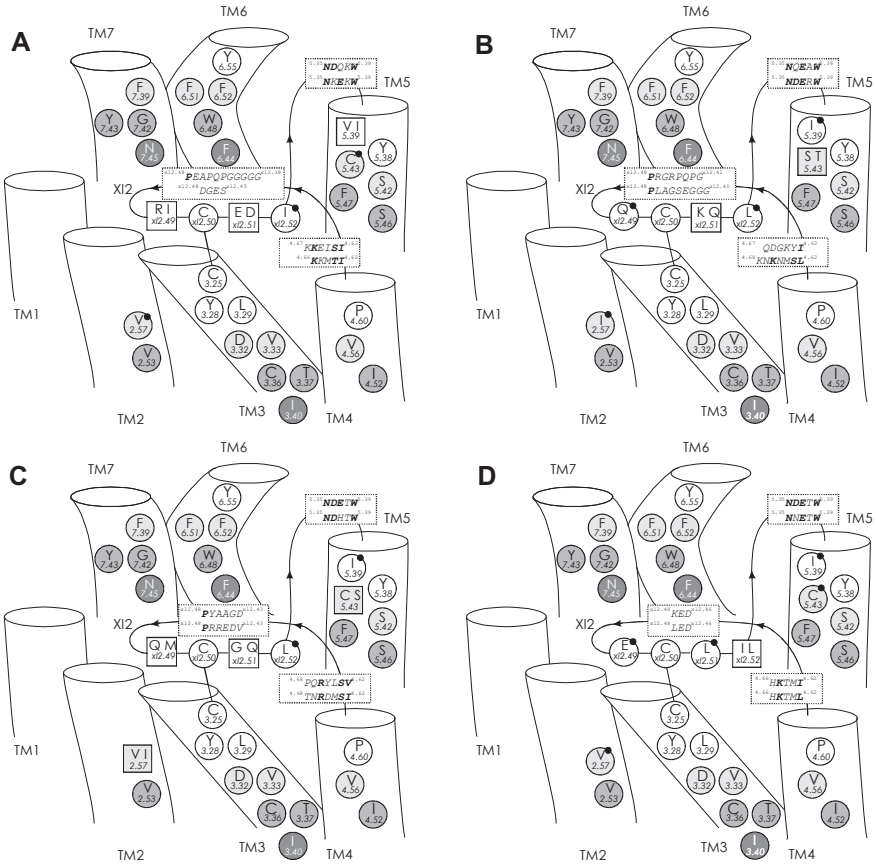


Figure 11. Schematic presentation of the putative ligand binding cavities of the human and zebrafish α_2-adrenoceptors based on the three-dimensional model structures. Only residues predicted to be exposed in the binding cavity are shown. Human versus zebrafish (A) α_{2A}, (B) α_{2B}, (C) α_{2C} and (D) the zebrafish α_{2Da} and α_{2Db} duplicates. Positional conservation and variation are indicated as follows: conserved positions in the compared receptors are circled and the amino acids are indicated in the single letter amino-acid code; a dot on the circle indicates that the position is not conserved across one or more of the paralogous receptors; variable positions in the compared receptors are boxed and the variation is indicated with the appropriate single letters (in panels A-C the amino acid in human is followed by that in zebrafish; in panel D the amino acid in α_{2Da} is followed by that in α_{2Db}). Residues whose location with respect to the proposed binding site is uncertain, belonging to XL2, are shown in dashed boxes. Shades of gray are used to indicate the location of amino acids with respect to the extracellular surface: light towards the surface and darker away from the surface. Taken from publication I.

5.2 Structural determinants involved in the interspecies difference of yohimbine analogues at human and mouse α_{2A}-adrenoceptors (II)

5.2.1 Comparison of the binding cavities of the human and mouse α_{2A}-adrenoceptors

In order to predict the amino acids that differ within the binding cavities, molecular models of the human and mouse α_{2A}-adrenoceptors were constructed based on the bovine rhodopsin crystal structure (Palczewski *et al.*, 2000) (Figure 12). Including the

expanded binding site proposal of Surgand *et al.* (2006), 34 amino acids in the TM domains were found to face the ligand binding cavity, and only one (5.43) of them differed between the human and mouse α_{2A}-adrenoceptors. The XL2 domain, thought to form an aromatic "lid" over the binding pocket, contains 25 amino acids but only four residues of these were thought to face the binding cavity, of which two (xl2.49 and xl2.51) were different between the human and mouse α_{2A}-adrenoceptors. Based on the model structure comparisons, the three amino acids that differ between the binding cavities of the mouse and human α_{2A}-adrenoceptors were replaced with the corresponding reciprocal amino acids by the use of site-directed mutagenesis, and the effects of the mutations on ligand binding were tested. A total of five receptor mutants were constructed: a human α_{2A}-adrenoceptor with two mutations in XL2 (Rxl2.49S and Exl2.51K) and the reciprocal mouse α_{2A}-adrenoceptor double mutant (Sxl2.49R and K2.51E); a human TM5 mutant (C5.43S) and the reciprocal mouse TM5 mutant (S5.43C); and a triple-mutated (S5.43, Sxl2.9R and K2.51E) mouse α_{2A}-adrenoceptor.

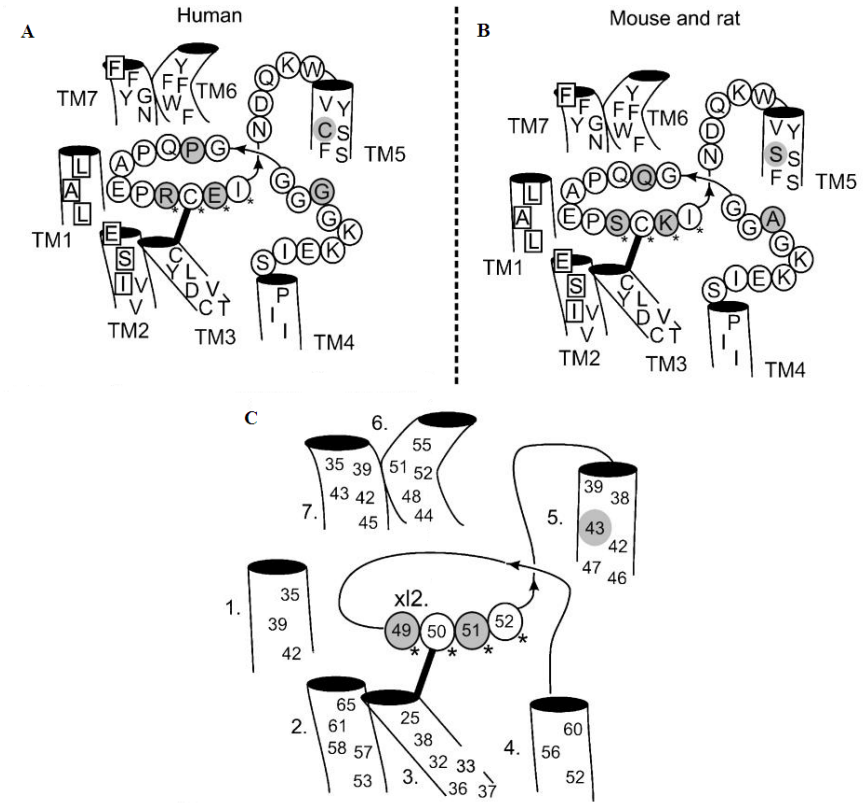


Figure 12. Schematic representations of the α_{2A}-adrenoceptors from (A) human, (B) mouse and rat, and (C) corresponding amino acid codes according to the Ballesteros and Weinstein (1995) numbering scheme. Amino acids facing the ligand binding cavity in the TM and XL2 domains are indicated with one-letter codes. Residues differing between the human and mouse receptors are indicated with grey. Four residues in XL2 suggested to face the ligand binding cavity are indicated with (*). Amino acids from the expanded binding site close to TM1 as proposed by Surgand et al. (2006) are boxed. Two cysteines at 3.25 and xl2.50 are connected by a disulphide bridge. Modified from II.

5.2.2 [^{3}H]RX821002 binding at human and mouse α_{2A}-adrenoceptors

The antagonist radioligand [^{3}H]RX821002 was found to have an about two-fold difference in its binding affinity towards the human and mouse α_{2A}-adrenoceptors (Table 10). The human α_{2A}-adrenoceptor bound [^{3}H]RX821002 with lower binding affinity than the mouse α_{2A}-adrenoceptor. This difference was reciprocally changed when the cysteine-serine difference at position 5.43 in TM5 was reversed. Otherwise, the binding affinity of [^{3}H]RX821002 at the mutant α_{2A}-adrenoceptors was observed to be in the same nanomolar range as found in the wild-type receptor orthologues.

Table 10. Binding affinities of [^{3}H]RX821002 and receptor densities in CHO cell membranes containing wild-type and mutated α_{2A}-adrenoceptors.

Receptor	K_d (nM)	B_{max} (pmol/mg protein)
hα_{2A} C/R/E (wild-type)	1.10 ± 0.17	29.5 ± 2.3
hα_{2A} C/S/K (xl2)	1.20 ± 0.12	14.5 ± 1.6
hα_{2A} S/R/E (TM5)	0.50 ± 0.02	43.3 ± 1.5
mα_{2A} S/S/K (wild-type)	0.54 ± 0.02	8.49 ± 0.03
mα_{2A} S/R/E (xl2)	0.93 ± 0.10	28.0 ± 5.3
mα_{2A} C/S/K (TM5)	1.88 ± 0.13	5.33 ± 0.10
mα_{2A} C/R/E (TM5 & xl2)	1.59 ± 0.28	2.57 ± 0.25

Receptor variants are named based on the amino acids at positions 5.43/xl2.49/xl2.51. Results are expressed as means ± s.e.m of three independent experiments. h = human, m = mouse

5.2.3 Effects of XL2 and TM5 substitutions on ligand binding profiles

The binding affinities (apparent K_i) of 10 antagonists and 3 natural agonists at wild-type human and mouse α_{2A}-adrenoceptors, as well as at five mutant receptors, were characterized using competition binding assays (Table 11). The natural α_2-adrenoceptor ligands, adrenaline, noradrenaline and dopamine, bound to the wild-type human and mouse α_{2A}-adrenoceptors with similar binding affinities in the following potency order: adrenaline > noradrenaline > dopamine. As quite often observed, the affinity values of agonists exhibited greater experimental variability – larger confidence intervals – than those of antagonists. This is partly explained by the effects of variation in the receptor expression levels, 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. This effect was also seen here where the expression levels were quite variable between the studied cell lines (see Table 11).

With respect to the antagonist ligands, yohimbine was observed to prefer the human wild-type α_{2A}-adrenoceptor by about 15-fold over the mouse orthologue, and for its chiral analogue rauwolscine, this ratio was even greater, about 20-fold. These observations agree with previous findings (Link *et al.*, 1992, Uhlèn *et al.*, 1998), but here, the binding affinities of another four antagonists were also observed to

discriminate between the human and mouse α_{2A}-adrenoceptors, but with more moderate effect sizes: WB4101 (seven-fold) and RS-79948-197 (four-fold) preferred the human α_{2A}-adrenoceptor, while both ARC239 and atipamezole preferred the mouse orthologue by somewhat less than four-fold.

Reciprocal mutations in XL2 and TM5 were observed to lead to reciprocal changes in the binding affinity profiles of the four antagonist ligands, rauwolscine, yohimbine, RS-79948-197 and WB4101, that preferred the human α_{2A}-adrenoceptor over the mouse receptor orthologue (Figure 13). However, for WB4101, only the mutation in the mouse α_{2A}-adrenoceptor at position S5.43C was observed to improve the binding affinity, but this conclusion must be viewed with caution because of large overlapping confidence intervals of the affinity estimates. With regard to the other three ligands, the double substitution Rxl2.49S and Exl2.51K in XL2 of the human α_{2A}-adrenoceptor increased significantly the K_i for rauwolscine (four-fold), yohimbine (six-fold) and RS-79948-197 (seven-fold). The reciprocal double mutation Sxl2.49R and Kxl2.51E in the mouse receptor led to opposite "gain-of-function" effects on the binding of these ligands as seen significant decreases in the K_i for rauwolscine (less than two-fold), yohimbine (four-fold) and RS-79948-197 (four-fold). For rauwolscine and yohimbine, but not for RS-79948-197, corresponding reciprocal effects were seen in receptors mutated at position 5.43 in TM5, as the C5.43S substitution in the human receptor led to an increase in the K_i for rauwolscine (eight-fold) and yohimbine (six-fold), whereas the reciprocal S5.43C substitution in the mouse receptor led to decreases in the K_i for rauwolscine (three-fold) and yohimbine (six-fold). When all three positions (S5.43C, Sxl2.49R and Kxl2.51E) were simultaneously mutated in the mouse α_{2A}-adrenoceptor, the affinities of yohimbine, rauwolscine and RS-79948-197 were observed to be comparable with those of the human wild-type α_{2A}-adrenoceptor: K_i was decreased for yohimbine (16-fold), rauwolscine (six-fold) and RS-79948-197 (six-fold), when compared to the wild-type mouse α_{2A}-adrenoceptor.

Table 11. Competition binding affinities of different ligands obtained with [^{3}H]RX821002 at human and mouse wild-type (WT) and point mutated α_{2A}-adrenoceptors expressed in CHO cells.

	Human α_{2A}			*Mouse α_{2A}*			
Ligand	*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 & 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)
L-657.743	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 K_i ratio	95	22	18	3	14	41	75

Results are expressed as apparent K_i (nM) and 95 % confidence intervals of three to five independent experiments. Ligands are grouped as 1) antagonists with more than 4-fold different affinity between the human and mouse wild-type receptors, 2) antagonists with less than 4-fold 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 vs. wild-type; $^{•}$ P<0.05; $^{••}$ P<0.01; $^{•••}$ P<0.001 human *vs.* mouse.

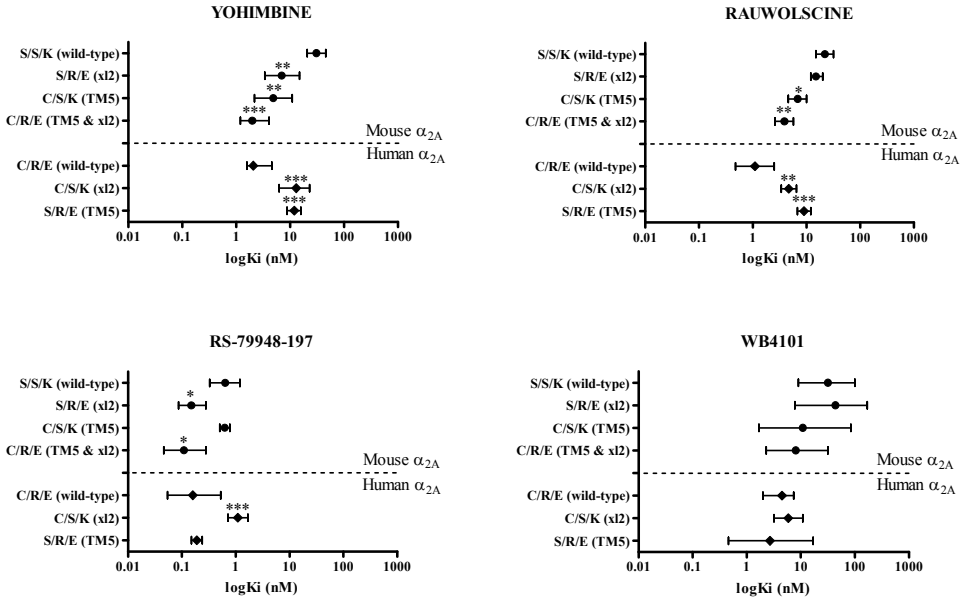


Figure 13. Comparison of the binding affinities of yohimbine, rauwolscine, RS-79948-197 and WB4101 towards the human and mouse α_{2A}-adrenoceptors and their mutants. Error bars represent 95 % confidence intervals of 3-5 independent experiments. Statistical significance compared to the wild-type receptor (unpaired t-tests): *P<0.05, **P<0.01, ***P<0.001. Modified from II.

5.3 Involvement of the first transmembrane domain of human α_2-adrenoceptors in the subtype-selectivity of bulky antagonists (III)

5.3.1 Construction of receptor chimaeras

At the start of the project, all receptor mutants (Figure 14) were planned to be prepared based on PCR and primers containing the desired mutations and an artificial restriction enzyme recognition site for subcloning into the pREP4 vector. However, only chimaeras based on the α_{2A}- and α_{2B}-adrenoceptors were constructed using PCR-based mutagenesis and two pairs of primers, designed for each subtype. The exchange of TM1 and the preceding amino terminal sequence was performed utilizing a conserved threonine-serine site at the intracellular end of TM1. Thus, reverse primers until the end of TM1 and forward primers from the start of the first intracellular loop were designed to contain an artificial SpeI-site; recognition sequence ACTAGT, coding for threonine-serine. In addition, the N- and C-terminal primers were designed to contain restriction sites for subcloning into the pREP4 expression vector. Finally, the PCR-amplified fragments were digested with the appropriate restriction enzymes and ligated into pREP4 for expression in mammalian cells.

After several failures in PCR-amplification of the C-terminal fragment of the α_{2C}-adrenoceptor, chimaeras based on this receptor subtype were constructed using the

Gene Editor™ *in vitro* Site-Directed Mutagenesis System. In this approach, the original nucleotide sequence encoding conserved threonine-serine amino acids at the end of TM1 were mutated to contain the sequence ACTAGT, a site for SpeI recognition. Subsequently, the α_{2C}-adrenoceptor-based chimaeras were constructed using the artificial SpeI-site and PCR-amplified TM1 (and N-terminal) fragments of the α_{2A}- and α_{2B}-adrenoceptors, and finally, the digested fragments were ligated and subcloned into the pcDNA3.1(+) expression vector.

Additional α_{2A}- and α_{2C}-adrenoceptor chimaeras with only TM1-region substitutions were constructed using the GeneEditor™ *in vitro* Site-Directed Mutagenesis System and the previous chimaeras as templates; the nucleotide sequence coding for the conserved tyrosine-serine pair at the end of the N-terminus was mutated to contain an NheI-site (recognition sequence GCTAGC, coding for alanine-serine). Subsequently, using this artificial NheI-site, the N-terminal fragments were digested, isolated from agarose gel, and ligated to the appropriate α_{2A}- and α_{2C}-adrenoceptor-based chimaeras. Thereafter, the artificial NheI-sites were restored to encode the original tyrosine-serine pair.

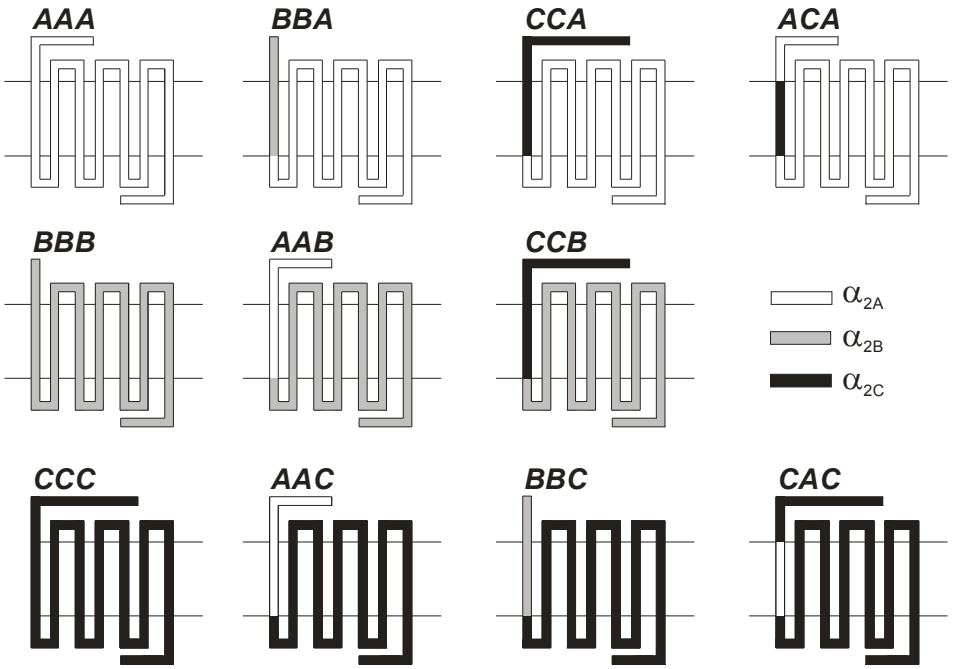


Figure 14. Schematic representation of the constructed α_2-adrenoceptor chimaeras. Receptors are named based on the origin of the sequence of the N-terminal segment, the TM1 domain and the body of the receptor, corresponding to the first, second and third letter, respectively. Modified from III.

5.3.2 Expression of α_2-adrenoceptor chimaeras in CHO cells

The cDNAs of eight chimaeric α_2-adrenoceptor constructs were transfected into CHO cells using lipid-based transfection mixtures. Cell cultures were screened for their capacity to bind the radioligand [^{3}H]RX821002 and the cell populations with the

highest levels of receptor expression were chosen for further binding experiments. Seven out of eight receptor constructs were successfully expressed in CHO cells, as evidenced by saturation binding experiments with [^{3}H]RX821002 (Table 12). For the wild-type receptors, [^{3}H]RX821002 had 5- to 10-fold lower affinity towards the human α_{2B}-adrenoceptor in comparison to the α_{2A}- and α_{2C}-adrenoceptors. This observation agrees with previous findings (O'Rourke *et al.*, 1994a, Deupree *et al.*, 1996, Uhlèn *et al.*, 1998). When the N-terminal and TM1 regions of the α_{2B}-adrenoceptor were replaced with the corresponding regions of the α_{2A}- and α_{2C}-adrenoceptors, the binding affinity of [^{3}H]RX821002 was increased 3-fold towards that of the wild-type α_{2A}- and α_{2C}-adrenoceptors. Reciprocal effects were seen in the α_{2A}-based chimaera containing the N-terminal and TM1 regions from the α_{2B}-adrenoceptor, *i.e.* an almost 3-fold decrease in affinity relative to the wild-type α_{2A}-adrenoceptor.

Table 12. Binding affinities of [^{3}H]RX821002 and receptor densities in recombinant CHO cell lines expressing wild-type and mutated human α_{2B}-adrenoceptor subtypes.

Receptor	K_d (nM)	B_{max} (fmol/mg protein)
α_{2A} *(AAA)*	0.45 ± 0.04	6700 ± 100
α_{2A} *BBA*	1.2 ± 0.7	400 ± 300
α_{2A} *CCA*	0.39 ± 0.06	5600 ± 500
α_{2A} *ACA*	0.27 ± 0.02*	19 000 ± 2 800
α_{2B} *(BBB)*	4.6 ± 1.0	1500 ± 300
α_{2B} *AAB*	1.4 ± 0.2*	1600 ± 100
α_{2B} *CCB*	1.6 ± 0.2*	6100 ± 500
α_{2C} *(CCC)*	0.87 ± 0.06	1800 ± 40
α_{2C} *AAC*	0.71 ± 0.04	470 ± 10
α_{2C} *BBC*	-	-
α_{2C} *CAC*	0.55 ± 0.15	710 ± 80

Receptors are named based on the exchanged domains (see Figure 14). Results are expressed as means ± s.e.m. of three independent experiments. Statistical significance of differences between the chimaeric receptors and the respective wild-type receptors were tested with Student's *t*-test: * $P<0.05$

Cells transfected with the α_{2C}-adrenoceptor-based construct containing TM1 and the N-terminus from the α_{2B}-adrenoceptor failed to show [^{3}H]RX821002 binding in the saturation binding experiments, in spite of several transfection experiments with several batches of plasmid DNA. To confirm the transfection of the construct, total RNA was isolated from transfected, antibiotic-resistant CHO cells and converted to cDNA. PCR amplification was performed with gene-specific primers designed to both sites used for nucleotide sequence cut-off (upper 5′-TCATGGGCGTGTTCGTGCTCTGCT-3′; lower 5′-ATCGCCGGAAATCCTGGTTGAAG-3′). Control reactions were run using RNA from non-transfected cells (Figure 15).

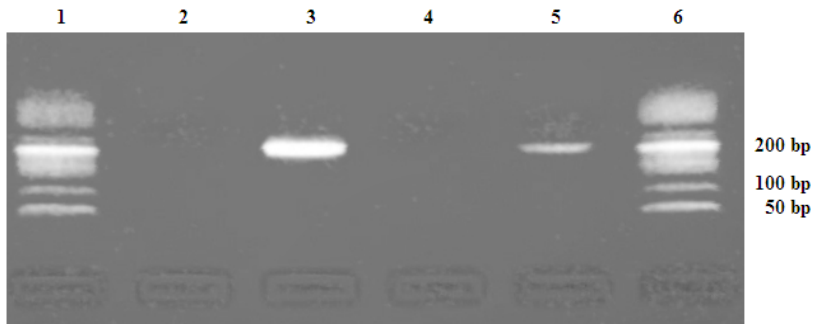


Figure 15. RT-PCR reactions using α_{2C} BBC gene-specific primers run on a 1 % agarose gel. Lanes 1 and 6: DNA size marker. Lane 2: PCR control (water used instead of template DNA) to prove that no DNA contamination exists. Lane 3: a 198-bp control fragment amplified from plasmid DNA (pcDNA3.1-α_{2C} BBC). Lane 4: RT-PCR using RNA isolated from non-transfected CHO-K1 cells. Lane 5: a 198-bp amplification fragment from RT-PCR using RNA isolated from α_{2C} BBC transfected CHO cells.

5.3.3 Characterization of antagonist binding profiles at wild-type and chimaeric α_2-adrenoceptors

The binding affinities (apparent K_i) of nine antagonist ligands at the seven receptor chimaeras and the three human wild-type α_2-adrenoceptors were characterized using competition binding assays and [^{3}H]RX821002 as radioligand (Table 13). For wild-type receptors, six ligands (ARC239, prazosin, spiperone, spiroxatrine, chlorpromazine and clozapine) showed significantly lower affinity towards the α_{2A}-adrenoceptor in comparison to the α_{2B}- and α_{2C}-adrenoceptor subtypes, as highlighted by the 10-100 -fold differences in the binding affinities. Only two of the ligands (chlorpromazine and spiroxatrine) were observed to significantly discriminate between the α_{2B}- and α_{2C}-wild-type receptors (see Table 2 in III). For three ligands (spiperone, spiroxatrine and chlorpromazine), the binding affinities were found to be significantly improved at the α_{2A}-adrenoceptor when TM1 and the N-terminus were replaced with the corresponding regions from the α_{2C}-adrenoceptor subtype. Also insertion of TM1 and the N-terminus of the α_{2B}-adrenoceptor into the α_{2A}-subtype significantly improved the binding affinities of two of these ligands (spiroxatrine and chlorpromazine) in comparison to the wild-type α_{2A}-adrenoceptor. Similar effects were also seen for ARC239 and prazosin, but the differences failed to reach statistical significance. Reciprocal effects were not seen in chimaeras based on the human α_{2B}- and α_{2C}-adrenoceptor subtypes. When only TM1 was imported into the α_{2A}-adrenoceptor from the α_{2C}-adrenoceptor, the binding affinities did not differ from those observed for the chimaera containing both TM1 and the N-terminus from the α_{2C}-adrenoceptor, indicating involvement of TM1 in the observed binding affinity changes.

Table 13. Competition binding affinities of nine antagonist ligands obtained with [^{3}H]RX821002 at wild-type (WT) and chimaeric α_2-adrenoceptors expressed in CHO cells.

	Human α_{2A}			
Ligand	*WT (AAA)*	*BBA*	*CCA*	*ACA*
ARC239	1600 (1000-2600)	450 (160-1400)	820 (600-1110)	760 (480-1200)
Prazosin	1500 (800-3100)	1400 (490-2400)	820 (600-1100)	910 (600-1500)
Spiperone	1200 (870-1600)	870 (450-1800)	560 (440-730)*	500 (320-770)**
Spiroxatrine	550 (240-1300)	69 (34-140)**	71 (39-130)**	91 (48-180)**
Chlorpromazine	600 (430-870)	160 (83-310)***	120 (56-260)***	160 (100-230)***
Atipamezole	2.1 (1.5-3.1)	2.0 (0.99-4.3)	1.2 (0.95-1.5)	1.1 (0.77-1.5)
Idazoxan	22 (15-31)	15 (2.7-45)	17 (13-24)	13 (8.3-22)
Clozapine	89 (54-150)	67 (32-150)	34 (17-67)	51 (35-77)
Rauwolscine	1.8 (0.78-4.5)	4.4 (2.0-9.8)	0.85 (0.40-2.1)	1.6 (0.82-3.1)

	Human α_{2B}		
Ligand	*WT (BBB)*	*AAB*	*CCB*
ARC239	150 (40-570)♦♦♦	240 (120-480)	98 (48-220)
Prazosin	47 (17-140)♦♦	200 (120-360)	170 (89-310)
Spiperone	12 (3.7-38)♦♦♦	26 (11-65)	14 (6.0-38)
Spiroxatrine	2.4 (1.2-5.0)♦♦♦	6.8 (1.8-28)	1.6 (0.64-5.9)
Chlorpromazine	43 (20-100)♦♦♦	53 (20-200)	46 (18-120)
Atipamezole	2.7 (0.56-14)	3.0 (1.7-5.6)	1.7 (0.80-4.0)
Idazoxan	24 (18-34)	37 (29-48)	30 (19-51)
Clozapine	12 (5.0-28)♦♦♦	18 (8.7-45)	6.7 (1.9-23)
Rauwolscine	1.1 (0.7-1.8)	1.2 (0.66-2.4)	2.7 (1.1-6.9)

	Human α_{2C}			
Ligand	*WT (CCC)*	*AAC*	*BBC*	*CAC*
ARC239	130 (66-260)♦♦♦	180 (86-380)	*n.d.*	77 (34-180)
Prazosin	45 (21-81)♦♦	110 (50-230)	*n.d.*	120 (55-260)
Spiperone	29 (9.2-81)♦♦♦	26 (10-75)	*n.d.*	39 (18-89)
Spiroxatrine	13 (5.9-28)♦♦♦	12 (5.8-27)	*n.d.*	18 (8.4-31)
Chlorpromazine	260 (150-350)♦♦	260 (110-720)	*n.d.*	230 (130-400)
Atipamezole	4.1 (2.0-9.0)	5.3 (4.8-8.3)	*n.d.*	10 (5.0-23)
Idazoxan	71 (20-251)	48 (21-110)	*n.d.*	18 (12-34)
Clozapine	6.5 (2.5-18)♦♦♦	5.1 (2.6-8.2)	*n.d.*	1.8 (0.2-17)
Rauwolscine	0.47 (0.21-1.2)	0.27 (0.12-0.63)	*n.d.*	0.39 (0.20-0.83)

Results are expressed as an apparent K_i (nM) and 95 % confidence intervals of three to six independent experiments; *n.d.* (not determined). Receptors are named based on the exchanged domains (see Figure 14). Statistical significances are shown with symbols (one) $P<0.05$; (two) $P<0.01$; (three) $P<0.001$. (*) chimaera *versus* wild-type α_{2A}; (♦) receptor wild-type *versus* wild-type α_{2A}.

6 DISCUSSION

6.1 Structural and pharmacological comparison of the orthologous α_2-adrenoceptors

The ligand binding characteristics of the mammalian α_2-adrenoceptor orthologues were known to be very similar (see *e.g.* Uhlèn *et al.*, 1998). This can be considered as another reflection of the highly conserved structure of the α_2-adrenoceptors among various mammalian species. The zebrafish is a teleost fish and, though only distantly related to mammals, has recently gained increasing interest as a model organism to study development and genetics, since many mutations in its genes cause phenotypes reminiscent of human diseases (Patton and Zon, 2001, Golling *et al.*, 2002, Shin and Fishman, 2002). Three genes coding for orthologues of the mammalian α_2-adrenoceptors and two genes coding for an additional duplicated α_2-adrenoceptor subtype have been cloned from zebrafish (Ruuskanen *et al.*, 2004). Although ~350 million years of evolution separate mammalian and zebrafish α_2-adrenoceptor genes from each other (Shin and Fishman, 2002, Ruuskanen *et al.*, 2004), the ligand binding characteristics of the human and zebrafish α_2-adrenoceptors were found to be surprisingly similar. This pharmacological conservation is perhaps constrained by evolution as all mammalian as well as zebrafish α_2-adrenoceptors recognise, bind and are activated *in vivo* by the same natural ligands, adrenaline and noradrenaline. However, there is no obvious reason why the binding affinities of synthetic ligands should also be similarly conserved, unless all ligands bind to the receptors in a similar mode. One explanation may be that the ligands examined were chosen mainly based on their affinity differences between the human α_2-adrenoceptor subtypes. Differences between receptor orthologues could have emerged had a broader range of ligands been used.

The similarity of the pharmacological profiles of the orthologous α_2-adrenoceptors was not totally unexpected, if one compares the model structures of the three human and five zebrafish α_2-adrenoceptors with each other. The model comparison revealed that only six of thirty-two amino acid residues vary within the proposed binding cavity (Figure 6 in I). The duplicated zebrafish α_{2D}-adrenoceptors are most similar with each other, having only a single conservative exchange (at position xl2.52) within the modelled binding cavity. In the receptor orthologues, three differences (at positions xl2.49, xl2.51 and 5.39) are present between the α_{2A}-adrenoceptors of human and zebrafish. For α_{2B}-adrenoceptors, only two residues (at positions xl2.51 and 5.43) are different between the human and zebrafish orthologues. The α_{2C}-adrenoceptor orthologues were found to be most divergent, having four variable residues (at positions 2.57, xl2.49, xl2.51 and 5.43) in the proposed binding site. The overall binding cavity of α_2-adrenoceptors seems to be evolutionarily strictly conserved as it is quite hydrophobic, with one-third of the side chains being aromatic and containing two polar regions in TM3 and TM5, important for the binding of small catecholic ligands.

In terms of pharmacology, the human and zebrafish α_2-adrenoceptors were clustered based on the binding affinities of 20 chemically divergent ligands using the

binary tree approach and PCA (Figure 2 in I). Here, the duplicated zebrafish α_{2D}-adrenoceptors clustered together, as did also the human and zebrafish α_{2A}-adrenoceptor orthologues. With respect to the α_{2B}-adrenoceptors, the pharmacology of the human receptor resembled more the characteristics of the duplicated zebrafish α_{2D}-adrenoceptor than those of its zebrafish α_{2B}-orthologue, although more amino acids (4-5) are variable between the human α_{2B}-adrenoceptor and the zebrafish α_{2D}-adrenoceptors than between the α_{2B}-orthologues, where only two amino acids differ. The α_{2C}-adrenoceptor orthologues appeared to be no more similar with each other than with the other human and zebrafish α_2-adrenoceptors. Obviously, as also evidenced here, clustering of receptors according to their ligand binding or other pharmacological properties is neither a sufficient nor a conclusive way to classify receptor subtypes, as compared with molecular phylogeny analyses. This is to some extent obvious as a small number of amino acid replacements may largely or totally explain the observed pharmacological differences. Therefore, caution must be exercised when conducting receptor subtype classifications according to pharmacological observations.

In spite of the generally conserved pharmacology of the human and zebrafish α_2-adrenoceptors, some differences have been observed in the binding affinities of certain compounds at orthologous α_2-adrenoceptors. The best-known pharmacological difference characterized for orthologous α_2-adrenoceptors is the binding affinity difference of yohimbine and rauwolscine towards α_{2A}-adrenoceptors from various species (Table 14). Mouse, rat, guinea-pig and bovine α_{2A}-adrenoceptors bind rauwolscine and yohimbine with clearly lower affinity in comparison to the orthologous receptors of human, pig, rabbit, chicken and zebrafish (Bylund *et al.*, 1988, Link *et al.*, 1992, O'Rourke *et al.*, 1994b, Svensson *et al.*, 1996, Uhlèn *et al.*, 1998, Naselsky *et al.*, 2001). Indeed, based on these pharmacological differences, the rodent α_{2A}-adrenoceptor was at one time misnamed as a fourth subtype, the α_{2D}-adrenoceptor (Simonneaux *et al.*, 1991). As rodents are frequently used as animal models in pharmaceutical development, it is extremely important to understand, also at the molecular level, the origin of their pharmacological differences from humans.

Table 14. Pharmacological comparison of α_{2A}-adrenoceptors from various animal species. K_i values (nM) derived from radioligand binding experiments.

	Human[(a)]	*Zebrafish*[(b)]	*Pig*	*Rabbit*	*Chicken*	*Mouse*[(a)]	*Rat*	*Bovine*
ARC239	1800	1800	365	100 000	133	500	749	352
Rauwolscine	1.1	1	0.37	11.3	0.25	22	19	9.5
Ratio	**1636**	**1800**	**986**	**8850**	**605**	**23**	**39**	**37**

Adapted from Bylund, 2005. [(a)] Derived from Study II; [(b)] Derived from Study I

Structural models of the α_{2A}-adrenoceptors from eight animal species revealed that most of the amino acid variability within the predicted binding cavity is located along XL2, while in the TM domains, only four amino acids (1.39, 3.33, 5.39 and 5.43) differed among the α_{2A}-orthologues (Figure 16). Of these, the serine-cysteine difference at position 5.43 was previously shown to account for some of the variation in yohimbine and rauwolscine binding affinities between the human and mouse α_{2A}-adrenoceptors (Link *et al.*, 1992, Cockcroft *et al.*, 2000). Across the entire species comparison, the presence of a cysteine or a serine at position 5.43 correlated with the receptor's affinity

for yohimbine, with serine being linked to lower affinity and cysteine to higher affinity. However, the chicken receptor represents an exception to this rule, as it has a serine at position 5.43 but a pharmacological profile similar to the human receptor, suggesting that also other regions of the binding site may have effects on yohimbine binding. Indeed, the chicken receptor differs from the mouse receptor within XL2 at several positions, as one amino acid downstream of xl2.49 is deleted in the chicken receptor, and glycine is found at xl2.49 whereas serine is present in the mouse receptor. This suggests that XL2 may also contribute to the binding of yohimbine at the chicken α_{2A}-adrenoceptor.

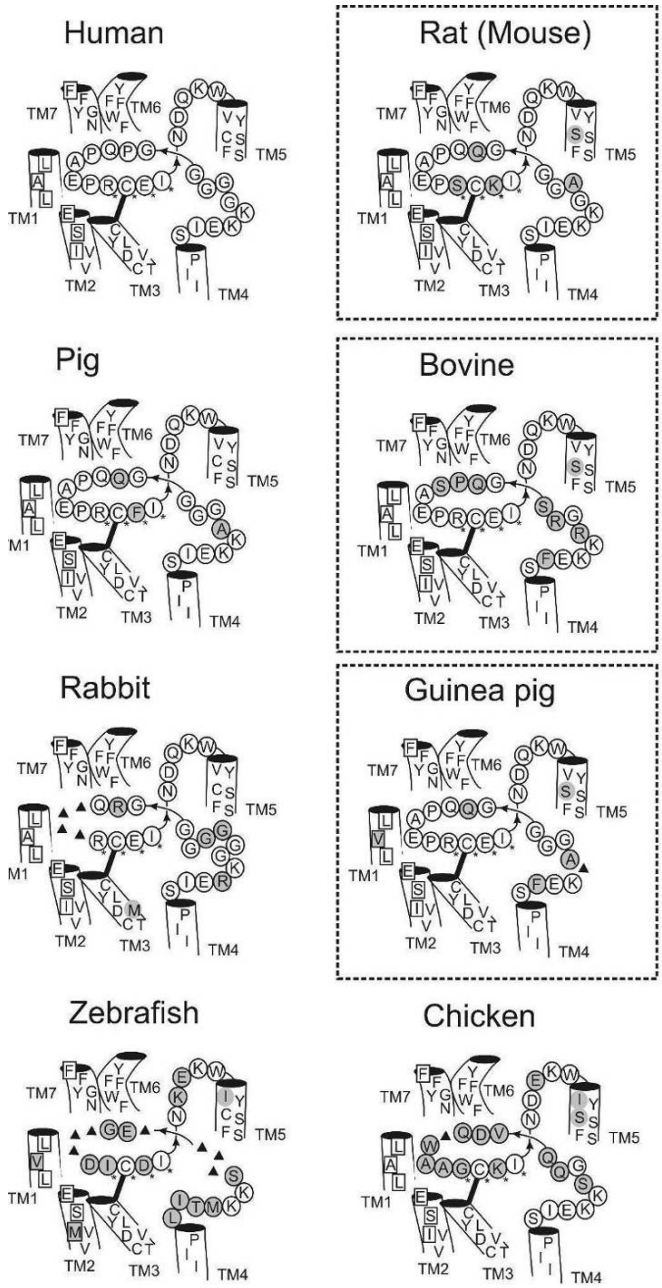


Figure 16. Schematic comparison of the α_{2A}-adrenoceptors from several species. Residues different from the human receptor are indicated with gray. Amino acid deletions are shown as triangles. Receptors with "mouse-like" low affinity for yohimbine are boxed. The residues in XL2 believed to face the binding cavity are indicated with stars.

Using site-directed mutagenesis targeted to residues at positions xl2.49 and xl2.51 of the human and mouse α_{2A}-adrenoceptors, we found that these positions influence the binding preference of yohimbine and rauwolscine of the human *versus* mouse α_{2A}-adrenoceptors. Reciprocal mutations for arginine/serine at xl2.49 and glutamate/lysine at xl2.51, as well as cysteine/serine at position 5.43 reversed the binding profiles of the human and mouse α_{2A}-adrenoceptors for yohimbine, rauwolscine and RS-79948-197, pointing to a role of XL2 in the determination of species-specific ligand binding profiles for yohimbine-like compounds. Nonetheless, the amino acid at position 5.43 is still believed to be a major determinant of the binding profile, as the human, rabbit, bovine and guinea-pig receptors are identical at xl2.49 and xl2.51 but have different pharmacological profiles. Of these, the bovine and guinea-pig receptors have a serine at position 5.43 and show mouse-like pharmacology, while the human and rabbit receptors have a cysteine at 5.43 and show human-like pharmacology. Yohimbine binding affinity thus appears to segregate only according to the residue present at position 5.43. However, the similarity of the residues at xl2.49 and xl2.51 cannot be interpreted as evidence that these amino acids are not involved in mediating the interactions also in these receptors.

In addition to the differences in XL2 and at position 5.43, some other amino acids facing the ligand binding cavity in the TM regions were also found to differ between the α_{2A}-orthologues. For example, the chicken and zebrafish receptors have an isoleucine (I) at position 5.39 instead of a valine (V) that is present in the other species, the rabbit receptor has a methionine (M) at position 3.33 instead of a valine in the others and the guinea-pig and zebrafish receptors have a valine at position 1.39 instead of an alanine (A). These differences may to some extent also contribute to the binding profiles of the receptors towards different ligands, but so far this has not been experimentally investigated.

6.2 The second extracellular loop forms part of the ligand binding site in α_2-adrenoceptors

Residues in the TM domains are generally held responsible for ligand binding in adrenoceptors, as they contain the main contact sites involved in the binding of the endogenous catecholamines, adrenaline and noradrenaline. However, with the recent appearance of crystal structures of β-adrenoceptors it has been established that the orthosteric ligand binding site of adrenoceptors extends from the conserved TM core to the extracellular surface where the extracellular regions form the top of the binding site (Cherezov *et al.*, 2007, Rasmussen *et al.*, 2007, Warne *et al.*, 2008). In β-adrenoceptors as well as in other crystal structures of GPCRs, the conserved cysteine at xl2.50 is attached with a disulphide bond to another cysteine, C3.25 in TM3, constraining the position of XL2 above the ligand binding site. As these cysteines are highly conserved among rhodopsin-like GPCRs, it is very likely that XL2 is folded similarly also in α_2-adrenoceptors. This would make the positions in the vicinity of the conserved Cxl2.50 exposed to the binding cavity and allow their involvement in ligand binding. In addition, the sequence alignment comparisons of orthologous and paralogous α_2-adrenoceptors (see *e.g.* Supplementary material in Ruuskanen *et al.*, 2004) support the

concept that not only the TM domains but also XL2 is conserved during evolution, as in many species a polar asparagine (D) is present at position xl2.53 and other polar residues such as arginine (R), glutamate (E), lysine (K) and glutamine (Q) are often found at xl2.49 and xl2.51, pointing to a functional role for this domain. Previously, in other monoamine receptors closely related to α_2-adrenoceptors, residues in XL2 have been implicated in ligand binding *e.g.* in the α_1-adrenoceptor subtypes A and B, where substitutions at xl2.51-xl2.53 affected the subtype-selectivity of phentolamine and WB4101 (Zhao *et al.*, 1996). In the dopamine D2 receptor, point mutations of xl2.49 and xl2.51-xl2.52 reduced the binding affinity of *N*-methylspiperone (Shi and Javitch, 2004). In the type-1D serotonin receptor, replacements in XL2 affected the binding of ketanserin (Wurch *et al.*, 1998, Wurch and Pauwels, 2000). As a follow-up to these studies, results from our xl2.49- and xl2.51-substituted human and mouse α_{2A}-adrenoceptors provided the first evidence that XL2 is involved in ligand binding also in α_2-adrenoceptors. Molecular models, based on either bovine rhodopsin or the human β_2-adrenoceptor, suggested that the ligand binding cavity of α_2-adrenoceptors is elongated and oriented horizontally with respect to the cell membrane, with XL2 folded above the binding cavity and forming part of the binding site, although the entire three-dimensional structure is likely to be more similar to the β-adrenoceptor structures than to rhodopsin. In the β-adrenoceptor structures (Cherezov *et al.*, 2007, Warne *et al.*, 2008), XL2 forms a hairpin that contains an α-helical segment and a second internal disulphide bridge that is not present in the α_2-adrenoceptor subtypes or in other crystal structures of GPCRs. Again in contrast to rhodopsin, in the β-adrenoceptor structures XL2 is located clearly deeper towards the centre of the binding cavity and is shifted away from TM5 towards TM1. As a consequence of this shift, in β_2-adrenoceptor-based receptor models xl2.49 is less exposed to the binding cavity than in rhodopsin-based models, suggesting that there is a relatively bigger impact for xl2.51 on binding affinity changes observed for the human and mouse α_{2A}-adrenoceptors. On the other hand, this shift also allows position xl2.54 to contribute to the binding cavity surface. However, an aspartic acid (D) at this position is rather conserved among α_{2A}-adrenoceptor orthologues as the zebrafish receptor makes the only exception to this by having a lysine (K) at xl2.54. Therefore this position cannot account for the interspecies differences in the binding of yohimbine.

Most available experimental evidence suggests that antagonists bind at least in part within the same orthosteric binding pocket where agonists bind in α_2-adrenoceptors, and that ligands containing a positively charged amine moiety form a direct ion-pair interaction with the conserved aspartic acid (D3.32) in TM3 (Frang *et al.*, 2001, Shi and Javitch, 2002). However, when yohimbine, rauwolscine and RS-79948-197 were docked into the orthosteric pocket of the human α_{2A}-adrenoceptor model, direct interactions with the amino acid residues at 5.43, xl2.49 and xl2.51 as well as F7.39 (Suryanarayana *et al.*, 1991) were possible only when the compounds were placed close to TM5, where direct ionic interactions with D3.32 became unlikely (Figure 17). This different binding mode of antagonists was also supported by other docking results when twelve antagonist ligands were automatically docked to rhodopsin-based α_{2A}-adrenoceptor models (see Xhaard *et al.,* 2005). Here, ion-pair formation with D3.32 would have required deviation of the models from the template structure, *e.g.* a rotation of TM3 to relocate D3.32 more centrally within the binding cavity, and creation of new

space near TM2/TM7 so that atoms of the antagonist ligands would not overlap with TM5. Thus, it was postulated that many antagonist ligands, though not the quinazolines, are unlikely to form ion-pairs with D3.32 (Xhaard *et al.,* 2005). An alternative binding mode for these antagonists was proposed, whereby the positively charged nitrogen of the antagonist ligands is stabilized by cation-π interactions with aromatic residues (*e.g.*, F6.51), and the antagonists simultaneously interact with D3.32 *via* carboxylate-aromatic interactions. In this proposal, position 5.43 may provide a better anchoring point for antagonist binding than D3.32. In addition, this hypothesis is also supported by the impairment of ligand binding seen for receptors mutated at D3.32 (Ho *et al.*, 1992, Porter *et al.*, 1996). Although the assumption of different binding modes for antagonists would enable better contact with XL2, which is in good agreement with the experimental results obtained for yohimbine, rauwolscine and RS-79948-197, this cannot be generalized to all antagonist ligands, *e.g.* as seen for MK-912. When this compound was docked on top of yohimbine, equally close to TM5, the ligand interfered with the receptor structure, especially with W6.48 in TM6, evidence of different binding modes for MK-912 and yohimbine (Figure 17d). This is consistent with the present experimental results, where the binding of MK-912 was affected neither by mutations at 5.43 nor at xl2.49/xl2.51.

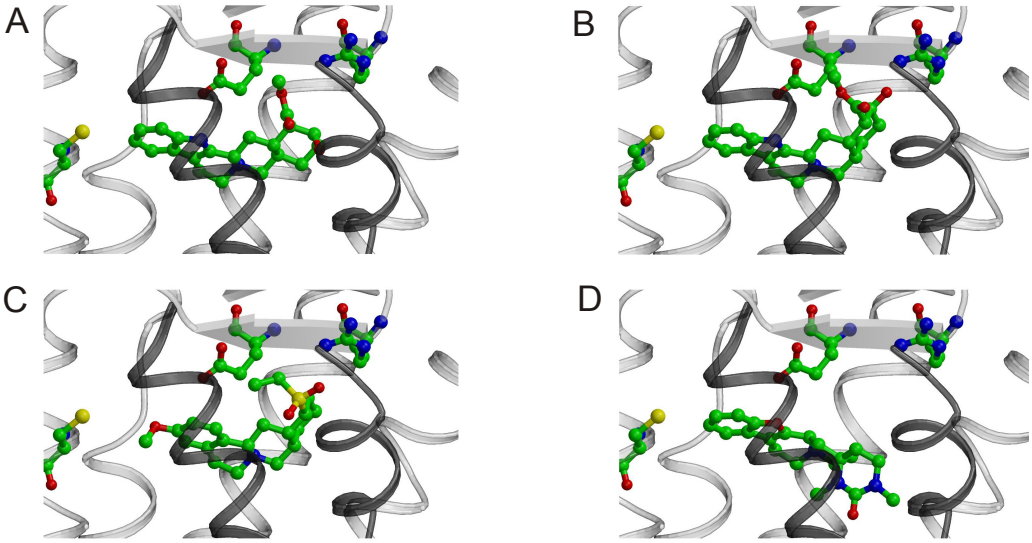


Figure 17. Close-up view of (A) yohimbine, (B) rauwolscine, (C) RS-79948-97 and (D) MK-912 docked into the molecular model of the human α_{2A}-adrenoceptor. TM6 and TM7 helices are shown in dark. Three residues found to affect the binding of yohimbine, rauwolscine and RS-79948-197 are shown; cysteine 5.43 (left of the ligand), glutamate xl2.51 (left above the ligand) and arginine xl2.49 (right above the ligand). Modified from paper II.

XL2 is thus involved in ligand binding to α_2-adrenoceptors and many other types of GPCRs. For drug discovery and development, XL2 is an ideal target, also for α_2-adrenoceptors, as it is highly variable in contrast to the TM domains. Due to the constrained position of XL2, especially the amino acids in the vicinity of the conserved cysteine at xl2.50 are likely to face the orthosteric binding site. In the human α_2-

adrenoceptor subtypes, the amino acids at these positions are rather variable: [R/Q/Q]xl2.49 ([human α_{2A}/human α_{2B}/human α_{2C}]), [E/K/G]xl2.51 and xl2.52 [I/L/L]. One would expect these differences to have at least some influence on the binding properties of certain ligands, especially antagonists. However, the experimental results obtained with single- and double-mutated human α_{2A}- and α_{2C}-adrenoceptors at positions xl2.49 and xl2.51 showed only marginal effects on the binding affinity of 19 ligands (unpublished results). The other positions along XL2 suggested to be exposed in the binding site, *e.g.* xl2.52 [I/L/L] and xl2.54 [D/Q/D], have so far not been evaluated experimentally.

In addition to ligand binding, XL2 was recently proposed to have a functional role in receptor activation. In the β_2-adrenoceptor structure, the carboxyl group of the polar Dxl2.51 forms a salt bridge with a positively charged lysine, K7.32, and ligands may have allosteric effects by influencing this interaction, as an agonist, an antagonist and an inverse agonist were found to stabilise distinct conformations of the extracellular loops (Bokoch *et al.*, 2010). For the α_{1B}-adrenoceptor, mutations at position 7.32 were also shown to affect the binding affinity of noradrenaline and adrenaline, evidence for the importance of this ion-pair interaction between XL2 and TM7 for the resting receptor conformation (Porter *et al.*, 1996). If this salt bridge exists also in α_2-adrenoceptors, it may have a functional role in regulation of receptor conformations and in addition be able to influence ligand binding. The corresponding amino acids are [R/H/G]7.32 and [E/K/G]xl2.51 in the human α_2-adrenoceptors, which would make an analogous salt bridge possible in the α_{2A}-subtype, but not in the α_{2B}- and α_{2C}-subtypes.

6.3 Indirect effects may influence pharmacological profiles

It is tempting to assume that an experimentally observed effect on ligand binding to a mutated receptor is a direct consequence of changed atomic contacts between the ligand and the amino acids exposed in the binding cavity of the receptor. However, a mutation may cause many indirect effects on the receptor's structure, *e.g.* affect protein folding, alter its post-translational modifications, or disrupt its cell surface expression (Shi and Javitch, 2002). Even the presence of a mutant receptor in the plasma membrane is not evidence of normal protein folding, as structural rearrangements do not always lead to intracellular retention. Mutations may also change side-chain volumes, charge distributions, hydrophobicity and other physico-chemical properties of the receptor which may indirectly affect its normal folding. Therefore, it can be extremely difficult to differentiate between the direct and indirect effects of mutations on receptor function.

The process of ligand binding is more dynamic than was considered in the era of classical receptor pharmacology, and the binding affinity is determined by more factors than the direct interactions of the ligand and the receptor. Even the dynamic flexibility of different segments of the receptor protein may have effects on ligand recognition. In addition, there are many examples of positive and negative allosteric modulators that can regulate binding affinity (Milligan and Smith, 2007). The presence of charged groups even outside of the binding pocket may affect the protonation state of compounds and in that way indirectly influence their binding to the receptor. Some

residues facing away from the binding cavity can undergo ionic interactions with other residues of the receptor, and ligands may have allosteric effects through influences on these interactions, as was recently reported for the β_2-adrenoceptor where Dxl2.51 forms a salt bridge with K7.32 (Bokoch *et al.*, 2010). It is actually possible that the effects of the present XL2 mutations on the binding of yohimbine analogues at human and mouse α_{2A}-adrenoceptors may be mediated through this ionic interaction instead of direct interactions between ligand and receptor. Position 7.32 in TM7 of the α_2-adrenoceptors has so far not been subjected to mutagenesis experiments, but this would be highly interesting from this point of view. Another example of allosteric effects on ligand binding is that mutations at position 2.50 of the α_{2A}-adrenoceptor lead to decreased agonist binding affinity, although this position is relatively far from the orthosteric agonist binding site (Ceresa and Limbird, 1994). In monoamine-binding GPCRs, the aspartate at 2.50 has been shown to form a sodium-binding site that plays a role in receptor activation and thus allows allosteric modulation of the receptor-ligand interaction (Neve *et al.*, 2001).

The competition binding results obtained with the chimaeric human α_2-adrenoceptors pointed to the involvement of TM1 in defining the specific pharmacological profile of the human α_{2A}-adrenoceptor for antagonists with a large molecular structure. However, molecular models indicated that TM1 has very limited exposure in the binding pocket, as it is distant from the orthosteric binding cavity. Actually, in the β_2-adrenoceptor-based α_2-adrenoceptor models, the distance of TM1 from TM5 and the orthosteric binding site is even longer than in rhodopsin-based models (26.5 Å *vs.* 23.5 Å, respectively) (see Figure 3 in III). Furthermore, access to TM1 is blocked by side-chain atoms from TM2 and TM7 (see Figure 4 in III). Thus, indirect effects are more likely to be involved rather than direct receptor-ligand interactions. It may be too speculative to try to define the exact mechanisms of such indirect effects, especially as no reciprocal effects were observed in the α_{2B}- and α_{2C}-adrenoceptor-based chimaeras. In addition to this study, there are also some other previous investigations on α_2-adrenoceptors where indirect effects could be involved rather than specific side-chain interactions. For example, substitution of five amino acids in the third cytoplasmic loop of the rat α_{2A}-adrenoceptor was shown to affect the binding affinity of several ligands including *e.g.* oxymetazoline and prazosin (Venkataraman *et al.*, 1997). Mutations of the serines at positions 2.61 in TM2 and 7.46 in TM7 reduced the stereoselective binding of the (-)-enantiomers of catecholamine agonists to the α_{2A}-adrenoceptor (Hehr *et al.*, 1997, Hieble *et al.*, 1998). It is possible that some of these findings may be explained by effects related to the charge distribution or overall shape of the binding pocket.

Although the recent crystal structures of some GPCRs have very significantly improved our understanding of structural determinants important for ligand binding, they have not so far produced real breakthroughs in GPCR drug discovery. This could be because these structures are static "snapshots" of receptor structure, and indeed, all of them represent inactive receptor conformations as they were determined while the receptor was complexed with antagonist ligands (the human β_2-adrenoceptor with timolol (Hanson *et al.*, 2008); the turkey β_1-adrenoceptor with cyanopindolol (Warne *et al.*, 2008); the human A_{2A} adenosine receptor with ZM241385 (Jaakola *et al.*, 2008); the human dopamine D3 receptor with eticlopride (Chien *et al.*, 2010); the CXCR4

chemokine receptor with IT1t (Wu *et al.*, 2010)) or inverse agonists (the human β_2-adrenoceptor with carazolol (Cherezov *et al.*, 2007, Rasmussen *et al.*, 2007, Rosenbaum *et al.*, 2007) and bovine rhodopsin covalently bound to 11-*cis* retinal (Palczewski *et al.*, 2000, Okada *et al.*, 2004)). There is no doubt that these structures are very useful for *in silico* drug design, but the need for additional structures, especially structures in an active state, is apparent. In addition, receptor structures in complex with other receptors (heterodimers) as well as structures which are linked with signaling molecules like G-proteins are needed for structure-based drug design. A good example of this is a recent molecular docking study where the human β_2-adrenoceptor crystal structure was used as the target and six new β_2-adrenoceptor-selective ligands were identified, all of them exhibiting inverse agonist activity (Kolb *et al.*, 2009). Therefore, when designing new compounds that should at the same time be selective for a certain receptor subtype and in addition have the desired functional activity, any information on the structural determinants of ligand binding, also including allosteric effects and other indirect effects, should be considered as potentially valuable.

7 CONCLUSIONS

The present ligand binding studies in combination with site-directed mutagenesis, molecular modelling and docking simulations of α_2-adrenoceptors indicated structural determinants that contribute to specific ligand binding and recognition at α_2-adrenoceptor subtypes from different animal species. These findings will hopefully promote the design and development of new α_2-adrenoceptor selective drug. The main results and conclusions were:

1. The human and zebrafish α_2-adrenoceptors have surprisingly similar ligand binding profiles in spite of their long evolutionary distance. Comparisons of the structural models of the human and zebrafish α_2-adrenoceptors indicate that amino acid differences especially in XL2 may contribute to the observed differences in ligand binding affinity.

2. Site-directed mutagenesis of the amino acids at positions 5.43, xl2.49 and xl2.51 reversed the binding profiles of the human and mouse α_{2A}-adrenoceptors for yohimbine, rauwolscine and RS-79948-197, pointing to a role of XL2 in the determination of species-specific ligand binding profiles.

3. Chimaeric α_2-adrenoceptors where TM1 was exchanged between the human α_2-adrenoceptor subtypes revealed that TM1 is involved in defining the specific pharmacological profile of the human α_{2A}-adrenoceptor. The binding affinities of three antagonists (spiperone, spiroxatrine and chlorpromazine) were significantly improved by TM1 substitutions in the α_{2A}-adrenoceptor. TM1 may thus influence the pharmacological profiles of the human α_2-adrenoceptors, but the observed effects are believed to be indirect rather than mediated by direct orthosteric receptor contacts.

ACKNOWLEDGEMENTS

This thesis work was carried out in the Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku. I want to express my gratitude to the Heads of the Department, Professors Mika Scheinin, Risto Huupponen, Markku Koulu and Erkka Syvälahti for the opportunity to work in such an excellent research facility.

I owe my deepest gratitude to Professor Mika Scheinin who has been the principal supervisor of my thesis work. I will never stop admiring his wisdom, calm and endless interest towards all kinds of knowledge, whether related to scientific or non-scientific issues. Mika has managed to keep me motivated throughout the project by encouraging me, especially during the hard times. His patience, optimistic attitude and relatively blind trust have been the elements that have carried this thesis to completion. I am grateful to Mika for many reasons, including the opportunity to visit Professor Marc Caron's famous research laboratory in Duke University in the summer of 2002 and to collaborate with several esteemed colleagues as well as for funding support, and his guidance in science as well as in different teaching tasks. I really feel privileged to have worked with him and to have learned to know him.

The Drug Discovery Graduate School (DDGS) and its coordinator Eeva Valve are acknowledged for providing financial support and arranging several travel opportunities as well as the chance to interact with many other young scientists.

I was very fortunate to have Professor Mark Johnson and his research group close to me. I thank Mark for his valuable direct contributions to the work and for his role as a member of my supervisory committee. It is a fact that without the Structural Bioinformatics Laboratory and the facilities and knowledge that Mark and his group have provided, this work would never have been completed.

The reviewers of this thesis, Docent Jarmo T. Laitinen and Docent Ulla Petäjä-Repo, are acknowledged for their valuable comments and constructive criticism. I wish to thank them for significantly improving the contents of this book. Dr. Ewen MacDonald is acknowledged for revision of the language of this thesis and for his encouraging comments.

My special thanks belong to Henri Xhaard and Jori Ruuskanen who have been my closest fellows from the beginning of my thesis studies. Henri, your contribution to all papers included in this thesis as well as the comments related to all kinds of issues in structural biology have been extremely valuable and didactic. It has been amazing to see how you zoom into the middle of a receptor and see what's here and there and what is actually relevant. Jori, your contribution and guidance in molecular biology and help in the planning of the binding experiments have been very important, especially when I first joined the laboratory. I also want to thank Jori for his good companionship while we were attending several international meetings as well as for being a member of my thesis committee.

All my other co-authors, Gloria Wissel, Matias Rantanen, Henry Karlsson, Ville-Veikko Rantanen, Minna Vainio, Anne Marjamäki, Karoliina Vuoriluoto, Siegfried Wurster and Tiina Salminen, are acknowledged for their valuable contributions to the

articles included in this thesis. Gloria, your input in the last sub-project was essential in getting the paper finally submitted for publication.

All fellows and members of the α_2-group, past and present, are acknowledged. This huge team with diverse backgrounds has offered a really multi-disciplinary template to create a worldwide collaboration network. It has been great to be able to work with you: Erik Aro, Susann Björk, Mia Engström, Veronica Fagerholm, Heini Frang, Huifang Ge, Paula Heinonen, Maya Holmberg, Anna Huhtinen, Maija Ivaska, Jaana Kallio, Eeva Kronqvist, Hanna Laine, Ilkka Lähdesmäki, Janne Lähdesmäki, Marja Nykänen, Tuire Olli-Lähdesmäki, Katariina Pohjanoksa, Jussi Posti, Hanna-Mari Raussi, Saku Ruohonen, Suvi Ruohonen, Jukka Sallinen, Amir Snapir, Mona Suikkari, Tomi Streng, Raisa Vuorinen, Ümit Özdogan, and all others I may not remember. Finally, from the α_2-group, I want to express my special thanks to Ms. Ulla Uoti, "mum" of the α_2-group, for all her care, enormous help and skilful technical assistance in the lab.

A big thanks to all the people in Farmis over the years. It is too difficult to remember you all as a list here, but you know who I mean. I want to express my special thanks to Ms. Anja Similä, Ms. Hanna Tuominen and Ms. Ulla Hurme for their patience and valuable secretarial assistance in office matters related to teaching tasks or administrative concerns. It has also been a pleasure to share my office with Sanna Tikka. Our unofficial talks about life and events outside of the lab and work have been a reason to come to work on many a Monday morning.

I want to thank all members of the Health Biosciences class of '98 at the University of Turku: Anna, Anna-Stiina, Anne, Hanna, Jenni B., Jenni V., Jonna, Jukka, Karoliina, Liisa, Mari, Matias, Niina, Riikka, Saku, Tiina, and Tuire. The spirit of our class has been indescribable. I am glad that this collegial attitude is still so strongly alive after the M.Sc. studies. I wish all of you success in the future.

I would also like to thank my best friends outside of the academic world, Toni, Risto and Pasi for your indispensable support and patience in listening when I have started to talk about things about which you could have no idea, and Antti for accommodating me and giving me a home several times during my research or other visits to Helsinki.

Dear mother, Arja Laurila, I want to thank you for the love and constant support that you have given to me during my life. You have always been present as a tower of strength when I have had difficulties. Your help in baby-sitting as well as in many practical things have made my life much easier in many ways. My siblings, Tatu, Anna and Lotta, I am happy that we have such close relationships, where all happiness and sorrows are shared. I want to thank you for your support and help in my life.

Last, but certainly not least, I am deeply grateful to the most important person in my life, my lovely daughter Venla. When I look at you, I really understand what is most important in life.

This thesis was financially supported by DDGS, the Finnish Cultural Foundation, the Finnish Society of Science and Letters, the Turku University Foundation, the Orion-Farmos Research Foundation, the Ida Montin Foundation and the Finnish-Norwegian Medical Foundation.

Turku, April 2011

Jonne Laurila

REFERENCES

Aantaa R, Jalonen J (2006). Perioperative use of α_2-adrenoceptor agonists and the cardiac patient. *Eur J Anaesthesiol.* **23:** 361-72.

Ahlquist RP (1948). A study of the adrenotropic receptors. *Am J Physiol.* **153:** 586-600.

Altenbach C, Greenhalgh DA, Khorana HG and Hubbell WL (1994). A collision gradient method to determine the immersion depth of nitroxides in lipid bilayers: Application to spin-labeled mutants of bacteriorhodopsin. *Proc Natl Acad Sci U S A.* **91:** 1667-71.

Altman JD, Trendelenburg AU, MacMillan L, Bernstein D, Limbird L, Starke K, *et al.* (1999). Abnormal regulation of the sympathetic nervous system in α_{2A}-adrenergic receptor knockout mice. *Mol Pharmacol.* **56:** 154-61.

Andersson KE, Stief C (2001). Oral α- adrenoceptor blockade as a treatment of erectile dysfunction. *World J Urol.* **19:** 9-13.

Angelotti T, Daunt D, Shcherbakova OG, Kobilka B and Hurt CM (2010). Regulation of G-protein coupled receptor traffic by an evolutionary conserved hydrophobic signal. *Traffic.* **11:** 560-78.

Arcangeli A, D'Alo C and Gaspari R (2009). Dexmedetomidine use in general anaesthesia. *Curr Drug Targets.* **10:** 687-95.

Arima J, Kubo C, Ishibashi H and Akaike N (1998). α_2-Adrenoceptor-mediated potassium currents in acutely dissociated rat locus coeruleus neurones. *J Physiol.* **508:** 57-66.

Attwood TK, Findlay JB (1994). Fingerprinting G-protein-coupled receptors. *Protein Eng.* **7:** 195-203.

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.

Balogh B, Szilagyi A, Gyires K, Bylund DB and Matyus P (2009). Molecular modelling of subtypes (α_{2A}, α_{2B} and α_{2C}) of α_2-adrenoceptors: A comparative study. *Neurochem Int.* **55:** 355-61.

Bes A, Eyssette M, Pierrot-Deseilligny E, Rohmer F and Warter JM (1988). A multi-centre, double-blind trial of tizanidine, a new antispastic agent, in spasticity associated with hemiplegia. *Curr Med Res Opin.* **10:** 709-18.

Birnbaumer L (1990). G proteins in signal transduction. *Annu Rev Pharmacol Toxicol.* **30:** 675-705.

Bockaert J, Pin JP (1999). Molecular tinkering of G protein-coupled receptors: An evolutionary success. *EMBO J.* **18:** 1723-9.

Bokoch MP, Zou Y, Rasmussen SG, Liu CW, Nygaard R, Rosenbaum DM, *et al.* (2010). Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature.* **463:** 108-12.

Bousquet P, Feldman J and Schwartz J (1984). Central cardiovascular effects of alpha adrenergic drugs: Differences between catecholamines and imidazolines. *J Pharmacol Exp Ther.* **230:** 232-6.

Boyajian CL, Loughlin SE and Leslie FM (1987). Anatomical evidence for α_2- adrenoceptor heterogeneity: Differential autoradiographic distributions of [^{3}H]rauwolscine and [^{3}H]idazoxan in rat brain. *J Pharmacol Exp Ther.* **241:** 1079-91.

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:** 248-54.

Brasili L, Cassinelli A, Angeli P and Melchiorre C (1986). Thiol group may be involved in the irreversible blockade of presynaptic α_2-adrenoreceptors by pyrextramine and benextramine in the isolated guinea pig ileum. *Life Sci.* **38:** 1633-40.

Brede M, Nagy G, Philipp M, Sorensen JB, Lohse MJ and Hein L (2003). Differential control of adrenal and sympathetic catecholamine release by α_2-adrenoceptor subtypes. *Mol Endocrinol.* **17:** 1640-6.

Bultmann R, Starke K (1993). Chloroethylclonidine: An irreversible agonist at prejunctional α_2-adrenoceptors in rat vas deferens. *Br J Pharmacol.* **108:** 336-41.

Bylund DB (2005). Alpha-2 adrenoceptor subtypes: Are more better? *Br J Pharmacol.* **144:** 159-60.

Bylund DB, Blaxall HS, Iversen LJ, Caron MG, Lefkowitz RJ and Lomasney JW (1992). Pharmacological characteristics of α_2-adrenergic receptors: Comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Mol Pharmacol.* **42:** 1-5.

Bylund DB, Rudeen PK, Petterborg LJ and Ray-Prenger C (1988). Identification of α_2-adrenergic receptors in chicken pineal gland using [^{3}H]rauwolscine. *J Neurochem.* **51:** 81-6.

Bylund DB, Toews ML (1993). Radioligand binding methods: Practical guide and tips. *Am J Physiol.* **265:** 421-9.

Ceresa BP, Limbird LE (1994). Mutation of an aspartate residue highly conserved among G-protein-coupled receptors results in nonreciprocal disruption of α_2-adrenergic receptor-G-protein interactions. A negative charge at amino acid residue 79 forecasts α_{2A}-adrenergic receptor sensitivity to allosteric modulation by monovalent cations and fully

effective receptor/G-protein coupling. *J Biol Chem.* **269:** 29557-64.

Cerione RA, Regan JW, Nakata H, Codina J, Benovic JL, Gierschik P, *et al.* (1986). Functional reconstitution of the α_2-adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J Biol Chem.* **261:** 3901-9.

Chen S, Lin F, Xu M and Graham RM (2002). Phe(303) in TMVI of the α_{1B}-adrenergic receptor is a key residue coupling TM helical movements to G-protein activation. *Biochemistry.* **41:** 588-96.

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:** 3099-108.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, *et al.* (2007). High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. *Science.* **318:** 1258-65.

Chien EY, Liu W, Zhao Q, Katritch V, Han GW, Hanson MA, *et al.* (2010). Structure of the human dopamine D_3 receptor in complex with a D_2/D_3 selective antagonist. *Science.* **330:** 1091-5.

Chotani MA, Mitra S, Su BY, Flavahan S, Eid AH, Clark KR, *et al.* (2004). Regulation of α_2-adrenoceptors in human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol.* **286:** H59-67.

Cockcroft V, Frang H, Pihlavisto M, Marjamäki A and Scheinin M (2000). Ligand recognition of serine-cysteine amino acid exchanges in transmembrane domain 5 of α_2-adrenergic receptors by UK 14,304. *J Neurochem.* **74:** 1705-10.

Cotecchia S, Kobilka BK, Daniel KW, Nolan RD, Lapetina EY, Caron MG, *et al.* (1990). Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J Biol Chem.* **265:** 63-9.

Dardonville C, Rozas I (2004). Imidazoline binding sites and their ligands: An overview of the different chemical structures. *Med Res Rev.* **24:** 639-61.

Daunt DA, Hurt C, Hein L, Kallio J, Feng F and Kobilka BK (1997). Subtype-specific intracellular trafficking of α_2-adrenergic receptors. *Mol Pharmacol.* **51:** 711-20.

Delmas P, Abogadie FC, Milligan G, Buckley NJ and Brown DA (1999). Betagamma dimers derived from G_o and G_i proteins contribute different components of adrenergic inhibition of Ca^{2+} channels in rat sympathetic neurones. *J Physiol.* **518:** 23-36.

Deslauriers B, Ponce C, Lombard C, Larguier R, Bonnafous JC and Marie J (1999). N-glycosylation requirements for the AT_{1a} angiotensin II receptor delivery to the plasma membrane. *Biochem J.* **339:** 397-405.

Deupree JD, Hinton KA, Cerutis DR and Bylund DB (1996). Buffers differentially alter the binding of [^{3}H]rauwolscine and [^{3}H]RX821002 to the α_2-adrenergic receptor subtypes. *J Pharmacol Exp Ther.* **278:** 1215-27.

Devedjian JC, Esclapez F, Denis-Pouxviel C and Paris H (1994). Further characterization of human α_2-adrenoceptor subtypes: [^{3}H]RX821002 binding and definition of additional selective drugs. *Eur J Pharmacol.* **252:** 43-9.

Dixon RA, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, Frielle T, *et al.* (1986). Cloning of the gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. *Nature.* **321:** 75-9.

Dorn GW,2nd, Oswald KJ, McCluskey TS, Kuhel DG and Liggett SB (1997). α_{2A}-Adrenergic receptor stimulated calcium release is transduced by G_i-associated $G_{\beta\gamma}$-mediated activation of phospholipase C. *Biochemistry.* **36:** 6415-23.

Dossin O, Mouledous L, Baudry X, Tafani JA, Mazarguil H and Zajac JM (2000). Characterization of a new radioiodinated probe for the α_{2C}-adrenoceptor in the mouse brain. *Neurochem Int.* **36:** 7-18.

Eason MG, Jacinto MT, Theiss CT and Liggett SB (1994). The palmitoylated cysteine of the cytoplasmic tail of α_{2A}-adrenergic receptors confers subtype-specific agonist-promoted downregulation. *Proc Natl Acad Sci U S A.* **91:** 11178-82.

Eason MG, Kurose H, Holt BD, Raymond JR and Liggett SB (1992). Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects. subtype-selective coupling of α_2C10, α_2C4, and α_2C2 adrenergic receptors to G_i and G_s. *J Biol Chem.* **267:** 15795-801.

Eason MG, Liggett SB (1995). Identification of a G_s coupling domain in the amino terminus of the third intracellular loop of the α_{2A}-adrenergic receptor. evidence for distinct structural determinants that confer G_s versus G_i coupling. *J Biol Chem.* **270:** 24753-60.

Eyssette M, Rohmer F, Serratrice G, Warter JM and Boisson D (1988). Multi-centre, double-blind trial of a novel antispastic agent, tizanidine, in spasticity associated with multiple sclerosis. *Curr Med Res Opin.* **10:** 699-708.

Fagerholm V, Grönroos T, Marjamäki P, Viljanen T, Scheinin M and Haaparanta M (2004). Altered glucose homeostasis in α_{2A}-adrenoceptor knockout mice. *Eur J Pharmacol.* **505:** 243-52.

Ferrè S, Navarro G, Casadò V, Cortès A, Mallol J, Canela EI et al. (2010). G protein-coupled receptor heteromers as new targets for drug development. *Prog Mol Biol Transl Sci.* **91:** 41-52.

Fox SL, Samson CR and Danzig MR (1979). Oxymetazoline in the treatment of allergic and non-infectious conjunctivitis. *J Int Med Res.* **7:** 528-30.

Frang H, Cockcroft V, Karskela T, Scheinin M and Marjamäki A (2001). Phenoxybenzamine binding reveals the helical orientation of the third

transmembrane domain of adrenergic receptors. *J Biol Chem.* **276:** 31279-84.

Fredriksson R, Lagerstrom MC, Lundin LG and Schiöth HB (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol.* **63:** 1256-72.

Freissmuth M, Waldhoer M, Bofill-Cardona E and Nanoff C (1999). G protein antagonists. *Trends Pharmacol Sci.* **20:** 237-45.

Garvey HL, Ling GM and Melville KI (1972). Cardiovascular actions of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) a new alpha-adrenergic receptor antagonist. *Eur J Pharmacol.* **18:** 148-58.

Ge H, Scheinin M and Kallio J (2003). Constitutive precoupling to G_i and increased agonist potency in the α_{2B}-adrenoceptor. *Biochem Biophys Res Commun.* **306:** 959-65.

Gentili F, Ghelfi F, Giannella M, Piergentili A, Pigini M, Quaglia W*, et al.* (2004). α_2-Adrenoreceptors profile modulation. 2. Biphenyline analogues as tools for selective activation of the α_{2C}-subtype. *J Med Chem.* **47:** 6160-73.

Golling G, Amsterdam A, Sun Z, Antonelli M, Maldonado E, Chen W*, et al.* (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat Genet.* **31:** 135-40.

Gongadze NV, Antelava NA and Kezeli TD (2008). Imidazoline receptors. *Georgian Med News.* **(160-161):** 44-7.

Halme M, Sjöholm B, Savola JM and Scheinin M (1995). Recombinant human α_2-adrenoceptor subtypes: Comparison of [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 as radioligands. *Biochim Biophys Acta.* **1266:** 207-14.

Hanson MA, Cherezov V, Griffith MT, Roth CB, Jaakola VP, Chien EY*, et al.* (2008). A specific cholesterol binding site is established by the 2.8 Å structure of the human β_2-adrenergic receptor. *Structure.* **16:** 897-905.

Happe HK, Coulter CL, Gerety ME, Sanders JD, O'Rourke M, Bylund DB*, et al.* (2004). α_2-Adrenergic receptor development in rat CNS: An autoradiographic study. *Neuroscience.* **123:** 167-78.

Hehr A, Hieble JP, Li YO, Bergsma DJ, Swift AM, Ganguly S*, et al.* (1997). Ser165 of transmembrane helix IV is not involved in the interaction of catecholamines with the α_{2A}-adrenoceptor. *Pharmacology.* **55:** 18-24.

Hein L, Limbird LE, Eglen RM and Kobilka BK (1999). Gene substitution/knockout to delineate the role of α_2-adrenoceptor subtypes in mediating central effects of catecholamines and imidazolines. *Ann N Y Acad Sci.* **881:** 265-71.

Heinonen P, Koulu M, Pesonen U, Karvonen MK, Rissanen A, Laakso M*, et al.* (1999). Identification of a three-amino acid deletion in the α_{2B}-adrenergic receptor that is associated with reduced basal metabolic rate in obese subjects. *J Clin Endocrinol Metab.* **84:** 2429-33.

Henderson R, Baldwin JM, Ceska TA, Zemlin F, Beckmann E and Downing KH (1990). Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J Mol Biol.* **213:** 899-929.

Henderson R and Schertler GF (1990). The structure of bacteriorhodopsin and its relevance to the visual opsins and other seven-helix G-protein coupled receptors. *Philos Trans R Soc Lond B Biol Sci,* **326**: 379-89.

Hengstmann JH, Gugler R and Dengler HJ (1979). Malignant pheochromocytoma. effect of oral alpha-methyl-p-tyrosine upon catecholamine metabolism. *Klin Wochenschr.* **57:** 351-5.

Hieble JP, Hehr A, Li YO and Ruffolo RR Jr (1998). Molecular basis for the stereoselective interactions of catecholamines with alpha-adrenoceptors. *Proc West Pharmacol Soc.* **41:** 225-8.

Hildebrand PW, Scheerer P, Park JH, Choe HW, Piechnick R, Ernst OP*, et al.* (2009). A ligand channel through the G protein coupled receptor opsin. *PLoS One.* **4:** e4382.

Ho BY, Karschin A, Branchek T, Davidson N and Lester HA (1992). The role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT_{1A} receptor: A site-directed mutation study. *FEBS Lett.* **312:** 259-62.

Holmberg M, Fagerholm V and Scheinin M (2003). Regional distribution of α_{2C}-adrenoceptors in brain and spinal cord of control mice and transgenic mice overexpressing the α_{2C}-subtype: An autoradiographic study with [^{3}H]RX821002 and [^{3}H]rauwolscine. *Neuroscience.* **117:** 875-98.

Hunter JC, Fontana DJ, Hedley LR, Jasper JR, Lewis R, Link RE*, et al.* (1997). Assessment of the role of α_2-adrenoceptor subtypes in the antinociceptive, sedative and hypothermic action of dexmedetomidine in transgenic mice. *Br J Pharmacol.* **122:** 1339-44.

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR*, et al.* (2008). The 2.6 angstrom crystal structure of a human A_{2A} adenosine receptor bound to an antagonist. *Science.* **322:** 1211-7.

Jaakola VP, Vainio M, Sen S, Rehn M, Heimo H, Scheinin M*, et al.* (2005). Intracellularly truncated human α_{2B}-adrenoceptors: Stable and functional GPCRs for structural studies. *J Recept Signal Transduct Res.* **25:** 99-124.

Janumpalli S, Butler LS, MacMillan LB, Limbird LE and McNamara JO (1998). A point mutation (D79N) of the α_{2A}-adrenergic receptor abolishes the antiepileptogenic action of endogenous norepinephrine. *J Neurosci.* **18:** 2004-8.

Jasper JR, Lesnick JD, Chang LK, Yamanishi SS, Chang TK, Hsu SA*, et al.* (1998). Ligand efficacy and potency at recombinant α_2-adrenergic receptors:

Agonist-mediated [^{35}S]GTPγS binding. *Biochem Pharmacol.* **55:** 1035-43.

Javitch JA, Fu D, Liapakis G and Chen J (1997). Constitutive activation of the beta2 adrenergic receptor alters the orientation of its sixth membrane-spanning segment. *J Biol Chem.* **272:** 18546-9.

Jensen AD, Gether U (2000). Assessing adrenergic receptor conformation using chemically reactive fluorescent probes. *Methods Mol Biol.* **126:** 345-61.

Johnson M (2001). β_2-Adrenoceptors: Mechanisms of action of β_2-agonists. *Paediatr Respir Rev.* **2:** 57-62.

Johnson MS, Overington JP (1993). A structural basis for sequence comparisons. an evaluation of scoring methodologies. *J Mol Biol.* **233:** 716-38.

Jones AM, Assmann SM (2004). Plants: The latest model system for G-protein research. *EMBO Rep.* **5:** 572-8.

Jones G, Willett P, Glen RC, Leach AR and Taylor R (1997). Development and validation of a genetic algorithm for flexible docking. *J Mol Biol.* **267:** 727-48.

Jones SB, Halenda SP and Bylund DB (1991). α_2-Adrenergic receptor stimulation of phospholipase A_2 and of adenylate cyclase in transfected chinese hamster ovary cells is mediated by different mechanisms. *Mol Pharmacol.* **39:** 239-45.

Jordan BA, Gomes I, Rios C, Filipovska J and Devi LA (2003). Functional interactions between μ opioid and α_{2A}-adrenergic receptors. *Mol Pharmacol.* **64:** 1317-24.

Kable JW, Murrin LC and Bylund DB (2000). In vivo gene modification elucidates subtype-specific functions of α_2-adrenergic receptors. *J Pharmacol Exp Ther.* **293:** 1-7.

Keefer JR, Kennedy ME and Limbird LE (1994). Unique structural features important for stabilization versus polarization of the α_{2A}-adrenergic receptor on the basolateral membrane of Madin-Darby canine kidney cells. *J Biol Chem.* **269:** 16425-32.

Keefer JR, Limbird LE (1993). The α_{2A}-adrenergic receptor is targeted directly to the basolateral membrane domain of Madin-Darby canine kidney cells independent of coupling to pertussis toxin-sensitive GTP-binding proteins. *J Biol Chem.* **268:** 11340-7.

Kenakin T (2004). Principles: Receptor theory in pharmacology. *Trends Pharmacol Sci.* **25:** 186-92.

Kobilka BK, Matsui H, Kobilka TS, Yang-Feng TL, Francke U, Caron MG*, et al.* (1987). Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science.* **238:** 650-6.

Koch WJ, Hawes BE, Inglese J, Luttrell LM and Lefkowitz RJ (1994). Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates $G_{\beta\gamma}$-mediated signaling. *J Biol Chem.* **269:** 6193-7.

Kohli U, Muszkat M, Sofowora GG, Harris PA, Friedman EA, Dupont WD*, et al.* (2010). Effects of variation in the human α_{2A}- and α_{2C}-adrenoceptor genes on cognitive tasks and pain perception. *Eur J Pain.* **14:** 154-9.

Kolaj M, Renaud LP (2001). Norepinephrine acts via α_2-adrenergic receptors to suppress N-type calcium channels in dissociated rat median preoptic nucleus neurons. *Neuropharmacology.* **41:** 472-9.

Kolakowski LF,Jr (1994). GCRDb: A G-protein-coupled receptor database. *Receptors Channels.* **2:** 1-7.

Kolb P, Rosenbaum DM, Irwin JJ, Fung JJ, Kobilka BK and Shoichet BK (2009). Structure-based discovery of β_2-adrenergic receptor ligands. *Proc Natl Acad Sci U S A.* **106:** 6843-8.

Kristiansen K (2004). Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: Molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther.* **103:** 21-80.

Kukkonen JP, Renvaktar A, Shariatmadari R and Akerman KE (1998). Ligand- and subtype-selective coupling of human α_2-adrenoceptors to Ca^{++} elevation in chinese hamster ovary cells. *J Pharmacol Exp Ther.* **287:** 667-71.

Kurose H, Arriza JL and Lefkowitz RJ (1993). Characterization of α_2-adrenergic receptor subtype-specific antibodies. *Mol Pharmacol.* **43:** 444-50.

Lagerström MC, Schiöth HB (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov.* **7:** 339-57.

Lähdesmäki J, Sallinen J, MacDonald E, Sirviö J and Scheinin M (2003). α_2-Adrenergic drug effects on brain monoamines, locomotion, and body temperature are largely abolished in mice lacking the α_{2A}-adrenoceptor subtype. *Neuropharmacology.* **44:** 882-92.

Lakhlani PP, MacMillan LB, Guo TZ, McCool BA, Lovinger DM, Maze M*, et al.* (1997). Substitution of a mutant α_{2A}-adrenergic receptor via "hit and run" gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo. *Proc Natl Acad Sci USA.* **94:** 9950-5.

Langer SZ (1976). The role of alpha- and beta-presynaptic receptors in the regulation of noradrenaline release elicited by nerve stimulation. *Clin Sci Mol Med Suppl.* **3:** 423-6.

Lanier SM, Downing S, Duzic E and Homcy CJ (1991). Isolation of rat genomic clones encoding subtypes of the α_2-adrenergic receptor. Identification of a unique receptor subtype. *J Biol Chem.* **266:** 10470-8.

Lee A, Wissekerke AE, Rosin DL and Lynch KR (1998). Localization of α_{2C}-adrenergic receptor immunoreactivity in catecholaminergic neurons in the rat central nervous system. *Neuroscience.* **84:** 1085-96.

Lefkowitz RJ, Cotecchia S, Samama P and Costa T (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci.* **14:** 303-7.

Lefkowitz RJ and Shenoy SK (2005). Transduction of receptor signals by β-arrestins. *Science.* **308:** 512-7.

Lehtonen JV, Still DJ, Rantanen VV, Ekholm J, Björklund D, Iftikhar Z*, et al.* (2004). BODIL: A molecular modeling environment for structure-function analysis and drug design. *J Comput Aided Mol Des.* **18:** 401-19.

Li J, Edwards PC, Burghammer M, Villa C and Schertler GF (2004). Structure of bovine rhodopsin in a trigonal crystal form. *J Mol Biol.* **343:** 1409-38.

Liapakis G, Simpson MM and Javitch JA (2001). The substituted-cysteine accessibility method (SCAM) to elucidate membrane protein structure. *Curr Protoc Neurosci.* **Chapter 4:** Unit 4.15.

Limbird LE (1988). Receptors linked to inhibition of adenylate cyclase: Additional signaling mechanisms. *FASEB J.* **2:** 2686-95.

Link R, Daunt D, Barsh G, Chruscinski A and Kobilka B (1992). Cloning of two mouse genes encoding α_2-adrenergic receptor subtypes and identification of a single amino acid in the mouse α_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 α_2-adrenergic receptor subtypes b and c. *Science.* **273:** 803-5.

Link RE, Stevens MS, Kulatunga M, Scheinin M, Barsh GS and Kobilka BK (1995). Targeted inactivation of the gene encoding the mouse α_{2C}-adrenoceptor homolog. *Mol Pharmacol.* **48:** 48-55.

Lohse MJ, Benovic JL, Codina J, Caron MG and Lefkowitz RJ (1990). β-Arrestin: a protein that regulates β-adrenergic receptor function. *Science.* **248:** 1547-50.

Lomasney JW, Cotecchia S, Lefkowitz RJ and Caron MG (1991). Molecular biology of alpha-adrenergic receptors: Implications for receptor classification and for structure-function relationships. *Biochim Biophys Acta.* **1095:** 127-39.

Lomasney JW, Lorenz W, Allen LF, King K, Regan JW, Yang-Feng TL*, et al.* (1990). Expansion of the α_2-adrenergic receptor family: Cloning and characterization of a human α_2-adrenergic receptor subtype, the gene for which is located on chromosome 2. *Proc Natl Acad Sci U S A.* **87:** 5094-8.

MacDonald E, Kobilka BK and Scheinin M (1997). Gene targeting--homing in on α_2-adrenoceptor-subtype function. *Trends Pharmacol Sci.* **18:** 211-9.

MacDonald E, Scheinin M (1995). Distribution and pharmacology of α_2-adrenoceptors in the central nervous system. *J Physiol Pharmacol.* **46:** 241-58.

MacMillan LB, Hein L, Smith MS, Piascik MT and Limbird LE (1996). Central hypotensive effects of the α_{2A}-adrenergic receptor subtype. *Science.* **273:** 801-3.

MacNulty EE, McClue SJ, Carr IC, Jess T, Wakelam MJ and Milligan G (1992). α_2-C10 adrenergic receptors expressed in rat 1 fibroblasts can regulate both adenylylcyclase and phospholipase D-mediated hydrolysis of phosphatidylcholine by interacting with pertussis toxin-sensitive guanine nucleotide-binding proteins. *J Biol Chem.* **267:** 2149-56.

Maehle AH (2004). "Receptive substances": John Newport Langley (1852-1925) and his path to a receptor theory of drug action. *Med Hist.* **48:** 153-74.

Makaritsis KP, Handy DE, Johns C, Kobilka B, Gavras I and Gavras H (1999). Role of the α_{2B}-adrenergic receptor in the development of salt-induced hypertension. *Hypertension.* **33:** 14-7.

Marjamäki A, Ala-Uotila S, Luomala K, Peräla M, Jansson C, Jalkanen M*, et al.* (1992). Stable expression of recombinant human α_2-adrenoceptor subtypes in two mammalian cell lines: Characterization with [^{3}H]rauwolscine binding, inhibition of adenylate cyclase and RNase protection assay. *Biochim Biophys Acta.* **1134:** 169-77.

Marjamäki A, Frang H, Pihlavisto M, Hoffrèn AM, Salminen T, Johnson MS*, et al.* (1999). Chloroethylclonidine and 2-aminoethyl methanethiosulfonate recognize two different conformations of the human α_{2A}-adrenergic receptor. *J Biol Chem.* **274:** 21867-72.

Marjamäki A, Luomala K, Ala-Uotila S and Scheinin M (1993). Use of recombinant human α_2-adrenoceptors to characterize subtype selectively of antagonist binding. *Eur J Pharmacol.* **246:** 219-26.

Marjamäki A, Pihlavisto M, Cockcroft V, Heinonen P, Savola JM and Scheinin M (1998). Chloroethylclonidine binds irreversibly to exposed cysteines in the fifth membrane-spanning domain of the human α_{2A}-adrenergic receptor. *Mol Pharmacol.* **53:** 370-6.

Mathias CJ, Luckitt J, Desai P, Baker H, el Masri W and Frankel HL (1989). Pharmacodynamics and pharmacokinetics of the oral antispastic agent tizanidine in patients with spinal cord injury. *J Rehabil Res Dev.* **26:** 9-16.

McCune SK, Voigt MM and Hill JM (1993). Expression of multiple alpha adrenergic receptor subtype messenger RNAs in the adult rat brain. *Neuroscience.* **57:** 143-51.

McNamara DM, MacGowan GA and London B (2002). Clinical importance of β-adrenoceptor polymorphisms in cardiovascular disease. *Am J Pharmacogenomics.* **2:** 73-8.

Melchiorre C, Gallucci P (1983). The irreversible alpha-blocker benextramine interacts with two different thiol groups. *Farmaco Sci.* **38:** 950-6.

Menon ST, Han M and Sakmar TP (2001). Rhodopsin: Structural basis of molecular physiology. *Physiol Rev.* **81:** 1659-88.

Michel AD, Loury DN and Whiting RL (1989). Differences between the α_2-adrenoceptor in rat submaxillary gland and the α_{2A}-and α_{2B}-adrenoceptor subtypes. *Br J Pharmacol.* **98:** 890-7.

Milligan G, Kostenis E (2006). Heterotrimeric G-proteins: A short history. *Br J Pharmacol.* **147:** 46-55.

Milligan G, Smith NJ (2007). Allosteric modulation of heterodimeric G-protein-coupled receptors. *Trends Pharmacol Sci.* **28:** 615-20.

Murakami M, Kouyama T (2008). Crystal structure of squid rhodopsin. *Nature.* **453:** 363-7.

Naselsky DP, Ashton D, Ruffolo RR,Jr and Hieble JP (2001). Rabbit α_2-adrenoceptors: Both platelets and adipocytes have α_{2A}-pharmacology. *J Pharmacol Exp Ther.* **298:** 219-25.

Neve KA, Cumbay MG, Thompson KR, Yang R, Buck DC, Watts VJ*, et al.* (2001). Modeling and mutational analysis of a putative sodium-binding pocket on the dopamine D_2 receptor. *Mol Pharmacol.* **60:** 373-81.

Nicholas AP, Pieribone V and Hökfelt T (1993). Distributions of mRNAs for α_2-adrenergic receptor subtypes in rat brain: An in situ hybridization study. *J Comp Neurol.* **328:** 575-94.

Niimura Y, Nei M (2006). Evolutionary dynamics of olfactory and other chemosensory receptor genes in vertebrates. *J Hum Genet.* **51:** 505-17.

Nobles M, Benians A and Tinker A (2005). Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. *Proc Natl Acad Sci U S A.* **102:** 18706-11.

Nordström KJ, Lagerström MC, Waller LM, Fredriksson R and Schiöth HB (2009). The secretin GPCRs descended from the family of adhesion GPCRs. *Mol Biol Evol.* **26:** 71-84.

Nyrönen T, Pihlavisto M, Peltonen JM, Hoffrèn AM, Varis M, Salminen T*, et al.* (2001). Molecular mechanism for agonist-promoted α_{2A}-adrenoceptor activation by norepinephrine and epinephrine. *Mol Pharmacol.* **59:** 1343-54.

Okada T, Fujiyoshi Y, Silow M, Navarro J, Landau EM and Shichida Y (2002). Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. *Proc Natl Acad Sci U S A.* **99:** 5982-7.

Okada T, Sugihara M, Bondar AN, Elstner M, Entel P and Buss V (2004). The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure. *J Mol Biol.* **342:** 571-83.

Oliveira L, Paiva PB, Paiva AC and Vriend G (2003). Sequence analysis reveals how G protein-coupled receptors transduce the signal to the G protein. *Proteins.* **52:** 553-60.

Olli-Lähdesmäki T, Kallio J and Scheinin M (1999). Receptor subtype-induced targeting and subtype-specific internalization of human α_2-adrenoceptors in PC12 cells. *J Neurosci.* **19:** 9281-8.

Olli-Lähdesmäki T, Scheinin M, Pohjanoksa K and Kallio J (2003). Agonist-dependent trafficking of α_2-adrenoceptor subtypes: Dependence on receptor subtype and employed agonist. *Eur J Cell Biol.* **82:** 231-9.

O'Rourke MF, Blaxall HS, Iversen LJ and Bylund DB (1994a). Characterization of [^{3}H]RX821002 binding to α_2-adrenergic receptor subtypes. *J Pharmacol Exp Ther.* **268:** 1362-7.

O'Rourke MF, Iversen LJ, Lomasney JW and Bylund DB (1994b). Species orthologs of the α_{2A}-adrenergic receptor: The pharmacological properties of the bovine and rat receptors differ from the human and porcine receptors. *J Pharmacol Exp Ther.* **271:** 735-40.

Paddleford RR, Harvey RC (1999). α_2-Agonists and antagonists. *Vet Clin North Am Small Anim Pract.* **29:** 737-45.

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:** 739-45.

Patton EE, Zon LI (2001). The art and design of genetic screens: Zebrafish. *Nat Rev Genet.* **2:** 956-66.

Peltonen JM, Nyrönen T, Wurster S, Pihlavisto M, Hoffrèn AM, Marjamäki A*, et al.* (2003). Molecular mechanisms of ligand-receptor interactions in transmembrane domain V of the α_{2A}-adrenoceptor. *Br J Pharmacol.* **140:** 347-58.

Peltonen JM, Pihlavisto M and ScheininM (1998). Subtype-specific stimulation of [^{35}S]GTPγS binding by recombinant α_2-adrenoceptors. *Eur J Pharmacol* **355:** 275-9.

Perälä M, Hirvonen H, Kalimo H, Ala-Uotila S, Regan JW, Akerman KE*, et al.* (1992). Differential expression of two α_2-adrenergic receptor subtype mRNAs in human tissues. *Brain Res Mol Brain Res.* **16:** 57-63.

Peterhoff M, Sieg A, Brede M, Chao CM, Hein L and Ullrich S (2003). Inhibition of insulin secretion via distinct signaling pathways in α_2-adrenoceptor knockout mice. *Eur J Endocrinol.* **149:** 343-50.

Pfeuffer T, Helmreich EJ (1988). Structural and functional relationships of guanosine triphosphate binding proteins. *Curr Top Cell Regul.* **29:** 129-216.

Philipp M, Brede ME, Hadamek K, Gessler M, Lohse MJ and Hein L (2002). Placental α_2-adrenoceptors control vascular development at the interface between mother and embryo. *Nat Genet.* **31:** 311-5.

Pohjanoksa K, Jansson CC, Luomala K, Marjamäki A, Savola JM and Scheinin M (1997). α_2-Adrenoceptor regulation of adenylyl cyclase in CHO cells: Dependence on receptor density, receptor subtype and current activity of adenylyl cyclase. *Eur J Pharmacol.* **335:** 53-63.

Porter JE, Hwa J and Perez DM (1996). Activation of the α_{1b}-adrenergic receptor is initiated by disruption

of an interhelical salt bridge constraint. *J Biol Chem.* **271:** 28318-23.

Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A*, et al.* (1998). Vertebrate genome evolution and the zebrafish gene map. *Nat Genet.* **18:** 345-9.

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC*, et al.* (2007). Crystal structure of the human β_2-adrenergic G-protein-coupled receptor. *Nature.* **450:** 383-7.

Regan JW, DeMarinis RM, Caron MG and Lefkowitz RJ (1984). Identification of the subunit-binding site of α_2-adrenergic receptors using [^{3}H]phenoxybenzamine. *J Biol Chem.* **259:** 7864-9.

Regan JW, Kobilka TS, Yang-Feng TL, Caron MG, Lefkowitz RJ and Kobilka BK (1988). Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. *Proc Natl Acad Sci U S A.* **85:** 6301-5.

Regan JW, Nakata H, DeMarinis RM, Caron MG and Lefkowitz RJ (1986). Purification and characterization of the human platelet α_2-adrenergic receptor. *J Biol Chem.* **261:** 3894-900.

Richman JG, Regan JW (1998). α_2-Adrenergic receptors increase cell migration and decrease F-actin labeling in rat aortic smooth muscle cells. *Am J Physiol.* **274:** 654-62.

Rios C, Gomes I and Devi LA (2004). Interactions between δ opioid receptors and α-adrenoceptors. *Clin Exp Pharmacol Physiol.* **31:** 833-6.

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS*, et al.* (2007). GPCR engineering yields high-resolution structural insights into β_2-adrenergic receptor function. *Science.* **318:** 1266-73.

Rosin DL, Talley EM, Lee A, Stornetta RL, Gaylinn BD, Guyenet PG*, et al.* (1996). Distribution of α_{2C}-adrenergic receptor-like immunoreactivity in the rat central nervous system. *J Comp Neurol.* **372:** 135-65.

Rudling JE, Kennedy K and Evans PD (1999). The effect of site-directed mutagenesis of two transmembrane serine residues on agonist-specific coupling of a cloned human α_{2A}-adrenoceptor to adenylyl cyclase. *Br J Pharmacol.* **127:** 877-86.

Ruffolo RR,Jr, Hieble JP (1994). α-Adrenoceptors. *Pharmacol Ther.* **61:** 1-64.

Ruffolo RR,Jr, Nichols AJ, Stadel JM and Hieble JP (1993). Pharmacologic and therapeutic applications of α_2-adrenoceptor subtypes. *Annu Rev Pharmacol Toxicol.* **33:** 243-79.

Ruuskanen JO, Xhaard H, Marjamäki A, Salaneck E, Salminen T, Yan YL*, et al.* (2004). Identification of duplicated fourth α_2-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.

Sallinen J, Haapalinna A, MacDonald E, Viitamaa T, Lähdesmäki J, Rybnikova E*, et al.* (1999). Genetic alteration of the α_2-adrenoceptor subtype c in mice affects the development of behavioral despair and stress-induced increases in plasma corticosterone levels. *Mol Psychiatry.* **4:** 443-52.

Sallinen J, Haapalinna A, Viitamaa T, Kobilka BK and Scheinin M (1998). Adrenergic α_{2C}-receptors modulate the acoustic startle reflex, prepulse inhibition, and aggression in mice. *J Neurosci.* **18:** 3035-42.

Sallinen J, Link RE, Haapalinna A, Viitamaa T, Kulatunga M, Sjöholm B*, et al.* (1997). Genetic alteration of α_{2C}-adrenoceptor expression in mice: Influence on locomotor, hypothermic, and neurochemical effects of dexmedetomidine, a subtype-nonselective α_2-adrenoceptor agonist. *Mol Pharmacol.* **51:** 36-46.

Salminen T, Varis M, Nyrönen T, Pihlavisto M, Hoffrèn AM, Lönnberg T*, et al.* (1999). Three-dimensional models of α_{2A}-adrenergic receptor complexes provide a structural explanation for ligand binding. *J Biol Chem.* **274:** 23405-13.

Samama P, Pei G, Costa T, Cotecchia S and Lefkowitz RJ (1994). Negative antagonists promote an inactive conformation of the β_2-adrenergic receptor. *Mol Pharmacol.* **45:** 390-4.

Sastre M, Garcia-Sevilla JA (1993). Opposite age-dependent changes of α_{2A}-adrenoceptors and nonadrenoceptor [^{3}H]idazoxan binding sites (I_2-imidazoline sites) in the human brain: Strong correlation of I_2 with monoamine oxidase-B sites. *J Neurochem.* **61:** 881-9.

Saunders C, Limbird LE (1999). Localization and trafficking of α_2-adrenergic receptor subtypes in cells and tissues. *Pharmacol Ther.* **84:** 193-205.

Savola JM, Ruskoaho H, Puurunen J, Salonen JS and Karki NT (1986). Evidence for medetomidine as a selective and potent agonist at α_2-adrenoreceptors. *J Auton Pharmacol.* **6:** 275-84.

Schambra UB, Mackensen GB, Stafford-Smith M, Haines DE and Schwinn DA (2005). Neuron specific α-adrenergic receptor expression in human cerebellum: Implications for emerging cerebellar roles in neurologic disease. *Neuroscience.* **135:** 507-23.

Scheibner J, Trendelenburg AU, Hein L, Starke K and Blandizzi C (2002). α_2-Adrenoceptors in the enteric nervous system: A study in α_{2A}-adrenoceptor-deficient mice. *Br J Pharmacol.* **135:** 697-704.

Scheinin M, Lomasney JW, Hayden-Hixson DM, Schambra UB, Caron MG, Lefkowitz RJ*, et al.* (1994). Distribution of α_2-adrenergic receptor subtype gene expression in rat brain. *Brain Res Mol Brain Res.* **21:** 133-49.

Schertler GF, Hargrave PA (1995). Projection structure of frog rhodopsin in two crystal forms. *Proc Natl Acad Sci U S A.* **92:** 11578-82.

Schiöth HB, Nordström KJ and Fredriksson R (2007). Mining the gene repertoire and ESTs for G protein-

coupled receptors with evolutionary perspective. *Acta Physiol (Oxf).* **190:** 21-31.

Schramm NL, McDonald MP and Limbird LE (2001). The α_{2A}-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. *J Neurosci.* **21:** 4875-82.

Schwartz DD, Clark TP (1998). Selectivity of atipamezole, yohimbine and tolazoline for α_2-adrenergic receptor subtypes: Implications for clinical reversal of α_2- adrenergic receptor mediated sedation in sheep. *J Vet Pharmacol Ther.* **21:** 342-7.

Schwartz TW, Frimurer TM, Holst B, Rosenkilde MM and Elling CE (2006). Molecular mechanism of 7TM receptor activation--a global toggle switch model. *Annu Rev Pharmacol Toxicol.* **46:** 481-519.

Serle JB (1994). Pharmacological advances in the treatment of glaucoma. *Drugs Aging.* **5:** 156-70.

Seuwen K, Magnaldo I, Kobilka BK, Caron MG, Regan JW, Lefkowitz RJ*, et al.* (1990). α_2-Adrenergic agonists stimulate DNA synthesis in chinese hamster lung fibroblasts transfected with a human α_2-adrenergic receptor gene. *Cell Regul.* **1:** 445-51.

Shi L, Javitch JA (2004). The second extracellular loop of the dopamine D_2 receptor lines the binding-site crevice. *Proc Natl Acad Sci U S A.* **101:** 440-5.

Shi L, Javitch JA (2002). The binding site of aminergic G protein-coupled receptors: The transmembrane segments and second extracellular loop. *Annu Rev Pharmacol Toxicol.* **42:** 437-67.

Shin JT, Fishman MC (2002). From zebrafish to human: Modular medical models. *Annu Rev Genomics Hum Genet.* **3:** 311-40.

Simonneaux V, Ebadi M and Bylund DB (1991). Identification and characterization of α_{2D}-adrenergic receptors in bovine pineal gland. *Mol Pharmacol.* **40:** 235-41.

Small KM, Brown KM, Forbes SL and Liggett SB (2001). Polymorphic deletion of three intracellular acidic residues of the α_{2B}-adrenergic receptor decreases G protein-coupled receptor kinase-mediated phosphorylation and desensitization. *J Biol Chem.* **276:** 4917-22.

Small KM, Forbes SL, Brown KM and Liggett SB (2000a). An Asn to Lys polymorphism in the third intracellular loop of the human α_{2A}-adrenergic receptor imparts enhanced agonist-promoted Gi coupling. *J Biol Chem.* **275:** 38518-23.

Small KM, Forbes SL, Rahman FF, Bridges KM and Liggett SB (2000b). A four amino acid deletion polymorphism in the third intracellular loop of the human α_{2C}-adrenergic receptor confers impaired coupling to multiple effectors. *J Biol Chem.* **275:** 23059-64.

Small KM, Liggett SB (2001). Identification and functional characterization of α_2-adrenoceptor polymorphisms. *Trends Pharmacol Sci.* **22:** 471-7.

Snapir A, Heinonen P, Tuomainen TP, Alhopuro P, Karvonen MK, Lakka TA*, et al.* (2001). An insertion/deletion polymorphism in the a α_{2B}-adrenergic receptor gene is a novel genetic risk factor for acute coronary events. *J Am Coll Cardiol.* **37:** 1516-22.

Snapir A, Mikkelsson J, Perola M, Penttilä A, Scheinin M and Karhunen PJ (2003). Variation in the α_{2B}-adrenoceptor gene as a risk factor for prehospital fatal myocardial infarction and sudden cardiac death. *J Am Coll Cardiol.* **41:** 190-4.

Soini SL, Duzic E, Lanier SM and Akerman KE (1998). Dual modulation of calcium channel current via recombinant α_2-adrenoceptors in pheochromocytoma (PC-12) cells. *Pflugers Arch.* **435:** 280-5.

Sprang SR, Chen Z and Du X (2007). Structural basis of effector regulation and signal termination in heterotrimeric galpha proteins. *Adv Protein Chem.* **74:** 1-65.

Stimmel GL, Dopheide JA and Stahl SM (1997). Mirtazapine: An antidepressant with noradrenergic and specific serotonergic effects. *Pharmacotherapy.* **17:** 10-21.

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:** 509-38.

Suryanarayana S, Daunt DA, Von Zastrow M and Kobilka BK (1991). A point mutation in the seventh hydrophobic domain of the α_2-adrenergic receptor increases its affinity for a family of β- receptor antagonists. *J Biol Chem.* **266:** 15488-92.

Suwa M, Ono Y (2009). Computational overview of GPCR gene universe to support reverse chemical genomics study. *Methods Mol Biol.* **577:** 41-54.

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

Szot P, Lester M, Laughlin ML, Palmiter RD, Liles LC and Weinshenker D (2004). The anticonvulsant and proconvulsant effects of α_2-adrenoreceptor agonists are mediated by distinct populations of α_{2A}-adrenoreceptors. *Neuroscience.* **126:** 795-803.

Taraviras S, Olli-Lähdesmäki T, Lymperopoulos A, Charitonidou D, Mavroidis M, Kallio J*, et al.* (2002). Subtype-specific neuronal differentiation of PC12 cells transfected with α_2-adrenergic receptors. *Eur J Cell Biol.* **81:** 363-74.

Tavares A, Handy DE, Bogdanova NN, Rosene DL and Gavras H (1996). Localization of α_{2A}- and α_{2B}-adrenergic receptor subtypes in brain. *Hypertension.* **27:** 449-55.

Teller DC, Okada T, Behnke CA, Palczewski K and 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:** 7761-72.

Timmons SD, Geisert E, Stewart AE, Lorenzon NM and Foehring RC (2004). α_2-Adrenergic receptor-mediated modulation of calcium current in neocortical pyramidal neurons. *Brain Res.* **1014:** 184-96.

Trendelenburg AU, Klebroff W, Hein L and Starke K (2001). A study of presynaptic α_2-autoreceptors in $\alpha_{2A/D}$-, α_{2B}- and α_{2C}-adrenoceptor-deficient mice. *Naunyn Schmiedebergs Arch Pharmacol.* **364:** 117-30.

Trendelenburg AU, Sutej I, Wahl CA, Molderings GJ, Rump LC and Starke K (1997). A re-investigation of questionable subclassifications of presynaptic α_2-autoreceptors: Rat vena cava, rat atria, human kidney and guinea-pig urethra. *Naunyn Schmiedebergs Arch Pharmacol.* **356:** 721-37.

Uhlèn S, Dambrova M, Näsman J, Schiöth HB, Gu Y, Wikberg-Matsson A*, et al.* (1998). 3H]RS79948-197 binding to human, rat, guinea pig and pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. comparison with MK912, RX821002, rauwolscine and yohimbine. *Eur J Pharmacol.* **343:** 93-101.

Uhlèn S, Porter AC and Neubig RR (1994). The novel α_2-adrenergic radioligand [^{3}H]-MK912 is α_{2C}-selective among human α_{2A}-, α_{2B}-and α_{2C}-adrenoceptors. *J Pharmacol Exp Ther.* **271:** 1558-65.

Venkataraman V, Duda T and Sharma RK (1997). A role for amino acid residues in the third cytoplasmic loop in defining the ligand binding characteristics of the α_{2D}-adrenergic receptor. *Mol Cell Biochem.* **177:** 125-9.

Vernier P, Cardinaud B, Valdenaire O, Philippe H and Vincent JD (1995). An evolutionary view of drug-receptor interaction: The bioamine receptor family. *Trends Pharmacol Sci.* **16:** 375-81.

Violin JD and Lefkowitz RJ (2007). β-Arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* **28:** 416-22.

Virtanen R (1989). Pharmacological profiles of medetomidine and its antagonist, atipamezole. *Acta Vet Scand Suppl.* **85:** 29-37.

Wang CD, Buck MA and Fraser CM (1991). Site-directed mutagenesis of α_{2A}-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol Pharmacol.* **40:** 168-79.

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R*, et al.* (2008). Structure of a β_1-adrenergic G-protein-coupled receptor. *Nature.* **454:** 486-91.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996). The cubic ternary complex receptor-occupancy model. III. resurrecting efficacy. *J Theor Biol.* **181:** 381-97.

Wikberg-Matsson A, Simonsen U (2001). Potent α_{2A}-adrenoceptor-mediated vasoconstriction by brimonidine in porcine ciliary arteries. *Invest Ophthalmol Vis Sci.* **42:** 2049-55.

Wilden U, Hall SW and Kühn H (1986). Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc Natl Acad Sci U S A.* **83:** 1174-8.

Winzer-Serhan UH, Raymon HK, Broide RS, Chen Y and Leslie FM (1997). Expression of α_2-adrenoceptors during rat brain development--II. alpha 2C messenger RNA expression and [3H]rauwolscine binding. *Neuroscience.* **76:** 261-72.

Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V*, et al.* (2010). Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science.* **330:** 1066-71.

Wurch T, Colpaert FC and Pauwels PJ (1999). G-protein activation by putative antagonists at mutant Thr373Lys α_{2A}- adrenergic receptors. *Br J Pharmacol.* **126:** 939-48.

Wurch T, Colpaert FC and 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:** 1088-96.

Wurch T, Pauwels PJ (2000). Coupling of canine serotonin 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes to the formation of inositol phosphates by dual interactions with endogenous $G_{i/o}$ and recombinant $G_{\alpha 15}$ proteins. *J Neurochem.* **75:** 1180-9.

Xhaard H, Nyrönen T, Rantanen VV, Ruuskanen JO, Laurila J, Salminen T *et al.* (2005). Model structures of α_2- adrenoceptors in complex with automatically docked antagonist ligands raise the possibility of interactions dissimilar from agonist ligands. *J Struct Biol.* **150:** 126-43.

Xhaard H, Rantanen VV, Nyrönen T and Johnson MS (2006). Molecular evolution of adrenoceptors and dopamine receptors: Implications for the binding of catecholamines. *J Med Chem.* **49:** 1706-19.

Zhang YQ, Limbird LE (2004). Hetero-oligomers of α_{2A}-adrenergic and μ-opioid receptors do not lead to transactivation of G-proteins or altered endocytosis profiles. *Biochem Soc Trans.* **32:** 856-60.

Zhao MM, Hwa J and Perez DM (1996). Identification of critical extracellular loop residues involved in α_1-adrenergic receptor subtype-selective antagonist binding. *Mol Pharmacol.* **50:** 1118-26.

Zurn A, Zabel U, Vilardaga JP, Schindelin H, Lohse MJ and Hoffmann C (2009). Fluorescence resonance energy transfer analysis of α_{2A}-adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol Pharmacol.* **75:** 534-41.