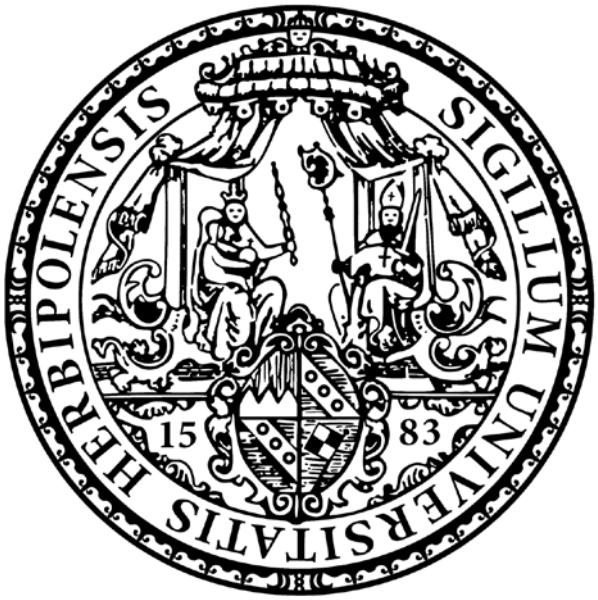

Investigating the Molecular Mechanism of Receptor Activation at Muscarinic Receptors by Means of Pathway-Specific Dualsteric Ligands and Partial Agonists

Molekularen Grundlagen der Rezeptoraktivierung von muskarinergen Acetylcholin Rezeptoren durch dualsteren Liganden und Partialagonisten

Doctoral thesis for a doctoral degree
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Table of Contents

List of Figures

List of Tables

Abbreviations

AC	adenylyl cyclase
ACh	Acetylcholine
AMP	adenosine monophosphate
AP-2	adaptor protein 2
ATP	adenosine triphosphate
β_2-AR	β_2 adrenergic receptor
BAL	British anti-Lewisite / 2,3-Dimercapto-1-propanol
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CTX	Cholera toxin
DAG	diacylglycerol
DMR	dynamic mass redistribution
EC50	effective concentration at half maximal activation
EL	extracellular loop
ER	endoplasmic reticulum
ERK	extracellular-regulated kinase
FDA	US Food and Drug Administration
FlAsH	Fluorescein arsenical hairpin binder
FLIM	fluorescent lifetime imaging
FLuc	Firefly luciferase
FRET	fluorescence resonance energy transfer
FSH	follicle-stimulating hormone
G-protein	GTP-binding protein
GAP	GTPase activating protein
GDP	guanosine diphosphate
GDI	G protein dissociation inhibitor
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein

GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
HD	helical domain
HOMO	highest occupied molecular orbital
IC	internal conversion
IL	intracellular loop
IP3	inositol trisphosphate
IR	infrared
ISC	intersystem crossing
JNK	c-Jun N-terminal kinases
LUMO	lowest unoccupied molecular orbital
M_1	muscarinic Acetylcholine receptor subtype 1
M_2	muscarinic Acetylcholine receptor subtype 2
M_3	muscarinic Acetylcholine receptor subtype 3
M_4	muscarinic Acetylcholine receptor subtype 4
M_5	muscarinic Acetylcholine receptor subtype 5
mAChR	muscarinic Acetylcholine receptor
MAPK	mitogen-activated protein kinase
MD	molecular dynamic simulation
mGluR1	metabotropic Glutamate receptor 1
ms	millisecond
MWC	Monod-Wyman-Changeux model
μOR	μ opioid receptor
nAChR	nicotinic Acetylcholine receptor
NAM	negative allosteric modulator
NLuc	NanoLuc luciferase
NP	nanoparticle
PAM	positive allosteric modulator
PDB	Protein Data Bank
PIP2	phosphatidylinositol 4,5-bisphosphate

PKC	protein kinase C
PLCβ	phospholipase C β
PNS	peripheral nervous system
PTL	photo-switchable tethered ligand
PTX	Pertussis toxin
QNB	3-quinuclidinyl-benzilate
ReAsH	resorufin arsenical hairpin binder
RET	resonance energy transfer
RFP	red fluorescent protein
RGS	regulators of G protein signaling
RLuc	*Renilla* luciferase
TR-FRET	time resolved FRET
TSH	thyroid-stimulating hormone
uaas	unnatural amino acids
UV	ultra violet

1 Introduction

Reception of ambient influences in order to provoke specific responses belongs to the central mechanisms of life and evolution. The majority of receipting biomolecules are protein structures composed of up to 20 different biogenic amino acids. Bacteria as well as Eukaryotes can be regarded as closed systems due to a membranous envelope predominantly consisting of phospholipids. Membrane spanning proteins have evolved in both domains to allow transport of small molecules across the membrane and perception of external stimuli.

1.1 G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) belong to the largest protein receptor family that is encoded in the human genome with more than 1200 members (Insel et al., 2012). These membrane-spanning proteins play an outstanding role in biomedical research and are thought to interact with 30% - 40% of all modern drugs (Flower, 1999; Hopkins and Groom, 2002; Santos et al., 2017). All GPCRs primarily interact with the guanosine triphosphate-binding protein (G-protein) family to mediate an extracellular stimulus into an intracellular response. The variety of extracellular interaction partners for GPCRs is diverse and thus GPCRs are not only involved in sensory processes like vision, olfaction and taste but also play an extraordinary role in neurobiology by interacting with neurotransmitters (e.g. acetylcholine, dopamine, glutamate) or in endocrinology by recognizing hormones (e.g. insulin, thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH)).

1.1.1 Classification and subdivision

In the past, various attempts were made to categorize GPCRs, based on ligand binding properties or physiological features. A commonly used and now widely accepted classification uses homology and functional properties to group all GPCRS in classes A-F. (Attwood and Findlay, 1994; Kolakowski, 1994). Here, receptors belonging to class D and E are solely non-human GPCRs.

Class	Attribution
A	Rhodopsin-like family
B	Secretin receptor family
C	Metabotropic glutamate receptor family
D	Fungal mating pheromone receptor family
E	Cyclic AMP receptor family
F	Frizzled/Smoothened receptor family

Table 1 GPCR Classification Based on Homology and Functionality

A second often used classification is based on phylogenetic analyses of the human genome and is called GRAFS-system (Fredriksson et al., 2003).

Class	Attribution	Member
G	Glutamate Family	15
R	Rhodopsin Family	701
A	Adhesion Family	24
F	Frizzled/Taste2 Family	24
S	Secretin Family	15

Table 2 GPCR Classification Based on Phylogenetic Genome Analyses

In this classification, the Rhodopsin family contains by far the largest number of receptors. Compared to the classification introduced before, the Rhodopsin family corresponds to class A. Within this family several structural properties are conserved, e.g. the DRY motive or the NSxxNPxxY motive (Kooistra et al., 2013).

1.1.2 Structural properties

All GPCRs consist of seven transmembrane helices (TM) that are connected via three extracellular loops (EL) and three intracellular loops (IL). In addition, the N-terminus of GPCRs expressed on the cell surface is exposed to the extracellular site whereas the C-terminus is exposed to the intracellular compartment (Figure 1.1). However, over the last decade detailed 3D structures using X-ray crystallography have added structural knowledge regarding GPCRs. The first GPCR crystallized was the inactive rhodopsin (Figure 1.1) (Palczewski et al., 2000). Until now, 46 different GPCRs have been crystallized and resolved, leading to 218 different structures. Most of the structures were resolved for class A GPCRs (196 structures of 39 different receptors) (Isberg et al., 2016).

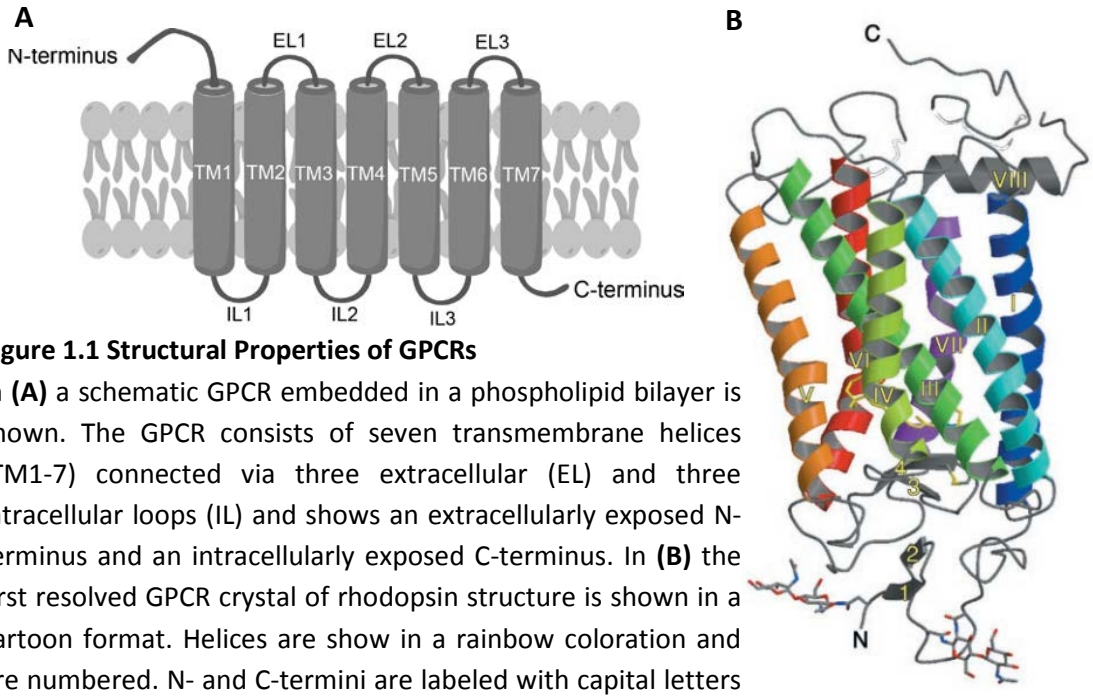

Figure 1.1 Structural Properties of GPCRs

In **(A)** a schematic GPCR embedded in a phospholipid bilayer is shown. The GPCR consists of seven transmembrane helices (TM1-7) connected via three extracellular (EL) and three intracellular loops (IL) and shows an extracellularly exposed N-terminus and an intracellularly exposed C-terminus. In **(B)** the first resolved GPCR crystal of rhodopsin structure is shown in a cartoon format. Helices are show in a rainbow coloration and are numbered. N- and C-termini are labeled with capital letters Due to historical reasons the receptor is displayed "upside down" when compared to non-rhodopsin illustrations (image taken and modified from Palczewski *et al.* 2000).

1.1.3 Cellular signaling of GPCRs

The physiological task of transmembrane receptors is both, the recognition of an extracellular stimulus and the transduction of this stimulus into a downstream response. Hence, GPCRs can be regarded as one of the central triggers for cellular signaling. In general, signaling can be described as a hierarchical cascade, which means that the initiating level can induce several different downstream responses and thus GPCRs show a temporally and spatially structured signaling pattern. Recognition of an extracellular stimulus induces a reorganization of the transmembrane helices within milliseconds. This conformational change is receptor specific, triggers G-protein activation, arrestin recruitment and binding and in the most cases internalization of the receptor (Bünemann et al., 2003; Miller and Lefkowitz, 2001). Considering these downstream processes, naturally the activation of GPCRs is of high interest for biomedical research and pharmaceutical companies (Hoffmann et al., 2005; Rochais et al., 2007; Vilardaga et al., 2003).

1.1.3.1 Heterotrimeric G protein

The interaction of the heterotrimeric G protein with the GPCR is the primary intracellular signaling step of all GPCRs. Heterotrimeric G proteins are composed of the α-, β- and γ-subunit in order of decreasing mass (Gilman, 1987; Northup et al., 1980). The Gα subunit consists of two domains. The helical domain (HD) is responsible for guanosine triphosphate (GTP) binding and the catalytically active Ras domain is responsible for GTP hydrolysis. Under physiological conditions the β and γ subunits form a complex via coiled coil interactions that is anchored in the plasma membrane. In the ground state (inactive state) guanosine diphosphate (GDP) is bound at the Gα subunit. Upon interaction with an active GPCR (which acts as a, guanine nucleotide exchange factor, GEF) mainly with the Ras domain of the G-protein, GDP is released followed by GTP binding (active state). After GTP binding a molecular reorganization of the G-protein heterotrimer takes place followed by signaling, involving effector protein interactions (Bünemann et al., 2003). Both Gα and Gβγ subunits can interact with different effector proteins. Promoted by a GTPase activating protein (GAP) GTP is hydrolyzed to GDP returning the α subunit to the inactive state (Dror et al., 2015; Flock et al., 2015; Rasmussen et al., 2011). Furthermore, G proteins are controlled by regulators of G protein signaling (RGS) and G protein dissociation inhibitors (GDIs) (Grundmann and Kostenis, 2017). RGS interaction can accelerate GTP hydrolysis by lowering the energy barrier and thus facilitates the inactivation of the G protein but can also intensify G protein signaling (Ross, 2008; Ross and Wilkie, 2000; Smith et al., 2009). The physiological importance of RGS is indicated by reports that found a deregulation of RGS in cancer or in bradyopsia (Cao et al., 2006; Nishiguchi et al., 2004). GDIs inhibit the GDP-GTP exchange and thus cause a downregulation of G protein signaling but there is known little about the effect on heterotrimeric G proteins (Grundmann and Kostenis, 2017).

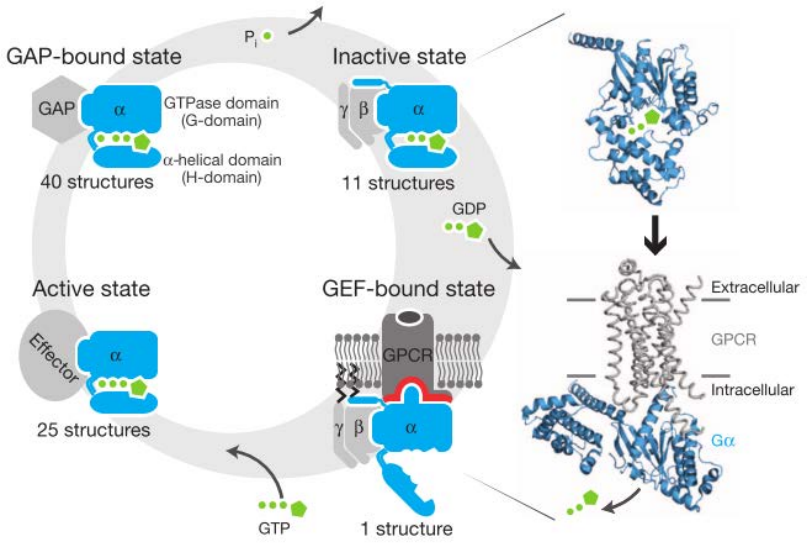

Figure 1.2 Ligand mediated GPCR activation and G-protein dependent signaling
In the inactive ground state, the heterotrimeric G protein is bound to GDP. Upon interaction with an active GPCR (GEF-bound state) GDP is released and GTP is bound representing the active state. In the active state the G protein subunits are dissociated and different effector molecules can interact with the subunits. In the GAP- bound state GTP gets hydrolyzed to GDP followed by a reassociation of the protein subunits and the G protein returns to the inactive state. On the right side a simulated GPCR Gα complex is shown (image taken and modified from Flock *et al.* 2015).

Until yet 16 different Gα and an equally diverse repertoire of Gβγ subunits were identified (Flock et al., 2017). Based on functional properties, the Gα subunits are subdivided in four classes. The understanding concerning the specificity of GPCR G protein interaction has changed during the last years. It seems that a GPCR can activate multiple G proteins and that the coupling of different species is mostly driven by two components, efficacy and kinetics (Masuho et al., 2015).

Gα	Effector protein	Cellular effect	Pharmacological modulation
G_s	Adenylate Cyclase	Activation of cAMP production	Cholera toxin (CTX)
G_i/G_o	Adenylate Cyclase	Inhibition of cAMP production	Pertussis toxin (PTX)
G_q	PLCβ	Increase in Ca^{2+}	YM254890 (Uemura et al., 2006); FR900359 (Schrage et al., 2015)
$G_{12/13}$	Rho-GTPases	Increase in kinase activity	

Table 3 G-protein Classification, Effector Proteins and Pharmacological Modulation

Two models were developed to explain how receptor and G-protein interact in the absence or in the presence of an agonist. The *collusion coupling* model describes that the GPCR activation upon ligand binding is the trigger for receptor G-protein interaction (Hein et al.,

2005). The *precoupling* model claims that an interaction of receptor and G-protein already exists without any agonist presence (Gales et al., 2006). Both described models agree that G-protein signaling is a highly dynamic process and more recent findings started to develop a multi-state model that is highly dependent on diffusion barriers, interaction hotspots and the cytoskeletal arrangements (Figure 1.3) (Sungkaworn et al., 2017). To this date, receptor G-protein interactions can be understood to appear randomly and mostly diffusion-driven in an unstimulated system. Upon receptor activation the interaction affinity increases, resulting in faster activation kinetics and leading to significant downstream signaling events. In fact, new data support partially both coupling models (Sungkaworn et al., 2017).

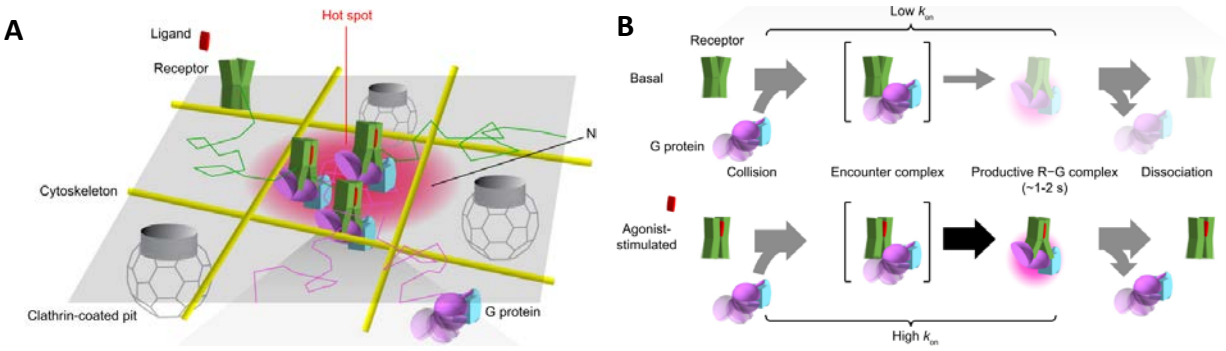

Figure 1.3 GPCR G Protein Interaction Model
In **(A)** a schematic cell surface is shown with ligand bound and ligand unbound GPCRs, G proteins, cytoskeleton structures (yellow) and clathrin coated pits (grey). Green and purple lines indicate trajectories of the single particle tracking application. In the center of the scheme an interaction hotspot is highlighted in red (image taken and modified from Sungkaworn *et al.* 2017). **(B)** compares an interaction model of ligand unbound (inactive) GPCRs and ligand bound (active) receptors. It is shown that GPCRs can interact with G proteins in the absence of a ligand on a basal level without activating G protein signaling. In contrast, ligand bound receptors do both, G protein binding and activation with high kinetics (image taken and modified from Sungkaworn *et al.* 2017).

1.1.3.2 Arrestin Binding and Internalization

During primary signaling via G-proteins the C-terminus and the IL3 of the activated receptor gets phosphorylated by GPCR kinases (GRKs) followed by arrestin binding (Figure 1.4). The phosphorylation process was described to be the rate limiting step that can take minutes. Afterwards arrestin binds the phosphorylated receptor within seconds (Krasel et al., 2005; Krasel et al., 2004). This terminates the primary signaling and is called homologous desensitization. In general, arrestins are subdivided into two classes, visual and non-visual arrestins. Visual arrestins (arrestin 1 and arrestin 4 (Kuhn, 1978; Kuhn et al., 1984; Pfister et al., 1985)) are expressed in the retina whereas non-visual arrestins (β-arrestin 1 and β-arrestin 2 (Benovic et al., 1987; Lohse et al., 1990)) are expressed ubiquitously in vertebrates. Both visual arrestins and β-arrestin 1 are expressed in the nucleus whereas β-arrestin 2 is expressed in the cytosol (Gurevich and Gurevich, 2006; Miller and Lefkowitz, 2001). The following part focuses on β-arrestin 2 and thus β-arrestin 2 will be simply called as “arrestin”. The GPCR arrestin interaction is understood as a multistep process in that

arrestin can adopt multiple conformations (Gurevich and Benovic, 1993; Schleicher et al., 1989; Shukla et al., 2014). It was shown, that the conformational change of arrestin encodes information about receptor ligand interactions and seems to be receptor and ligand specific (Lee et al., 2016; Nuber et al., 2016). The physiological role of arrestin is not only the termination of the primary G-protein signaling, but also the initiation of secondary signaling pathways. After arrestin has bound to the receptor it serves as an adaptor protein and can recruit several interaction partners like the adaptor protein 2 (AP-2) and clathrin (Figure 1.4). These two proteins promote the internalization of the still active receptor into clathrin coated pits that are pinched of by the molecule dynamin (Laporte et al., 1999; Miller and Lefkowitz, 2001). In addition, the receptor arrestin complex can modulate several members of the mitogen-activated protein kinase family (MAPK) like the c-Jun N-terminal kinases (JNKs) and the extracellular-regulated kinases (ERKs). MAPKs represent an important cellular signaling pathway that ultimately affects gene transcription and expression. After a decent time arrestin dissociates from the receptor and recent studies showed a delayed conformational relaxation of arrestin. This finding could indicate physiological relevance of arrestin after dissociating from the receptor (Nuber et al., 2016). In Addition, the first GPCR arrestin structure has been recently resolved (Kang et al., 2015; Zhou et al., 2017). A different subject of intensive arrestin research is the question of how the phosphorylated receptor and arrestin communicate to induce effector dependent signaling responses as well as tissue and cell type specific responses (Bahouth and Nooh, 2017). It was found that the first level of regulation in signaling occurs via the GRK interaction because in contrast to the high number of GPCRs there are only seven different GRKs and only GRK 2, 3, 5 and 6 are ubiquitously expressed (Nobles et al., 2011; Violin and Lefkowitz, 2007). Distinct GRKs seems to establish different phosphorylation pattern on the same receptor resulting in a complex cellular response. Furthermore, it was found that the phosphorylation pattern can be ligand

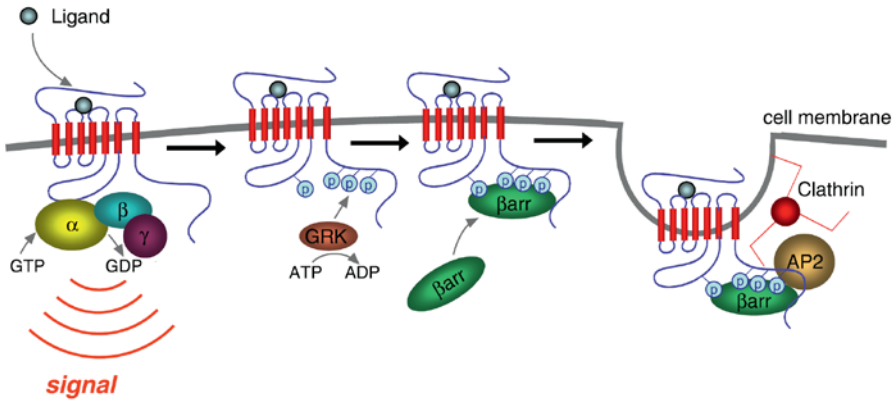

Figure 1.4 Arrestin Binding and Internalization
This image represents a temporal overview of GPCR signaling and desensitization triggered by agonist interaction. After the G protein binding and activation, the receptor gets phosphorylated by a GRK mainly at the C-terminus followed by arrestin binding. Arrestin can scaffold AP2 and clathrin resulting in an invagination of the cell membrane into clathrin coated pits. The pits are pinched off by dynamin (not shown) (image taken and modified from Irannejad & von Zastrow 2014).

specific and varies between different cell types and tissues (Butcher et al., 2011; Torrecilla et al., 2007). In addition, it was shown that an agonist-regulated receptor dephosphorylation needs to be considered as a regulatory mechanism (Butcher et al., 2011). Taking these findings together the barcode theory was established to describe the specificity of arrestin, GRK and GPCR interactions (Bahouth and Nooh, 2017).

1.1.3.3 Endosomal Compartmentation and Recycling

Initially, agonist induced endocytosis of GPCRs was understood to primarily desensitize the GPCR signaling (Waldo et al., 1983). Due to the facts that the described internalization process is too slow for the observed desensitization kinetics (Lohse et al., 1990) and stochastically the number of internalized receptors does not agree with the percentage of desensitization (Roth et al., 1991), phosphorylation and arrestin binding was understood as the main desensitization trigger rather than the internalization process itself (Lohse, 1993). In combination with the scaffolding properties of arrestin and the activation of MAPK pathways, ideas were developed concerning compartmentation and endosomal signaling (Daaka et al., 1998; Luttrell et al., 1999). The first evidence for G_s-protein-mediated signaling from intracellular compartments was found in 2009 (Calebiro et al., 2009; Ferrandon et al., 2009) and later described as a biphasic model of signaling (Irannejad et al., 2013). Recently it was shown, that the intracellular signaling can also occur in the trans Golgi network (Godbole et al., 2017). However, once a GPCR has entered the endosomal compartment the receptor gets either recycled to the membrane or degraded in lysosomes.

1.1.4 Receptor Dynamics

In the past, GPCRs dynamics were regarded as a two-state model that exist either in an active or in an inactive conformation. This was derived on the one hand by using rhodopsin as a model receptor and on the other hand because of inadequate techniques that could not provide detailed insights into receptor activation dynamics. Nowadays, rhodopsin can be regarded as an exception rather than the rule, because the *cis-trans* isomerization of retinal upon photon absorption appeared to be exclusive for the on and off state of rhodopsin. The

Figure 1.5 Molecular Simulation of GPCR Motions
The figure shows a molecular dynamic simulation of an agonist bound adrenergic receptor at the atomic level. Shown is the structural rearrangement of the helices due to the transition of the active receptor to the inactive state. The highlighted helix in red represents TM6 showing the biggest displacement and thus indicates the degree of mobility of GPCRs in general (image was taken and modified from Latorraca *et al.* 2017).

understanding of GPCR dynamics benefited during the last years from the tremendous efforts that were made in X-ray crystallography and in bioimaging. Especially the direct comparison of the active and inactive states of the β2 adrenergic receptor (β_2AR) (PDB: active=3SN6 / inactive=2RH1) (Cherezov et al., 2007; Rasmussen et al., 2011), the muscarinic Acetylcholine receptor (mAChR) subtype 2 (M_2) (PDB: active=4MQS / inactive=3UON) (Kruse et al., 2012; Kruse et al., 2013) and the μ opioid receptor (μOR) (PDB: active=5C1M / inactive=4DKL) (Huang et al., 2015; Manglik et al., 2012) changed substantially the understanding of receptor activation states. In combination with molecular dynamic simulations (MD) GPCRs can be regarded as highly dynamic structures and more as a liquid than as a solid phase with a constant motion at an atomic level (Figure 1.5) (Latorraca et al., 2017). For class A GPCRs the most prominent structural rearrangements occur at the intracellular coupling interface within TM5-7. The biggest motion is performed by TM6 that is rotating and swinging nearly 14 Å. In contrast TM5 and TM7 perform a slightly inward movement accompanied by a rotation. Recent studies revealed that even in the absence of an effector, GPCRs can adopt various conformations of different free energies without favoring a distinct conformation (Manglik et al., 2015). The addition of an agonist stabilizes a conformation characterized by a free energy valley and consequently the probability for the receptor to stay in this conformation increases significantly (Figure 1.6).

This multistate perspective can also describe different effectors such as antagonists, inverse agonists and partial agonists. The physiological kinetics of the transition from the inactive to an active state was investigated intensively in the past. Rhodopsin was the first studied GPCR and an activation time of 1-2 ms have been described (Lohse et al., 2014). Other class A GPCRs have intensively been studied with fluorescent energy transfer (FRET) studies that revealed activation kinetic of 30-50 ms for different receptors, i.e. α2-adrenergic, β1-adrenergic and M3-muscarinic (Hoffmann et al., 2005; Maier-Peuschel et al., 2010; Rochais et al., 2007; Vilardaga et al., 2003; Ziegler et al., 2011). For class C GPCRs that function as a dimer like the metabotropic glutamate receptor 1 (mGluR1) intra- and intersubunit kinetics of ≈50 ms and ≈30 ms have been described (Hlavackova et al., 2012).

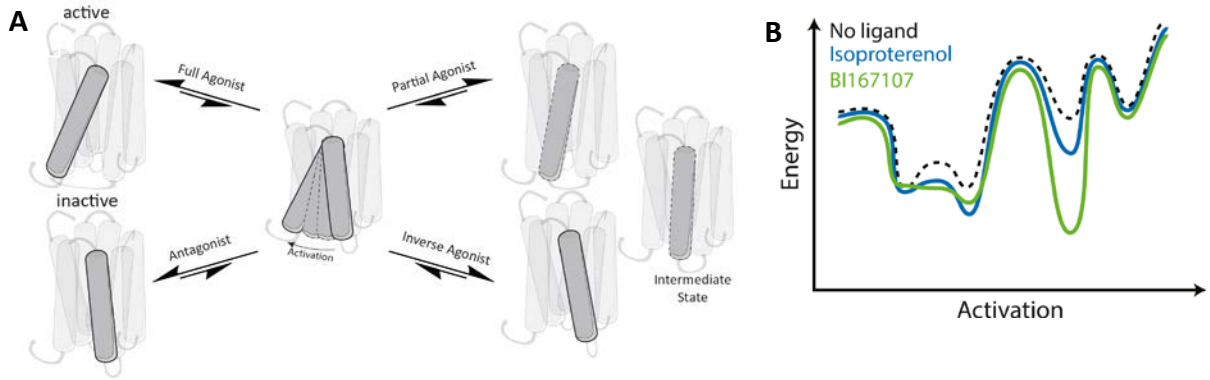

Figure 1.6 Different States of Receptor Activation

In (**A**) a steady state concept of receptor dynamics is displayed. It describes that a receptor can adopt diverse different conformations even in the ligand unbound state. The resting time in a distinct state is determined by the probability. Upon ligand binding the probability of resting in a distinct state increases displayed by a conformational change (image was taken and modified from Kauk & Hoffmann 2017). (**B**) shows the energy level of different activation states. The grey curve represents an unbound receptor whereas the green and blue curves represents agonist bound receptors. Both ligands stabilize the active receptor conformation indicated by lowering different energy level (image was taken and modified from Manglik *et al.* 2015).

1.1.5 Scientific and Therapeutic Relevance of GPCRs

The five protein families that represents the most druggable targets are ion channels, kinases, nuclear hormone receptors, proteases and GPCRs (Hauser et al., 2017). 33-34 % (475 drugs) of all by the US Food and Drug Administration (FDA) approved therapeutics act on GPCRs indicating the therapeutic relevance of this protein family (Figure 1.7). These therapeutics account for 27 % of the global market share of therapeutic drugs which corresponds to an equivalent of US$ 890 billion for the period from 2011 to 2015. During the past five years, 69 new drugs have been approved which target GPCRs and these numbers have been predicted to even increase over time. Structural knowledge about GPCRs and receptor dynamics has changed in the last five to ten years and therapeutics that were derived from structure based drug design are about to enter clinical trials (Hauser et al., 2017; Santos et al., 2017). From the scientific point of view, the number of available scientific publications could indicate the relevance of a topic for the research community. Here, the number of publications increased dramatically since the 1970s until 2015 by a factor bigger than 500 (Figure 1.7). In addition, several Nobel Prizes have been awarded to scientists dealing with GPCR research. The Nobel Prize for Physiology and Medicine was awarded to Earl Sutherland for his discoveries concerning "the mechanisms of the action of hormones" in 1971. Sir James W. Black, Gertrude B. Elion and George H. Hitchings received

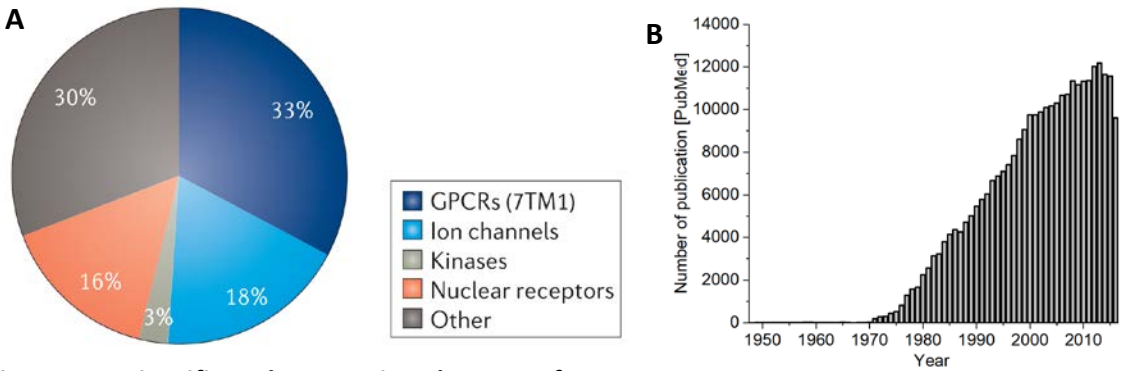

Figure 1.7 Scientific and Economic Relevance of GPCRs

The pie chart in (**A**) displays the proportion of small molecule drugs targeting the major protein families. It is shown that GPCRs represent by far the biggest druggable protein family in the human body (image was taken and modified from Santos *et al.* 2017). The diagram in (**B**) shows the development of publication on NCBI-PubMed dealing with GPCRs since 1945 (the graph was prepared by the author from data provided by NCBI PubMed for publications containing the catchword G protein-coupled receptor).

the Nobel Prize in 1988 for their discoveries of "important principles for drug treatment". In 1994 Alfred G. Gilman and Martin Rodbell were awarded the Nobel Prize in Physiology or Medicine “For their discovery of G-proteins and the role of these in signal transduction in cells”. 2000 Arvid Carlsson, Paul Greengard and Eric Kandel were honored for their discoveries concerning "signal transduction in the nervous system" and in 2004 Richard Axel and Linda B. Buck got the Nobel Prize for their discoveries of "odorant receptors and the organization of the olfactory system". In 2012 Robert Lefkowitz and Brian Kobilka shared the Nobel Prize in Chemistry “For studies of G-protein-coupled receptors”. This high number of academic distinctions over the last decades can also be seen as a convincing indication for the scientific relevance of GPCR research.

1.2 Muscarinic Acetylcholine Receptor Family

Discovered in 1915 by Henry Hallett and later described as “Vagusstoff” by Otto Loewi, acetylcholine (ACh) was the first described neurotransmitter (Loewi, 1922). Today it is known that ACh is one of the major neurotransmitters in the central nervous system (CNS) and the peripheral nervous system (PNS) mainly interacting with two membrane receptor classes, the nicotinic acetylcholine receptors (nAChRs) and the muscarinic acetylcholine receptors (mAChRs) (Wess, 2004). nAChRs act as membrane cation channels that bind to acetylcholine whereas mAChRs belong to the aminergic class A GPCRs. There are five subtypes of mAChRs (M_1-M_5) that show sequential homology but can be distinguished by their expression pattern and signaling priorities (Caulfield, 1993; Caulfield and Birdsall, 1998; Wess, 1996).

1.2.1 The Five Subtypes

The five muscarinic subtypes can be subdivided into two classes. The M_1, M_3 and M_5 receptors predominantly couple to the G_q protein family, whereas M_2 and M_4 couple primarily to the G_i protein family (Figure 1.8) (Wess et al., 2007). The signal transduction by G_q results in an activation of the membrane bound phospholipase Cβ (PLCβ). The lipase hydrolyzes the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into cellular second messenger molecules, namely soluble inositol trisphosphate (IP3) and membrane bound diacylglycerol (DAG) two cellular second messenger molecules. IP3 interacts with IP3 receptors at the endoplasmic reticulum (ER) resulting in a calcium ion (Ca^{2+}) release. Ca^{2+} can influence several other signaling pathways, e.g. by calmodulin binding. DAG activates protein

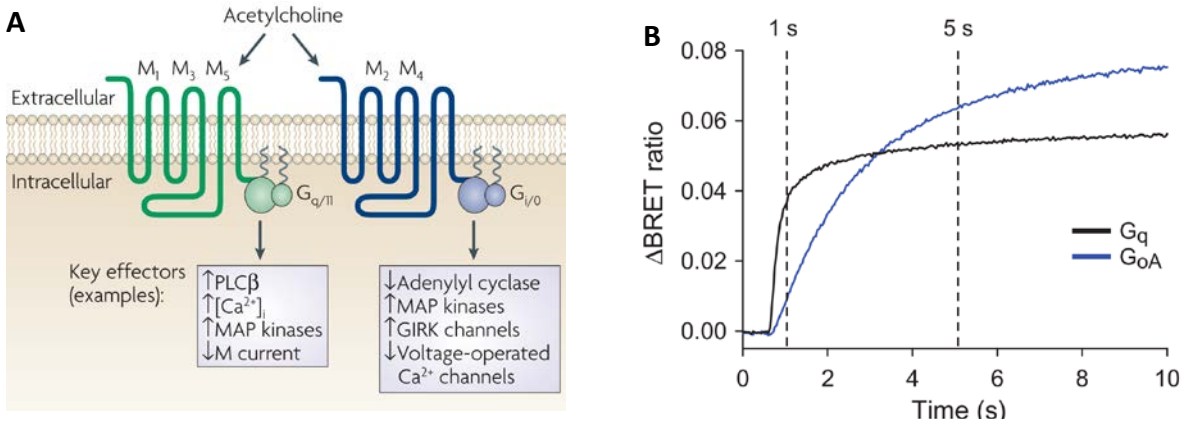

Figure 1.8 Signaling Specificities of the Five Muscarinic Subtypes

In (**A**) the signaling specificities of the five muscarinic subtypes is displayed. The odd numbered subtypes couple primarily to Gq proteins whereas the even numbered subtypes couple primarily to Gi proteins (figure was taken and modified from Wess *et al.* 2007). The graph displayed in (**B**) show the signaling behavior of the M_3 receptor. Upon receptor activation the receptor couple to Gq and induces a very fast and prominent Gq signal. Interestingly on long term the receptor shows a slow Go coupling that at some point shows an even higher efficacy than the Gq pathway (figure was taken and modified from Masuho *et al.* 2015).

kinase C (PKC), a long acting kinase that can modulate the activity of several other signaling pathways via phosphorylation of substrate molecules. G_i protein activation mainly results in an inhibition of the adenylate cyclase (AC). The AC is an enzyme the catalyzes the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). It can also activate, albeit to small extent, the PLC resulting in a IP3 and DAG production (Wess et al., 2007). Recent studies have shown that GPCRs do not necessarily couple to one G protein exclusively. G-protein coupling was described to be a compromise of an efficacy factor and an affinity factor. For the M_3 it was reported that the G_q response is the fastest or the primary G-protein coupling but there seems to be an additional long-term coupling to G_o with a slow kinetic but with a very high efficacy (Figure 1.8) (Masuho et al., 2015). In agreement to that it was also reported before, that the M_1 does not solely couple to G_q but also to G_s. Concerning the M_2 it is known that it can couple to both Gi and Gs proteins and due to that muscarinic receptors, especially the M_2 became a model for studying biased agonism and dualsteric ligands (Bock et al., 2016; Bock et al., 2014; Bock et al., 2012).

1.2.2 Physiological Relevance

In the past, different studies showed the ubiquitous presence of mAChRs with overlapping expression distributions indicating a crucial role in the CNS and PNS (Levey, 1993; Wolfe and Yasuda, 1995). M_1, M_4 and M_5 subtypes are mainly expressed in the CNS whereas M_2 and M_3 are present in both the CNS and in the PNS. The M_1 subtype is abundantly expressed in forebrain tissues including striatum, cerebral cortex and hippocampus. These brain regions are thought to be involved in learning and memory processes. In addition, recent studies provide evidence that the M_1 is of importance for myelination and demyelination processes (Mei et al., 2016; Plemel et al., 2017). In peripheral regions, the M_2 is mostly expressed in the heart, in smooth muscles and in skin tissues, mediating negative chronotropic effects and facilitating smooth muscle contractility (Brodde and Michel, 1999; Eglen et al., 1996; Wess, 2004). Additionally, the M_2 is expressed in the CNS in the hypothalamus and the spinal marrow and seems to be involved in body temperature regulation and analgesic responses (Gomeza et al., 1999a). In the PNS, the M_3 subtype is mainly expressed in smooth muscles and glands suggesting involvement in muscle contraction for example in the urinary bladder, pupillary muscles or in the airway and glandular secretion (Eglen et al., 1996; Gautam et al., 2006; Matsui et al., 2000). In the CNS, the M_3 is mainly expressed in the hypothalamus and is thought to play a role in the regulation of appetite (Yamada et al., 2001). However, this finding was controversially discussed because a second group reported the M_3 to be essential for salivary secretion during eating (Nakamura et al., 2004). Hence, the reduced appetite reported previously was addressed to the unbalanced nutrition with dehydrated food. The M_4 subtype is preferentially expressed in the forebrain (Levey, 1993; Wess, 1996). There is little known about the physiological relevance of this subtype due to the overlapping expression pattern with the M_2 and the same primary signaling via Gi protein but it is thought to be involved in analgesic effects and in the regulation of dopamine release in neurons and thus in locomotion (Gomeza et al., 1999b; Zhang et al., 2002). The M_5

receptor is predominantly expressed in the CNS in both neuronal and non-neuronal cells. It is the only mAChR that was detected in dopaminergic neurons of the midbrain and was described to modulate the dopamine release. Additionally, the M_5 is expressed in peripheral and cerebral blood vessels and was described to mediate vasorelaxing effects (Bonner et al., 1988; Eglen and Nahorski, 2000; Phillips et al., 1997; Weiner et al., 1990). However, it has been very challenging to address physiological effects to distinct subtypes mainly due to a lack of orthosteric ligands that can selectively activate or inactivate a specific receptor subtypes. In addition, the overlapping receptor expression distributions in many tissues increased the complexity of this work (Wess, 2004; Wess et al., 2007). Most of the data were obtained by investigating phenotypic characteristics in single or double knock out mice (Thomsen et al., 2017).

1.2.3 Structural Comparison

The five mAChR subtypes belong to class A GPCRs and thus show the general structural properties of GPCRs. The basic structural knowledge was gained by radio ligand binding, mutational analysis and sequence analysis studies (Abdul-Ridha et al., 2014; Gregory et al., 2007; Hulme et al., 2003a; Hulme et al., 2003b; Vogel et al., 1997; Wess et al., 1992). All mAChRs have a relatively large IL3 domain and it was shown, that the N-terminal portion of the loop is involved in G-protein coupling and G-protein selectivity (Lechleiter et al., 1990). The ligand binding appears to be mediated by a conserved Asp ($D^{3.32}$) residue in TM3 that forms a charged interaction with the ligands positively charged amino head group. Interestingly, this kind of charged interaction exists for all aminergic GPCRs, e.g. adrenergic, dopamine or histamine receptors (Kooistra et al., 2013; Wess et al., 1992). Other motifs that are highly conserved in GPCRs and thus can be found for example in rhodopsin or adrenergic receptors is the DRY motive in TM3 and the NPXXY motive in TM7. Both motifs are described to stabilize the active state of the receptors and to be of general relevance for receptor activation. Early reports showed a high sequence similarity between the five mAChRs especially for the TM domains. Because of that, the orthosteric ligand binding region that is formed by the TM domains appears to be nearly identical for all five subtypes. This resulted in big difficulties for the development of ligands that selectively bind to distinct receptor. N- and C-terminus of the receptors are less conserved and the loop regions show only minor similarity.

1.2.4 Crystal Structure

Over the last ten years, enormous efforts were made to resolve 3D structures of GPCRs via X-ray crystallography. To this date five different crystal structures of mAChRs are available. The tiotropium bound inactive M_1 structure was resolved in 2016 by replacing the highly flexible IL3 with a T4 lysozyme and removing glycosylation sites (Thal et al., 2016). Similar modifications were done in 2012 for obtaining the inactive 3-quinuclidinyl-benzilate (QNB) bound M_2 structure (Haga et al., 2012). The active iperoxo-bound M_2 structure was resolved by using an active state selective G-protein mimetic nanobody in 2013 (Kruse et al., 2013). In 2012 the tiotropium bound inactive M_3 structure could be resolved by using the T4 lysozyme fusion strategy (Kruse et al., 2012). The inactive tiotropium bound M_4 structure was solved in 2016 by replacing the IL3 with a minimal T4 lysozyme and the truncation of the N-terminus (Thal et al., 2016). Till today the M_5 receptor structure has not been resolved. The direct comparison of the four inactive receptor structures revealed a very high similarity of the tertiary structure for the whole mAChR family especially in the TM domains. Whether the minor differences in the ECL domains or in the ICL domains are of significance is still a matter of debate (Figure 1.9). Since the introduced protein modifications or crystal packing

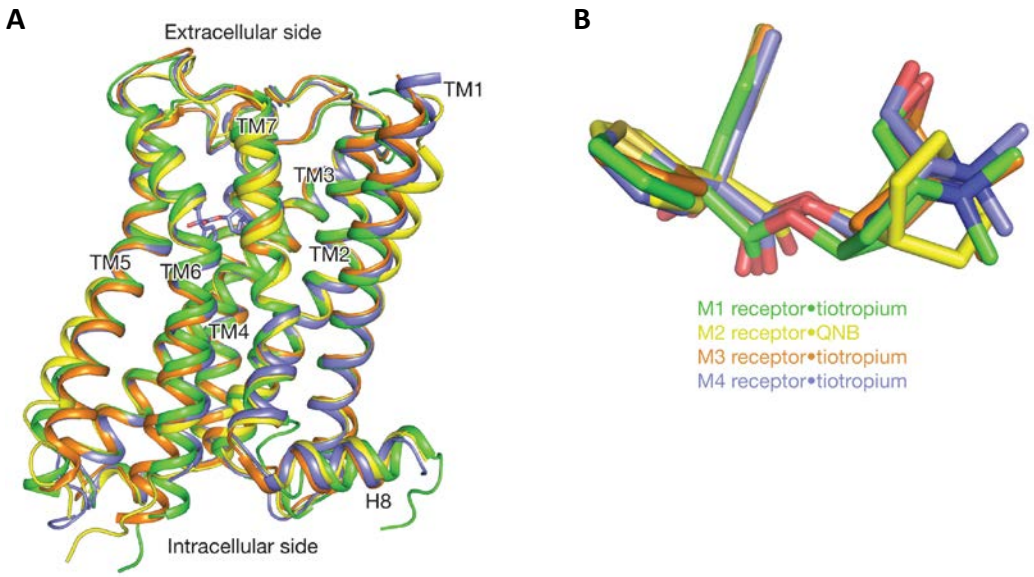

Figure 1.9 Structural Comparison of the mAChR subtypes

Figure (**A**) represents an overlay of the crystal structures of the M_1-M_4 receptors bound to an antagonist. The helices are rainbow colored and numbered. Interestingly, the overall structure of the inactive receptors appears to be nearly identical. The structural information about the IL 3 is not resolved for all four subtypes (image was taken and modified from Thal *et al.* 2016). (**B**) compares the position of the orthosteric bound antagonist. The structural superposition indicates a nearly equal ligand conformation in the inactive receptor structure. This can be seen as a strong indication for a nearly identically shaped orthosteric binding pocket for all receptor subtypes (image was taken and modified from Thal *et al.* 2016).

interactions could in theory induce similar effects (Thal et al., 2016). The nearly identical binding positions of the co-crystallized inverse agonists tiotropium/QNB confirms the homologous shape of the orthosteric binding pocket (Figure 1.9) (Thal et al., 2016). Another interesting structural property for muscarinic receptors is the aromatic lid structure that is located above the orthosteric binding site. This lid is described to be in an open conformation for an inactive receptor and to close during the receptor activation after ligand binding (Kruse et al., 2013). Formed by three tyrosine residues from the helical domains TM3, TM6 and TM7 the lid structure seems to be a unique characteristic for mAChRs when compared to other aminergic GPCRs. The closure of the aromatic lid was described to be essential for receptor activation and mutational studies described that the partial or complete removal of the lid results in a substantial loss of receptor activity (Gregory et al., 2007; Vogel et al., 1997; Wess et al., 1992). Until now the structural comparison is only possible for inactive mAChRs and thus all observations that have been made are true only for the inactive state. This does not preclude the possibility of differences in tertiary structure or conformational differences upon receptor activation (Thal et al., 2016).

1.2.5 Activation Mechanism of mAChRs

The direct comparison of active and inactive receptor structures is essential for developing models for the receptor activation process and are thus indispensable for drug research (Latorraca et al., 2017). This comparison can be made for a variety of GPCRs but concerning mAChRs solely for the M_2 subtype (Kruse et al., 2012; Kruse et al., 2013). However, due to the sequence homology in the mAChR family a distinct similarity between the subtypes can be considered concerning the activation process. The key feature of receptor activation is the outward movement of TM5 and TM6 creating a pocket on the intracellular site. Here, the interaction of active receptor and G protein takes place. The displacement of TM6 is bigger than 10 Å. The outward movement of the TM domains on the cytoplasmic side is reflected by an inward movement of the TM domains on the extracellular side and thus a narrow geometry above the bound ligand is formed (Figure 1.10). Next, reformations of the interaction networks of the DRY motive in TM3 and the NPXXY motive in TM7 occur, leading to a slight rotation of TM3 and an unwinding of TM7. This is of importance, because now residues in TM3 can stabilize the receptor ligand interaction and thus the active conformation. In parallel, the movement of TM3, TM6 and TM7 enables polar interactions between Tyr104$^{3.33}$, Tyr403$^{6.51}$ and Tyr426$^{7.39}$ resulting in the closure of the tyrosine lid over the agonist (Figure 1.10).

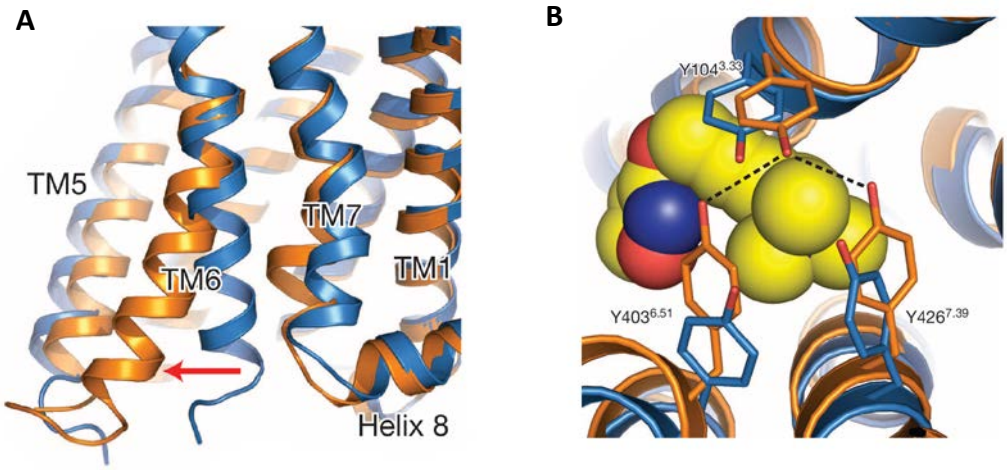

Figure 1.10 Structural Comparison of the Active and Inactive M_2 Structure
In figure (**A**) the helical rearrangement upon receptor activation can be seen. Blue helices represent the inactive receptor structure whereas orange helices represent the active M_2 structure. The image clearly show that the structural rearrangement occurs mainly in the helices 5-7 whereas helix 8 barely moves (image was taken and modified from Kruse *et al.* 2014). Figure (**B**) shows a top down perspective into the orthosteric binding pocket occupied by iperoxo. The aromatic lid formation can be clearly seen. The three tyrosine residues $Y^{3.33}$, $Y^{6.51}$ and $Y^{7.39}$ show an open conformation in the inactive (blue) state and a closed conformation that is stabilized by a hydrogen bond in the activated (orange) state (image was takes and modified from Kruse *et al.* 2014).

1.2.6 Importance of mAChRs for Drug Development

Muscarinic receptors are important key regulators in the human body for diverse somatic functions and thus are important targets for treating or investigating diverse pathophysiological conditions. Already today numerous mAChR-targeted therapeutics are of clinical relevance (Svoboda et al., 2017). With respect to glaucoma therapy, the agonist carbachol and the partial agonist pilocarpine are used to reduce the intra ocular pressure. The antagonist atropine and tropicamide are of relevance for ophthalmology by regulating the dilation of the pupil. For treating an overactive bladder, antagonists like tolterodine, oxybutynin and sesoterodine are used (Abramov and Sand, 2004; Tzefos et al., 2009). Additionally, for treating urge urinary incontinence the antagonists darifenacin and solifenacine are in use. The most relevant clinical application for muscarinic effectors is the treatment of chronic obstructive pulmonary disease (COPD), a lung disease. In this regard, Tiotropium is currently used as a long-acting bronchodilator due to its pharmacological properties as a high affinity M_3 antagonist. A second used drug is ipratropium, a short acting antagonist. Both drugs are commonly used in inhalers (Keating, 2012; Restrepo, 2007). There are two mAChR antagonists (Biperiden and Trihexyphenidyl) applied in the therapy of Parkinson especially for treating symptoms like tremors and rigidity (Brocks, 1999; Svoboda et al., 2017). Recently, the antagonist scopolamine was described to exhibit antidepressant properties (Witkin et al., 2014). The use of anticholinergic drugs has often been questioned because of the numerous side-effects such as a dry mouth or problems with urinary retention, cognitive impairment, blurred vision or even psychosis and addiction (Desmarais

et al., 2012; Ogino et al., 2014). This is mainly due to the abundant expression pattern and the homology of the ligand binding pocket between the mAChRs. However, despite of risks and side effects antimuscarinic drugs provide a huge potential for clinical applications, if dosed with care (Svoboda et al., 2017). mAChRs are regarded as promising targets for a variety of diseases for which current treatments are insufficient or not available (Kruse et al., 2014). For instance, currently there are no effective drugs in preventing Alzheimer's disease. Here several studies have reported the importance of the M_1 receptor as a potential target (Davis et al., 2010; Medeiros et al., 2011). In addition, targeting the M_1 and M_4 subtypes in brain tissues with the partial agonist xanomeline was described to be potentially clinically useful for the treatment of schizophrenia (Dencker et al., 2011; Shekhar et al., 2008). Several subtypes were reported to provide a promising therapy for treating drug abuse or drug addiction (Schmidt et al., 2011; Thomsen et al., 2012; Wess et al., 2007). Next, the M_1 has been described to play an important role for demyelination and myelin reformation (Mei et al., 2016; Plemel et al., 2017). This finding is of interest in the future therapy for multiple sclerosis. M_3 receptors that are expressed in pancreatic β-cells have been described to play an important role in insulin secretion and thus a selective M_3 activation is thought to have impact on type 2 diabetes treatment (Jain et al., 2013; Kong et al., 2010). There is also evidence, that certain muscarinic subtypes play important roles in cancer formation. It was shown, that M_3 deficient mice showed reduced cell proliferation and tumor number and that M_1 knock out mice show decreased prostate cancer invasion (Magnon et al., 2013; Raufman et al., 2008; Raufman et al., 2011).

1.3 Allostery

The concept of proteins showing indirect interactions between distinct specific binding sites was formalized in 1965 as the Monod-Wyman-Changeux (MWC) model (Monod et al., 1963; Monod et al., 1965). Based on this model allostery is defined as an effect produced by the simultaneous binding of at least two molecules in two different regions of a protein, without having any kind of direct interaction (Kenakin, 2010). The effector binding induces a molecular/allosteric transition reflected by a reversible conformational change in the protein structure. At this point it can be discriminated between homotropic allosteric effects that can be found between identical ligands (e.g. Bohr-effect that describes cooperativity of oxygen binding by hemoglobin) and heterotropic allosteric effects that can be found between different ligands (Monod et al., 1965). Monod described the existence of more than one potential binding site (allosteric site) next to the binding site of the endogenous substrate or agonist (orthosteric site). Initially described in the field of enzymology and later also for GPCRs, allostery can nowadays be extended to probably all bigger protein families including GPCRs, ligand-gated ion channels, voltage-gated ion channels, nuclear hormone receptors and receptor tyrosine kinases (Changeux and Christopoulos, 2016; Christopoulos, 2014; Clark and Mitchelson, 1976; Lüllmann et al., 1969). In this circumstance, it is possible to describe from a more generous point of view the GPCR G protein interaction as a heterotropic allosteric phenomenon (May et al., 2007). The research field of allosteric

modulators that can alter the orthosteric ligands binding affinity and efficacy is currently a very prevailing field with numerous innovations. In addition, the gained knowledge is of high clinical relevance because in contrast to the orthosteric binding region, allosteric binding regions are less conserved. Due to that selective receptor subtype binding of allosters was reported in the past. The possibility of selectively targeting a distinct receptor can contribute to desired therapeutic effects and the reduction of side effects (Christopoulos, 2014).

1.3.1 Allosteric Modulation

In agreement with the MWC model, GPCR targeting allosteric modulators interact with another distinct location when compared to the endogenous orthosteric binding region. Interestingly, this binding region highly depends on the chemical properties of the allosteric ligand and can differ among different modulators and different receptors (Congreve et al., 2017; De Amici et al., 2010). The alloster can bind to the receptor in the presence or absence of an orthosteric ligand. The simultaneous binding of alloster and orthoster is described as a ternary complex formation and thus is formalized in the allosteric ternary complex model (ATCM) (Figure 1.11). In this ternary complex a reciprocal binding cooperativity between alloster and orthoster is exhibited and in general there is a discrimination between positive, neutral and negative cooperativity (May et al., 2007). Positive allosteric modulators (PAMs) can enhance the binding or the maximum effect of an orthoster and show a cooperativity factor α>1. This is reflected in a left-shifted binding curve or concentration response curve and can show higher efficacy values. Negative allosteric modulators (NAMs) can also be described as noncompetitive inhibitors due to a negative binding or activation cooperativity

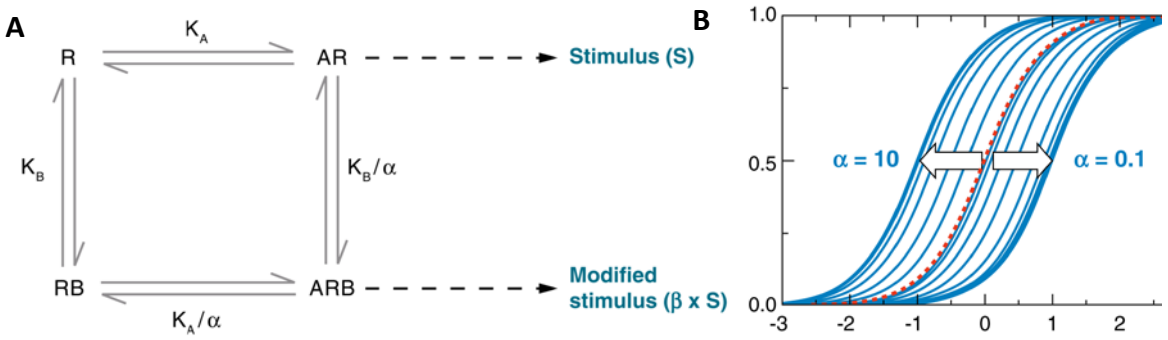

Figure 1.11 Allosteric Modulation of GPCRs

Figure (**A**) shows the simple ATCM that can be used to describe GPCR (R) orthoster (A) interaction in the presence or absence of an allosteric modulator (B). A receptor can either bind an orthoster and form a stimulating complex (AR) with a dissociation constant K_A or an allosteric modulator and form a non-stimulating complex RB with a dissociation constant K_B. The two complexes can now be bound by the third component to form the stimulating ternary complex ARB with the corresponding dissociation constant modified by a cooperativity factor α (the figure was taken and modified from May *et al.* 2007). In figure (**B**) the effect of α towards a concentration response relationship is shown. Positive cooperativity displayed by α>1 results in a clear left shift of the curve. Negative cooperativity characterized by α<1 results in a clear right shift of the curve. Allosteric modulators with an α=1 do not affect the concentration response correlation and are thus named neutral allosteric modulators (image was taken and modified from May *et al.* 2007).

(De Amici et al., 2010). With a cooperativity factor α<1 binding or activation curves show a significant right shift and can show a reduced efficacy. Neutral or silent allosteric modulators have no effect on binding or activation properties of the orthosteric ligand and thus show a cooperativity factor of α=1 (Figure 1.11). Cooperative effects of allosteric modulators are highly sensitive to the conformational change of the receptor and thus to the bounds orthosteric ligand. This effect is called probe dependency (Jakubik et al., 1997). In addition, the degree of modulating a receptors activity is limited and can reach a saturation (ceiling effect). This means that a fully activated receptor cannot show an increased activation by adding a PAM or a NAM addition can never lead to a full displacement of the orthosteric ligand.

1.3.2 Bivalent Ligands

For modern drug discovery orthosteric and allosteric ligands can show partially beneficial properties but also partially disadvantageous properties. Thus, it seems reasonable to covalently link two pharmacophores and create a new molecule aiming to profit mainly from desired characteristics and to exclude undesired effects. The so-called class of bivalent ligands represent a very interesting therapeutic design for modern drug development and pharmacological research. The most convincing nomenclature of bivalent ligands have been introduced by Bock & Mohr in 2013 (Bock and Mohr, 2013). Bivalent ligands consist of two covalently linked pharmacophores and consist either of two identical (homobivalent) or different (heterobivalent) pharmacophoric subunits. Heterobivalent ligands or bitopic ligands can now be further subdivided into three subclasses due to the binding properties of the molecule. Bitopic ligands can show monovalent binding at two different target structures at different timepoints, bitopic binding to two different target structures at the same time or dualsteric binding to the same target structure simultaneously (Bock and Mohr, 2013).

1.3.3 Dualsteric Ligands

The concept of dualsteric ligands for mAChRs was introduced for the first time mAChR in 2006 by Desingrini *et al.* who reported a hybrid consisting of the high affinity orthosteric agonist iperoxo and a M_2 selective allosteric ligand W84 or naphmethonium (Disingrini et al.,

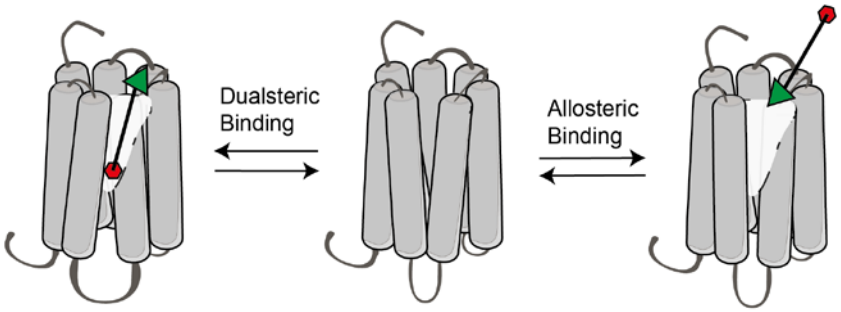

Figure 1.12 Dynamic Ligand Binding Model for Dualsteric Ligands
The displayed model describes different receptor binding poses of dualsteric ligands. It is indicated that binding to a GPCR can either occur in a dualsteric, receptor activating mode or in a purely allosteric, inactive mode.

2006). In 2009 Antony *et al.* fused a highly potent orthosteric ligand to different allosteric modulators (Antony et al., 2009). This new ligand design could induce receptor specific activation profiles by interacting with the orthosteric and allosteric site simultaneously. mAChRs have always been a model system for investigating not only allostery but also dualsteric ligands because the M_2 subtype was the first GPCR described as being sensitive to allosteric modulation (Lüllmann et al., 1969) and intensive efforts were spent towards mutational analysis of the orthosteric and allosteric binding region and thus the structural and dynamic understanding for these receptors was advanced despite of not having a crystal structure (Mohr et al., 2010). The binding mode of dualsteric ligands must be distinguished from the ATCM model because here two independent molecules do no longer exist. The dynamic ligand binding model describes multiple binding states for dualsteric ligands like the dualsteric binding mode, purely allosteric binding mode or purely orthosteric binding mode (Figure 1.12) (Antony et al., 2009; Mohr et al., 2010; Valant et al., 2008). Both moieties contribute to the dynamic ligand binding in a different role. The orthosteric interaction provides the receptor activation due to high affinity binding and the allosteric interaction provides subtype selectivity and modulation characteristics towards the orthosteric interaction. Due to the binding equilibrium described by the dynamic ligand binding model, the majority of dualsteric ligands behave as partial agonists (Chen et al., 2015). Recent studies provided evidence that also the linker properties can have impact on activation kinetics and efficacy and thus can be regarded as a third moiety in dualsteric ligands (Agnetta et al., 2017; Matucci et al., 2016; Messerer et al., 2017). The field of dualsteric ligands is very promising for the development of future therapeutics displaying selective binding and activation properties.

1.3.4 Biased Agonism

Biased agonism describes an agonist that preferentially activates a downstream signaling pathway whereas another agonist in the same system preferentially activates a second or several downstream signaling pathways (Figure 1.13) (Michel and Charlton, 2018). This observation was made the first time in 1998 for a peptide ligand (Jarpe et al., 1998). Since then various scientific terms like functional selectivity, asymmetrical signaling or functional dissociation were developed describing the same phenomenon (Kilts et al., 2002; Urban et al., 2007; Whistler et al., 1999). The molecular basis for this can be found in the receptor ligand interaction process since different ligands are able to stabilize different receptor conformations (Manglik et al., 2015; Staus et al., 2016). This can then be reflected in a different profile of G protein coupling or in an altered ratio of different downstream pathways (Kenakin and Christopoulos, 2013; Masuho et al., 2015). However, understanding biased agonism is of relevance for future drug design and clinical applications. Opioids are frequently used as analgesics but can lead to respiratory depression, tolerance and constipation, effects that are linked to the recruitment of arrestin. Here, a drug that would prefer G protein signaling over arrestin recruitment is thought to be a promising strategy for future therapy (Bohn et al., 1999; Groer et al., 2007). Signaling properties can also be biased

by allosteric modulation (Kenakin and Christopoulos, 2013). This is of specific interest for the rational design of dualsteric ligands because with this strategy not only subtype selectivity can be achieved but also a desirable downstream signaling profile can be designed (Bock et al., 2014; Bock et al., 2012).

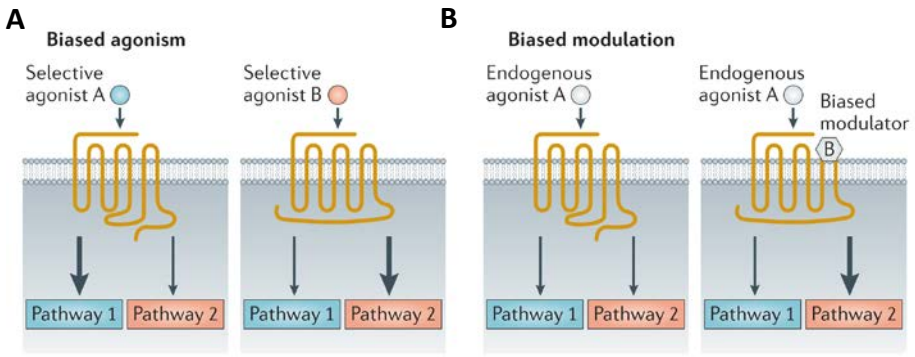

Figure 1.13 Biased Signaling on GPCRs
Here different origins of biased signaling are shown. Biased agonism as displayed in (**A**) results of different agonists preferring different downstream signaling events. This is mainly due to specific ligand receptor interactions. Biased modulation as shown in (**B**) is the result of a modulator binding event. Under normal conditions the unbiased endogenous agonist (A) does not prefer a distinct downstream pathway. Upon modulator binding signaling preferences are shifted towards one pathway (image was taken and modified from Kenakin & Christopoulos 2013).

1.4 Bioimaging Tools in Biomedical Research

1.4.1 Fluorescence

The ground state of a molecule is characterized as a low energy state of high stability. In this state all electrons exist in bonding orbitals (π) except for lone pairs. By introducing electromagnetic energy into the molecule, e.g. by light illumination, electrons can be exited into unoccupied anti-bonding orbitals (π*) of higher energy. This transition occurs from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) and is also possible for electrons in non-bonding orbitals (n). The energy difference between LUMO and HOMO defines the excitation wavelength of the light. There are different ways for returning into the ground state. The transition between the different electronic states is illustrated by the Jablonski energy diagram and explained by the Franck-Condon principle. The different energy states are arranged vertically and the spin multiplicity is grouped horizontally (Figure 1.14 Jablonski Energy DiagramFigure 1.14).

Under normal conditions an electron rests in a singlet ground state (S_0). After an electron absorbs a high energy photon the system reaches an electronically and vibrationally excited singlet state (S_n; v_n; 10^{-15} s). The relaxation from high energy states occurs mostly by internal conversion (IC), a nonradiative transition (10^{-12} s). This process involves the dissipation of energy from the molecule to its surroundings. The relaxation from the first singlet (S_1) state can occur by IC but also by emitting a photon. This phenomenon is called fluorescence and can also be described as a singlet-singlet transition and occurs within 10^{-9} s. A third way of relaxation is described by intersystem crossing, a transition to a state with a different spin multiplicity. The relaxation from the triplet state back to the singlet ground state belongs to the forbidden transitions. Since such transitions are less likely, they are delayed. This phenomenon is called phosphorescence and can take from milliseconds up to minutes. The energy of the emitted fluorescent photon is always smaller than the energy of the absorbed photon mainly due to IC processes. This leads to a spectral shift to a higher wavelength (red-shift) and is called Stokes shift.

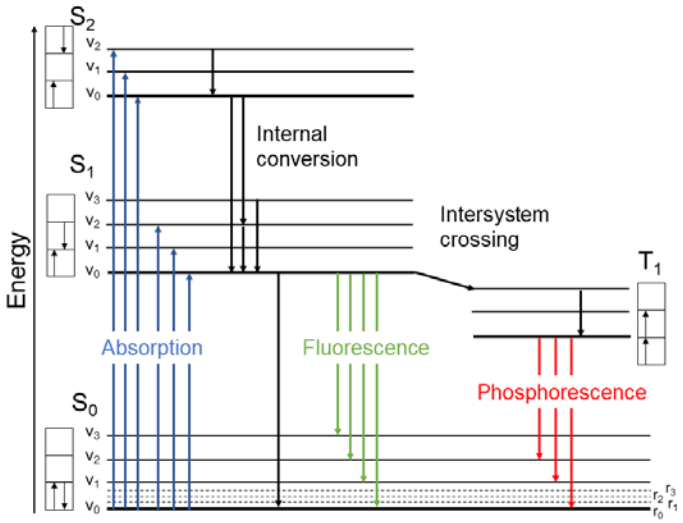

Figure 1.14 Jablonski Energy Diagram
The Jablonski diagram illustrates the electronic states of a molecule and can be used to explain the transition of electrons between different energy states. Upon light absorption an electron resting in the stable ground state (S_0) gets excited to a higher energy state (S_1 /S_2). Relaxation can now occur via internal conversion, emitting a photon (fluorescence) or by intersystem crossing that can also lead to a photon emission (phosphorescence).

1.4.1.1 Fluorescence Based Bioimaging

The direct visualization of cellular processes is one of the key components in biomedical research. Here, the discovery and the isolation of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was one of the important milestones and can be seen as a starting point for the field of fluorescence based bioimaging (Frommer et al., 2009). The fusion of GFP at a genetical level to a second target protein results is a fusion protein providing strong and visible fluorescence without any additional cofactors (Heim and Tsien, 1996). Described and initially characterized the first time in 1962 by Shimomura et al. GFP is composed of 238 amino acids (27 kDa) and shows a β-barrel structure made of eleven β-sheets (Figure 1.15) (Ormo et al., 1996; Shimomura et al., 1962; Yang et al., 1996). The chromophore (p-Hydroxybenzylidenimidazolinon) is buried inside the β-barrel and autocatalytically formed by the three amino acids Serin65, Tyrosin66 and Glycin67 located in an α-helical secondary structure. The autocatalytic process is divided into three steps cyclization, dehydration and aerial oxidation (Frommer et al., 2009).

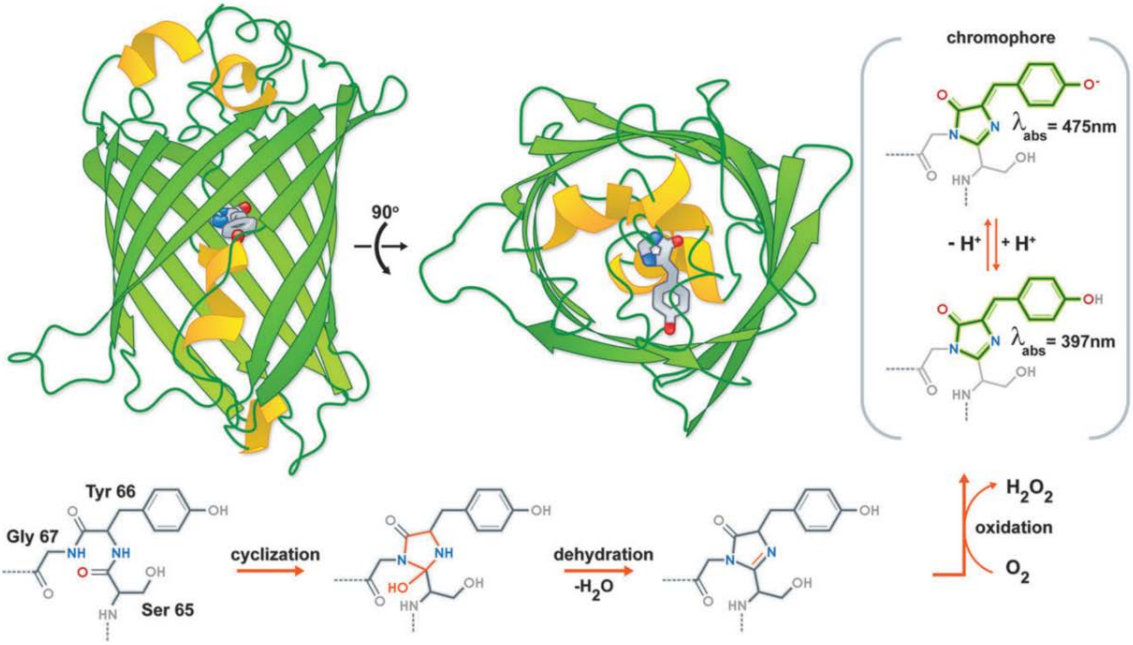

Figure 1.15 Green Fluorescent Protein

GFP is a beta-barrel structured 27 kDa protein containing a central chromophore. In the upper panel a schematic structure of GFP is given, showing a side view and a top down view. In addition, the chromophore formation is displayed. The formation is a three-step process autocatalytically done by three nearby amino acids (Ser65, Tyr66, Gly67). Absorption properties of GFP are dependent on chromophoric protonation states resulting in two absorption maxima in the excitation spectrum (the image was taken and modified from Frommer *et al.* 2009).

The excitation spectrum of GFP shows a dominant absorption maximum at 395 nm and a small absorption maximum at 470 nm. The emission spectrum shows a sharp peak at 505 nm with a shoulder at around 540 nm. The introduction of various mutations in the periphery of the chromophore resulted in altered absorption and emission spectra and thus today GFP and its derivatives can cover the entire visible spectrum (Miyawaki et al., 2003). In addition, the introduction of mutations also led to increased pH stability, temperature stability, rapid folding, monomeric configuration, higher photon yields and improved photo-stability. The isolation of the red fluorescent protein (RFP) from corals (*Discosoma*) extended the range of fluorescent proteins to the red and far-red spectra. Especially far-red shifted fluorescent proteins are of interest for tissue imaging because of the tissue penetrating properties of light with a longer wavelength (Miyawaki et al., 2003).

1.4.1.2 Protein Labeling Techniques

The discovery of GFP and its variants had a tremendous impact on bioimaging over decades. Nowadays, the field has reached an accuracy that the reliability of data relies critically on the ability to introduce a reporter probe precisely at the desired location without perturbing the protein or receptor functions (Tian et al., 2017). Therefore, the size of a GFP molecule that shows free rotation characteristics can be of disadvantage and thus the direct introduction into a protein of interest can always raise doubts. There are various alternatives for using

fluorescent proteins but all of them show individual strengths and weaknesses to be aware of. First, immunofluorescent based approaches using specific antibody antigen interactions are possible alternatives but due to the size of the antibody, the effort necessary to establish them and the possible alteration in protein function and integrity might lead to the conclusion that antibodies may not be the tool of choice. Additionally, membrane proteins like GPCRs that are embedded in cellular membranes are not completely accessible to the antibody. In contrast nanobodies (≈15 kDa) are much smaller than antibodies (≈ 150 kDa) and have emerged as a more promising tool not only for immune-imaging but also for crystallization purposes. Second, chemoenzymatic labeling approaches make use of the highly specific enzyme substrate interaction by using evolutionized enzymes and fluorescently modified substrates, e.g. SNAP-, CLIP- and HALO-tag (Gautier et al., 2008; Keppler et al., 2003; Los et al., 2008; Pober et al., 1978; Stryer, 1978). This technique can provide orthogonal labeling and a huge variety of different fluorophores. Unfortunately, the substrates are costly and can cross cellular membranes only with low efficiency which makes them rather unsuitable for intracellular assays. A third relevant approach is the introduction of unnatural amino acids (uaas) into a protein sequence that can be used for labeling purposes after translation (Huber and Sakmar, 2014; Noren et al., 1989; Pless and Ahern, 2013). In short, this technique uses modified stop codons as target site on DNA level. During protein biosynthesis a modified suppressor tRNAs gets inserted that can be chemically modified afterwards. The main drawback of this method are the reaction conditions that need to be applied to physiological systems and often show cytotoxicity.

1.4.1.2.1 Fluorescein Arsenical Hairpin Binder

The fluorescein arsenical hairpin binder (FlAsH) tag technology provides numerous advantages compared to the techniques described before. These small organic fluorophores (≈ 700 Da) bind to tetracysteine motifs of variable length that can be genetically introduced into a protein sequence (Figure 1.16). This means that a labeling protocol needs to be introduced in contrast to a genetically encoded fluorescent protein (Adams et al., 2002; Griffin et al., 1998). Interestingly, the FlAsH or the related redshifted variant ReAsH (resorufin arsenical hairpin binder) are provided in membrane permeable, non-fluorescent complexes with ethanedithiol (Tian et al., 2017). The high affinity interaction of FlAsH with cysteine motifs can leads to unspecific binding when labeling was performed with an immature protocol (Stroffekova et al., 2001). Optimized protocols can avoid unspecific binding and make the application even more specific and powerful (Hoffmann et al., 2010). In addition, protocols have been developed that allow an orthogonal labeling strategy by using FlAsH and ReAsH simultaneously (Figure 1.16) (Zürn et al., 2010). Intensive efforts led to a further development of arsenic hairpin binders resulting in a full coverage of the visible spectrum by diverse derivatives. By modifying the tetracysteine binding motif CCPGCC to flnCCPGCCmep and hrkCCPGCCktf higher quantum yields, improved the affinities and fluorescence intensities were obtained (Martin et al., 2005). FlAsH shows comparable optical properties to the yellow fluorescent protein (YFP). Thus, it was introduced into

pharmacologically relevant receptor sensors and lead to improved pharmacological properties compared to the established sensors. This was shown for fluorescent and bioluminescent sensors but will be described more precisely in the section about resonance energy transfer techniques (Bourque et al., 2017; Hoffmann et al., 2005; Nuber et al., 2016; Sleno et al., 2016). When compared to other fluorescent proteins, the main beneficial properties of the FlAsH technology can be seen in its small size, site specific introduction and its improved imaging properties.

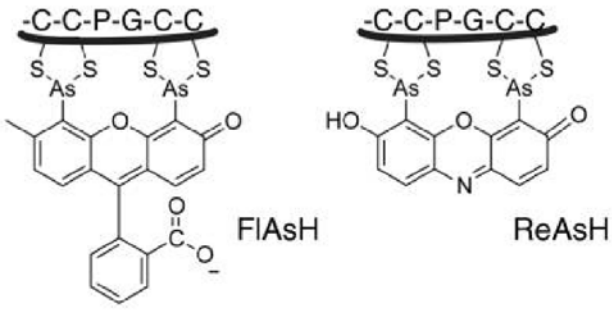

Figure 1.16 Arsenic Hairpin Binder
Shown is the chemical structure of the two most often used arsenic hairpin binder. The binding sequence consisting of six amino acids is genetically encoded. Thus, prior using these fluorophores a labeling procedure needs to be applied. Both fluorophores belong to peptide tag-based fluorescent probes (image was taken and modified from Tian *et al.* 2017).

1.4.2 Resonance Energy Transfer Techniques

1.4.2.1 Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a physical phenomenon described by Theodor Förster in 1948 for the first time and thus is also called Förster resonance energy transfer (FRET) (Forster, 1948). FRET is a nonradiative energy transfer between a donor fluorophore and an acceptor fluorophore via dipole-dipole coupling and depends mainly on three parameters. First, a spectral overlap of the donor emission and the acceptor excitation spectra is necessary. Second, FRET is distance dependent and can be mediated at distances between 10 Å to 100 Å. At lower distances a direct electron exchange can occur (Dexter energy transfer) and at higher distances the transfer efficiency is nearly 0. Third, the relative orientation of the dipole moments is of importance. The highest energy transfer efficiency is possible for parallel dipole moments (Forster, 1948; Jares-Erijman and Jovin, 2003; Lohse et al., 2012). The distance (R) between the two fluorophores and the Förster-radius (R_0) are two crucial points for FRET efficiency. R_0 describes the distance between two fluorophores where the energy transfer rate is exactly 0.5 and is dependent on the spectral overlap and thus on the donor-acceptor pair (Figure 1.17). FRET can be measured in three different ways via sensitized emission, acceptor photobleaching or fluorescent lifetime imaging (FLIM). From a molecular point of view FRET can be measured either as intra- or intermolecular FRET. Intermolecular FRET is used for protein-protein interaction studies and thus both interaction partners are fluorescently labeled. Here, it is of importance to take care of equimolar expression rates to avoid artefacts (Lohse et al., 2012). This circumstance is automatically assured for intramolecular FRET sensors with genetically encoded fluorescent proteins. Measuring FRET by applying FLIM provides the advantages that the fluorophore's

expression rate is of minor interest for data acquisition and that only the fluorescence intensity of the donor needs to be monitored. (Bastiaens and Squire, 1999). FLIM can be measured by observing the time domain or the frequency domain. The time domain is measured by using a pulsed laser excitation of the donor, where the pulse of the laser is much shorter than the lifetime of the fluorophore and the intensity decay of the donor represents the primary data (Figure 1.17). The frequency domain is measured by exciting the donor with sinusoidal modulated light. The emission of the donor fluorescence occurs with the same sinusoidal modulated information but with a reduced modulation depth and a shifted phase that serves as a readout (Bastiaens and Squire, 1999). The presence of an acceptor fluorophore affects the readout parameter in both cases and the information difference can be interpreted as a FRET effect.

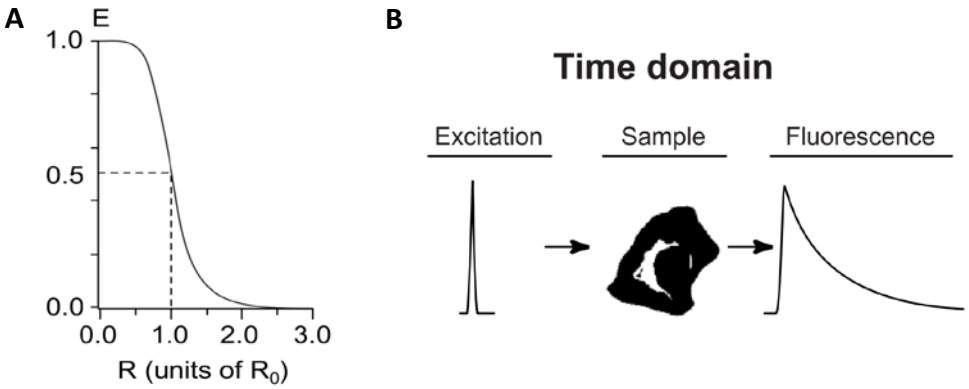

Figure 1.17 Bases of FRET and FLIM
(**A**) shows the relation between the distance of the fluorophores (R) and the efficiency of the energy transfer (E). As can be seen the energy transfer is highly distance dependent to a factor R^{-6}. The distance showing an energy transfer of 0.5 is called the Förster radius (F_0). Next to distance the spectral overlap of the fluorophores and the fluorophore dipole orientation is important for FRET efficiency (image taken and modified by Bastiaens *et al.* 1999). (**B**) describes the principle of FLIM. Once the energy state of a fluorophore is excited the fluorescence lifetime defines the average time the electron remains in the excited state. This donor fluorescence decay time can be significantly altered by an acceptor molecule via FRET. FlIM application only observe the donor fluorescence over time (image taken and modified by Bastiaens *et al.* 1999).

Approaching FRET by sensitized emission enables to monitor protein dynamics in case of kinetics, ligand specificity and efficacy. Therefore, the intensities of donor and acceptor fluorophore need to be monitored over time and a FRET signal is clearly defined by an antiparallel movement of the intensity parameters (Figure 1.18). Donor and acceptor fluorescence represent the primary data and are usually converted and displayed as a ratio (acceptor/donor). Before using this approach, it is necessary to include controls for equimolar expression rates, bleed-through from the donor into the acceptor channel and false excitation rates. A third approach that is frequently applied is time resolved FRET (TR-FRET). TR-FRET uses the fluorescent properties of lanthanoids but does strictly not belong to FRET techniques because there is no singlet-to-singlet transition and thus no fluorescence but relies on the same fundamental mechanism (Selvin, 2002). The advantages of TR-FRET

can be seen in a larger measurable range with a lower background information and almost no dependence on the dipole orientation compared to conventional FRET. Increasing the FRET efficiency by modifying the experimental environment has been the goal for researchers for many years. First, combining multiple donor molecules to increase the overall fluorescent information in the studied system and thus to obtain increased readout information (Hemmig et al., 2016; Olejko and Bald, 2017; Tanenbaum et al., 2014). Other approaches made use of measuring FRET in proximity to nanoparticles or used nanoapertures and nanoantenna (Aissaoui et al., 2017; Ghenuche et al., 2014; Ghenuche et al., 2015). These approaches showed promising effects on the FRET ratio but mostly adverse effects on the FRET efficiency. A recent study could show that not only a nanostructure is necessary for increasing the FRET efficiency but also the specific orientation of the fluorophores towards each other (de Torres et al., 2016).

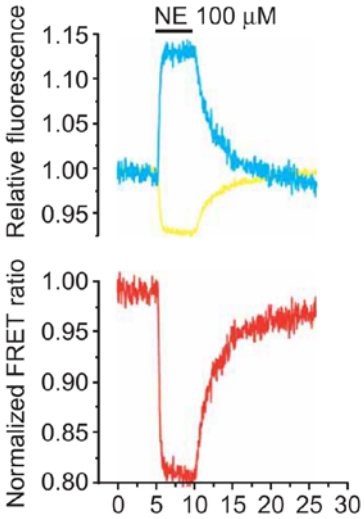

Figure 1.18 Sensitized Emission
Measuring FRET via sensitized emission demands the accurate readout of both the donor and the acceptor fluorescence over time and thus this two information represents the primary data. The division of acceptor by donor fluorescence results in the FRET ration. The image was taken and modified from Hoffmann *et al.* 2005 and represents an α_{2a}-adrenergic receptor FRET sensor. Upon stimulation with norepinephrine (NE) as indicated by black bars the conformational change of the receptor was red out via FRET. A clear antiparallel movement in acceptor and donor channel could be observed with in milliseconds.

1.4.2.2 FRET Sensors and GPCRs

FRET applications play a central role in biomedical research because they are applicable in human *in vivo* systems under near physiological conditions and can provide a high spatial and temporal resolution (Lohse et al., 2008; Lohse et al., 2007; van Unen et al., 2015). Especially in the field of GPCR research they proved to be a useful tool for intramolecular applications like receptor sensors or downstream signaling (Hoffmann et al., 2005; Nikolaev et al., 2004; Nuber et al., 2016; Stumpf and Hoffmann, 2016; Vilardaga et al., 2003). Receptor FRET sensors provided a significant contribution to biomedical research by allowing to monitor receptor conformations, kinetics or investigating different types of agonism (Vilardaga et al., 2005; Ziegler et al., 2011). In addition, several intermolecular FRET sensors have been described e.g. for monitoring G protein dissociation/rearrangement, to understand functional receptor dimers or arrestin recruitment (Bünemann et al., 2003; Goedhart et al., 2011; Hein et al., 2006; Hlavackova et al., 2012; Janetopoulos et al., 2001; Olofsson et al., 2014; van Unen et al., 2016). The most frequently used FRET pairs are CFP/YFP or CFP/FlAsH but also other fluorophore pairs like GFP/mCherry have been

reported. The receptor FRET sensors mostly contain one fluorophore in IL-3, because TM5 and TM6 undergo the biggest conformational changes in GPCRs upon activation and the second fluorophore is most often attached to the C-terminus. In general, the design of FRET sensors is target specific and supported by structural knowledge gained by X-ray crystallography. The major advantages of using the FRET sensor technology is the high spatial and temporal resolution of the experiments that can give new insights into cellular or subcellular dynamics. Additionally, the experiments can be performed under near physiological conditions and thus can provide a decent contribution to biomedical research (Boute et al., 2002; Kauk and Hoffmann, 2018).

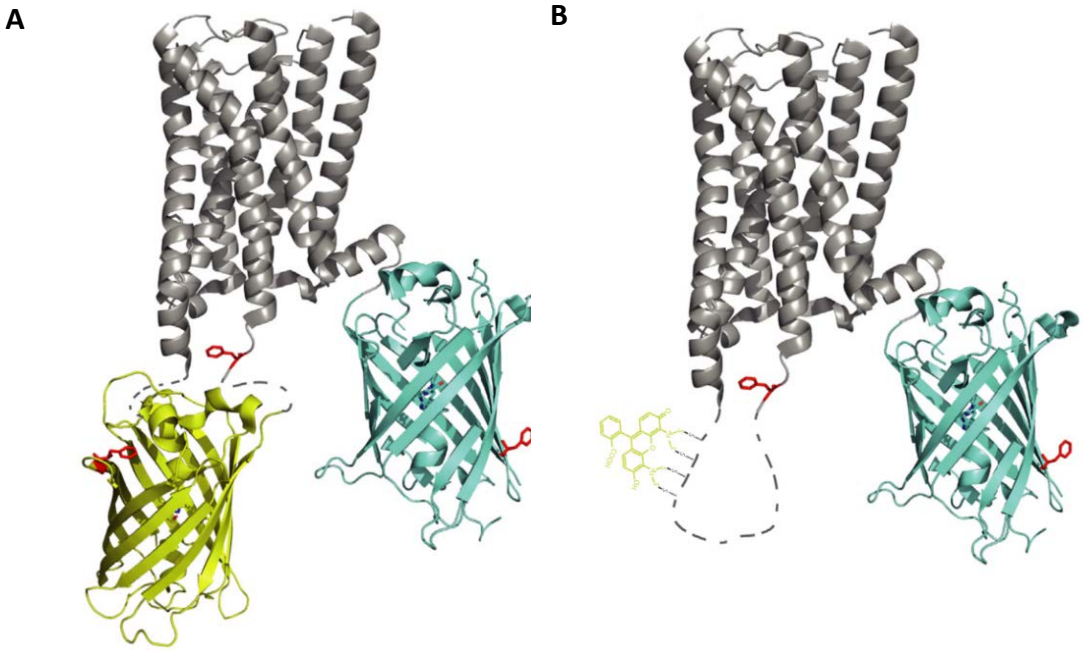

Figure 1.19 Receptor FRET Sensors
In Figure (**A**) a schematic representation of the first generation GPCR FRET sensor is shown. The fluorophores CFP and YFP are genetically encoded in the receptor construct. The CFP is attached to the C-terminus and the YFP is inserted in the truncated IL3. These sensors can be regarded as a breakthrough in case of receptor sensor design but mostly showed an altered downstream signaling due to the big size of the fluorescent tags (image was taken and modified from Kauk & Hoffman 2018). In contrast in (**B**) a GPCR FRET sensor of the second generation is displayed, using FlAsH and CFP and fluorescent tags. When compared to YFP, FlAsH is almost 40 times smaller in molecular size. These sensors showed in different reports a downstream signaling comparable to the wild type receptor (image was taken and modified from Kauk & Hoffmann 2018). The red highlighted phenylalanine residues serve as a reference for the displayed molecule size.

1.4.2.3 Bioluminescence Resonance Energy Transfer

Bioluminescent resonance energy transfer (BRET) is a naturally occurring intrinsic process that has evolved as a communication strategy for example in sea creatures like jellyfish (*Aequorea victoria*) or sea pansy (*Renilla reniformis*) (Widder, 2010). Similar to FRET, BRET is also a nonradiative energy transfer via dipole-dipole interactions between a donor and an

acceptor molecule. The major difference is the presence of a luciferase, a photon emitting enzyme, in BRET assays (Pfleger and Eidne, 2006). The acceptor molecule is a fluorescent molecule e.g. a GFP or a small synthetic molecule. The limitations of FRET and BRET are very comparable due to spectral overlap and distance dependence (Lohse et al., 2012). The two commonly used luciferases are the *Renilla* luciferase (RLuc / 36 kDa) and the Firefly luciferase (FLuc / 61 kDa) that provide rather low photon intensities. The development of the NanoLuc luciferase (NLuc) from the deep-sea shrimp *Oplophorus gracilirostris* resulted in an increase of luminescence of more than 100-fold (compared to RLuc and FLuc) and a signal half-life time of longer than 2h (Hall et al., 2012). In addition to this, the improvement of the substrate furimazine represents a milestone for BRET applications in biomedical research. In general, BRET is a useful application for studying protein-protein interaction and concerning GPCR research, BRET was applied for studying receptor oligomerization, as conformational sensors and for single cell imaging (Bourque et al., 2017; Gales et al., 2005; Goyet et al., 2016; Machleidt et al., 2015; Scholler et al., 2017). The variety of this method in combination with different labeling approaches is also shown by the recently described ligand binding approaches for GPCRs and receptor tyrosine kinases (Stoddart et al., 2015; Stoddart et al., 2018). The main advantages in using the BRET technology can be seen in the relatively simple requirements towards the instrumentation. For BRET, no external light source is necessary resulting in reduced background information and in less information deficit over time and a better signal to noise ratio. This makes BRET suitable for studying protein-protein interactions also because luciferases were actively engineered towards longer emission half-life times. The major disadvantages of BRET can be seen in the lower spatial and temporal resolution of the technique when compared to FRET. This and the lower intensity of the luciferases make BRET a less favorable system for imaging purposes (Boute et al., 2002; Kauk and Hoffmann, 2018).

1.4.3 Photo-Pharmacology

The remote activation of drugs by light in a non-invasive manner at a very specific site of action is one of the major aims of photo-pharmacology. In contrast, most of the currently used bioactive compounds show permanent activation or inhibition of a target structure and systemic activity (Lerch et al., 2016). There are different requirements on the target

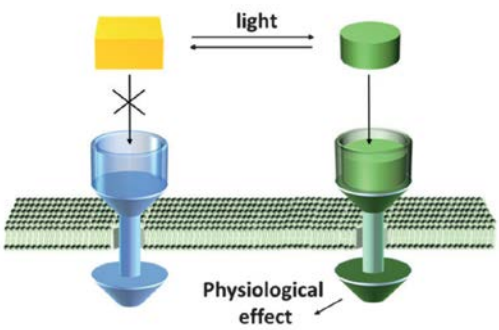

Figure 1.20 Photo-Pharmacology
The concept of photopharmacology implies that the pharmacological activity of a distinct drug is either triggered or diminished by the absorption of light. Furthermore, photoswitches are characterized by a repetitive switching process between an active and an inactive state (image was taken and modified from Lerch *et al*. 2016).

structures concerning a potential therapy with photoactivatable drugs and hence photo-druggability mostly relies on three factors. First, the target should show sensitivity towards the light induced structural properties of the photo-pharmacophore. Second, the disease should have a distinct localization like local inflammation or a defined tumor tissue. Third, the target structure should be accessible to light. Light as an external remote control has several advantages for instance a high level of spatiotemporal resolution, it is in general non-invasive, sterile, a precise regulation of intensity and frequency is possible and it is inert concerning most elements of living systems (Szymanski et al., 2013). Moreover, there is a strict correlation between wavelength and tissue penetration depth. The accessibility by light is one of the major differences that need to be dealt with when comparing druggability with photo-druggability (Cheng et al., 2007; Lerch et al., 2016; Owens, 2007). The definition of photo-druggability contains a classification of organs concerning the ease with which light can be delivered to them. Class 1 represents easily accessible organs like eyes or skin that can be reached non-invasively. Class 2 and class 3 are formed by tissues that can be reached with low invasive efforts like mouth and throat, the respiratory system, lymph nodes or bones lying directly under the skin. Class 4 and class 5 represents target structures that can be reached via minor and major incisions like liver, ovaries, brain and bone marrow (Lerch et al., 2016). One way of introducing light sensitivity into a biomolecule is through the insertion of molecular photo-switches.

Azobenzene are well studied molecular switches that are characterized by a *cis* and *trans* isomerization, where the *trans* conformation represents the more stable isoform. The isomerization into the *cis* conformation appears upon irradiation with UV light (≈ 320 nm). The reverse isomerization occurs thermally or can be induced by irradiating the molecule with visible light (≈ 460 nm). Noteworthy, the photo-physical properties of the final molecule are highly dependent on the substituents and on the solvent and thus need to be determined for every molecule and every application (Szymanski et al., 2013). Concerning GPCR pharmacology, a variety of interesting developments have been reported during the last years. In 2013 the groups of D. Trauner and E. Isacoff reported a photo-switchable tethered ligand (PTL) for metabotropic glutamate receptors (mGluRs) (Levitz et al., 2013). The ligand consisted of glutamate as the ligand, an azobenzene group and a maleimide group that was fused to the extracellular site of the receptor by using click chemistry. This approach was successful for both a photo-agonist and a photo-antagonist. Both ligands showed rapid, reversible and reproducible properties (Leippe et al., 2017; Levitz et al., 2013). A second interesting application of photo-pharmacology is the optical control of PAMs or NAMs. The first switchable NAM was reported in 2014 for the $mGluR_5$ (Pittolo et al., 2014). This ligand was characterized in a cellular and in an animal system with very interesting light inducible changes in behavior. The follow up reports published by the same group extended the portfolio of switchable NAMs for the $mGluR_5$ and extended the applicability to a second animal model (Font et al., 2017; Gomez-Santacana et al., 2017). Related to this, photo-activatable allosteric ligands have been described for mAChR receptors that upon UV light illumination bind irreversibly to the receptor and thus display a continuous influence (Davie

et al., 2014a; Davie et al., 2014b). The first photo-switchable dualsteric ligand was described in 2017 (Agnetta et al., 2017). Here, isoform dependent changes in receptor conformations as well as tunable characteristics for downstream signaling events have been described the first time. Next to photo-switchable ligands the ligand uncaging application is of potential relevance in photo-pharmacology. Here, the biologically active compound is fused to photo-removable protecting group ("caging group") (Deiters, 2010; Kaplan et al., 1978). The most common protecting groups are of *ortho*-nitrobenzyl, quinoline, dibenzofuran or coumarin origin. By irradiating the protecting group with non-photodamaging light (>360 nm) a single switching process can be performed that results either in an increase or decrease of activity, dependent on the ligand design. The relevance for GPCR research was demonstrated by studying intracellular signaling behavior of the $mGluR_5$ performed with caged glutamate (Jong and O'Malley, 2017). In addition, this method has high potential for studying GPCR activation kinetics because it allows releasing higher amounts of ligand simultaneously in very close proximity to a receptor.

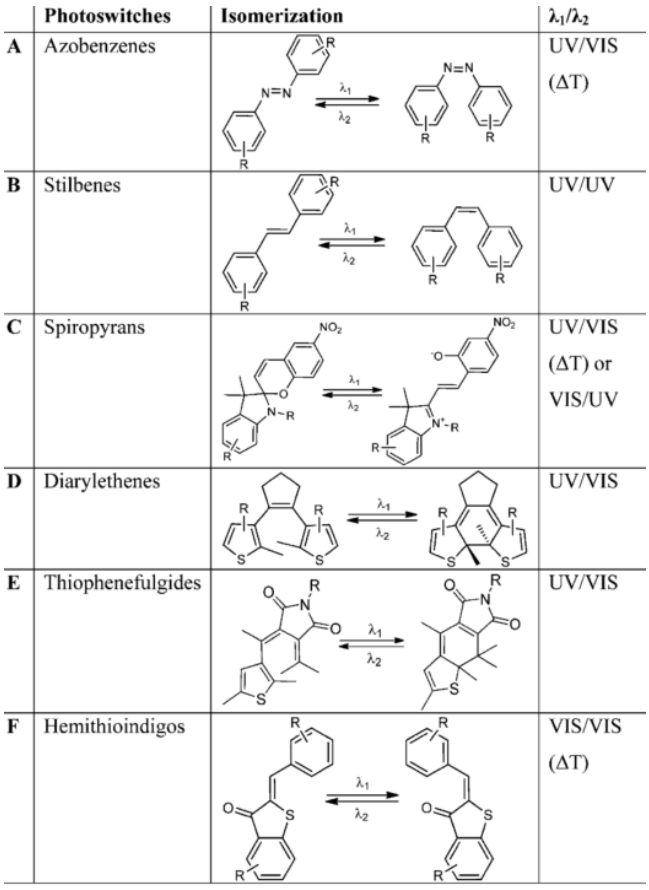

Figure 1.21 Molecular Photoswitches

The displayed table gives a detailed overview of different photo-active groups that have been used in the past for generating photoswitchable molecules. In addition, the corresponding wavelengths (λ_1/λ_2) for inducing the isomerization process are displayed. The most frequently used photoactive group are azobenzenes that are switched with UV light into the *cis* state whereas radiation with visible light leads to a *trans* conformation of the molecule (image was taken and modified by Szymański *et al.* 2013).

2 Aim of the Work

One major aim of this thesis was to improve the overall understanding of ligand receptor interaction dynamics for the muscarinic Acetylcholine receptor family (mAChRs) with focus on dualsteric ligands interacting with the receptor subtype 1 (M_1). In order to achieve this, a fluorescence resonance energy transfer (FRET) receptor sensor needed to be designed by introducing a fluorescein arsenical hairpin binder (FlAsH) binding sequence into the third intracellular loop (IL3) and a cyan fluorescent protein (CFP) to the receptor C-terminus. Next, this M_1 receptor FRET sensor was supposed to be characterized for downstream signaling and agonist mediated receptor activation.

The pharmacological class of interest was chosen to be rationally designed dualsteric ligands. It was planned to study different agonists and corresponding derivatives for their capability to induce a conformational change at the receptor sensor and thus to activate the receptor. In addition, different sets of dualsteric ligands showing variations in the allosteric, orthosteric and linker moiety should be characterized concerning receptor activation. Here, not only different combination of allosteric and orthosteric moieties should be of interest but also the role of the linker should be investigated in detail. In addition, the assay design should be optimized towards gaining improved insights for the effects of distinct molecular entities of dualsteric ligands concerning receptor activation. Additionally, promising ligands should be tested for subtype selectivity. It was furthermore planned to establish a reciprocal workflow between pharmacological characterization and medicinal chemistry to perform drug design based on structure activity relationships. At an advanced point it was planned to use the gained knowledge about dualsteric ligand design in combination with photo-pharmacological approaches. This new class of photo-switchable ligands should afterwards be characterized at the M_1 receptor with bioimaging tools. Next, it should be aimed to prepare the ground for future pharmacological studies at the entire mAChR family to enable future studies of promising compounds on a larger scale.

Besides pharmacological ligand characterizations it was planned to search for innovative approaches to increase the applicability of FRET in general. Newly developed microscopy coverslips were considered as a promising starting point. To study the FRET efficiency different microscopy settings should be applied and validated next to different configurations and orientations of coverslips.

In summary, although this thesis project is originated in pharmacological sciences, this study was planned with a high degree of interdisciplinarity and multiple contact points to different fields of research. Hence, scientific overlap should be searched and cooperation should be used to reach the aims of this work.

3 Published Research Article

3.1 FRET Studies of Quinolone-Based Bitopic Ligands and Their Structural Analogues at the Muscarinic M1 Receptor

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([#] these authors contributed equally)
ACS Chemical Biology, 2017 Mar 17;12(3):833-843

This study presents the synthesis and characterization of two different sets of rationally designed dualsteric ligands concerning their M_1 receptor activation properties. In addition, structural analogues of the hybrids were used to obtain detailed insights into the activation mechanism of dualsteric ligands. The characterization was done by using a newly designed receptor FRET sensor for the M_1 receptor. The first set of dualsteric ligands consisted of a benzyl quinolone carboxylic acid (BQCA) derived allosteric moiety (dBQCA) and an iperoxo as orthosteric building block. The second set contained ACh, the endogenous agonist, as orthosteric building block. The two moieties were connected through a polymethylene chain consisting of four, six, eight or ten methylene groups.

The M1-I3N-CFP is a conformational sensitive receptor FRET sensor and was designed by introducing a FlAsH binding sequence beneath the TM5 domain and by fusing a CFP to the C-terminus of the receptor. The functional characterization of the FRET receptor sensor was done by a calcium release assay, resulting in an indistinguishable sensor physiology compared to the wild type receptor. In addition, the characterization of the endogenous agonist acetylcholine and the synthetic full agonist iperoxo showed comparable characteristics to previous reports (Chen et al., 2015).

To investigate the effect of a covalently attached poly-methylene chain towards the agonistic properties of an orthoster a set of ten iperoxo derivates were characterized with the M1-I3N-CFP sensor. The results clearly showed a gradual decrease of the maximal receptor activation until iperoxo-C3. Derivatives with an even longer substitution showed no detectable receptor activation. In a competition experiment it was shown, that iperoxo and iperoxo-C6 can compete for the orthosteric binding region. This led to the conclusion that iperoxo-C6 behaves as an antagonist and is was concluded that the methylene chain elongation results in a loss of agonistic and a gain of antagonistic properties. Related effects were shown for chain modified ACh derivatives.

The probe dependent positive allosteric modulation of dBQCA towards iperoxo was shown in an innovative ligand addition experiment, in accordance with the literature (Ma et al., 2009). The characterization of the iperoxo/dBQCA hybrids resulted in a previously undescribed bell-shaped activation pattern. The iperoxo-C4-dBQCA hybrid resulted in 14.5 % receptor activation whereas the C6 derivative showed a higher receptor activation of 23.7 %. This represented the maximal activation for the iperoxo/dBQCA hybrids indicating that a chain length of six carbon atoms preferably connects orthosteric and the here targeted

allosteric binding regions. A further elongation of the linker moiety resulted in a loss of receptor activation for the iper-C8-dBQCA derivative (15.4 %). Hence, these experiments revealed partial agonistic behavior for the tested compounds in fully agreement with the literature (Antony et al., 2009; Bock et al., 2012; Chen et al., 2015; Valant et al., 2008). Interestingly, the Iperoxo-C10-dBQCA hybrid resulted in an inverse FRET signal of -32 % compared to iperoxo indicating a significantly different conformational change also in comparison to the other hybrids. The second set of hybrids consisting of ACh and dBQCA showed a comparable antiparallel FRET signal for the hybrids containing a C8 and a C10 carbon linker. The origin of the signal was validated against the inverse agonists atropine and tiotropium to rule out that the signal occur from a constitutively active receptor sensor. The negative results confirmed that the inverse signal derived by the hybrid ligand represents a different conformational change.

To deceiver the origin of the most significant dualsteric signal, the hybrid iperoxo-C6-dBQCA was investigated in a newly established fragment based ligand approach. Therefore, the single building blocks were applied individually or in a mixture of fragments. It was shown that dBQCA induces a small signal but iperoxo-C6 did not show a change in FRET. When applying a dBQCA/iperoxo-C6 mixture a robust FRET signal was induced with the same intensity compared to the corresponding hybrid. Interestingly, the fragment based signal showed a reduced activation kinetic. Thus, this study is the first report of a fragment based screening for dualsteric ligands.

In addition, it was shown that the hybrid iper-C6-dBQCA clearly discriminate between muscarinic subtypes. Therefore, a M_3 receptor sensor that was published before has been used (Hoffmann et al., 2012). The hybrid characterization clearly showed no conformational change at the M_3 sensor in contrast to partial agonistic behavior at the M_1 sensor. This represents the first dualsteric ligand showing preferential activation for M_1 over M_3.

3.2 A Photoswitchable Dualsteric Ligand Controlling Receptor Efficacy

Luca Agnetta[#], Michael Kauk[#], Maria Consuelo Alonso Canizal, Regina Messerer, Ulrike Holzgrabe, Carsten Hoffmann and Michael Decker

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This study represents the first chemical description and pharmacological characterization of a photo-switchable dualsteric ligand for mAChRs. The here reported ligand design was inspired by the results presented above and by the work of Chen et al. 2015 (Chen et al., 2015; Messerer et al., 2017). Based on these reports the highly potent orthosteric agonist iperoxo was modified with an azobenzene group resulting in the photo-iperoxo molecule and the linker moiety of an iperoxo/dBQCA hybrid was replaced by an azobenzene group resulting in the BQCAAI molecule. The physicochemical characterization of the compounds via chromatography showed that the isomerization equilibrium can be shifted to the *cis*

isoform by irradiating the molecule with UV light and to the *trans* isoform by exposing the ligands to white light or heat.
The pharmacological characterization of the two compounds was done with the previously reported M1-I3N-CFP receptor FRET sensor, a FRET sensor sensitive for Gq activation and a fluorescence sensor sensitive for calcium release. The characterization of photo-iperoxo revealed antagonistic properties. In contrast, the dualsteric photo-switch BQCAAI was described as a partial agonist in the *trans* isoform and as an antagonist in the *cis* isoform.
Interestingly, the *trans* isoform of BQCAAI induced a 25 % FRET signal at the M1-I3N-CFP sensor when compared to iperoxo. This signal intensity is nearly superimposable with the signal induced by the iperoxo-C6-dBQCA hybrid (23,7 %) (Messerer et al., 2017). The comparison of the activation kinetics of the two hybrids revealed a significantly slower receptor activation by the BQCAAI molecule. To check whether this could be attributed to the sterically demanding and hydrophobic linker a new iperoxo/dBQCA hybrid was designed (RM405). The RM405 hybrid also showed a reduced activation kinetic confirming the assumption that the linker moiety of dualsteric ligands play an important role in receptor activation.
Next, it was investigated whether the light dependent isomerization process of the BQCAAI molecule can be used to tune the ligand efficacy of the photo-switchable ligand in an *in vivo* experiment. Therefore, the illumination of the FRET sensor was reduced from 10 Hz to 1Hz and thus the light exposure to the sensor and the ligand was reduced by 90 %. This change in illuminating settings significantly changed the Gq activation of the less stable *cis* isoform by more than 30 % whereas the activation properties of the *trans* isoform remained unaltered. This finding served as a proof of concept that photo-switchable ligands might show a dimmable efficacy dependent on the used light portion.

3.3 Enhanced Fluorescence Resonance Energy Transfer in G Protein-Coupled Receptor Probes by Nano-Coated Microscopy Coverslips

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This research article represents the methodological proof of principle for enhancing the FRET efficiency in an established *in vivo* assay by using plasmonic surfaces. Therefore, standard microscopy coverslips were coated with a gold layer of 20 nm thickness. The effect of the gold coated coverslips towards the FRET efficiency was investigated by using the M1-I3N-CFP FRET sensor.
At first, the FRET efficiency of the sensor was testes in the presence or absence of the gold layer. Therefore, a chemical bleaching of the receptor sensor with 2,3-Dimercapto-1-propanol (British anti-Lewisite (BAL)) was performed. BAL was described as an antidot for acute arsenic poisoning and thus can remove FlAsH from its binding site of the receptor

sensor. The removal of the FRET acceptor resulted in an increase in donor (CFP) fluorescence and this effect was afterwards recalculated into a FRET efficiency. The FRET efficiency of the M1-I3N-CFP on normal glass coverslips was reported to be 16.3 %. Performing the same experiment on gold coated coverslips resulted in a FRET efficiency of 21.6 % and thus to an increase of 30%. This was further optimized by changing the coverslip configuration. In the until yet used normal configuration the excitation photons as well as the emitted photons need to cross the glass respectively the gold coverslip twice. In case of glass this leads to a negligible loss off information. In contrast, the optical properties of the gold layer resulted in a significant loss of photons due to absorbance. By changing the normal upright coverslip configuration to a flipped coverslip configuration this loss of information could be avoided and thus the FRET efficiency was determined to be 26.3 %. Out of that it was concluded, that gold particles in proximity to the M1-I3N-CFP sensor can increase the FRET. These data were validated and reproduced by acceptor photobleaching experiments.
To show that these observations can be reproduced in a pharmacological experiment iperoxo dependent conformational changes were investigated with the M1-I3N-CFP sensor. Therefore, dynamic stimulation experiments were performed on normal glass and on gold coated coverslips. Afterwards the normalized signal intensity of a full receptor activation was compared. The signals obtained from gold coated coverslips showed a nearly 60 % increase in FRET when compared to the signals from none coated coverslips and thus reproduced the experimental results from the bleaching experiments.
In summary, this study represents the first report of an enhanced FRET efficiency by plasmonic nanostructures in a detection system of physiological relevance.

3.4 Intramolecular and Intermolecular FRET Sensors for GPCRs – Monitoring Conformational Changes and Beyond

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This review article represents a detailed overview of the current state of the art in GPCR FRET sensor design and different cutting-edge applications of FRET in biomedical research. To understand the difficulties of designing a GPCR FRET sensor labeling techniques are highlighted for the site specific fluorescent tagging of proteins. Here, not only the latest developments were presented but also already established and useful techniques. Thus, a critical comparison of historically lined up receptor sensors was made.
Furthermore, different fields were presented in that the application of GPCR FRET sensors contributed to state of the art research. The high spatial and temporal resolution of this technique could contribute significantly over the last decades to unravel the dynamic motions of GPCRs. It helped not only to understand drug receptor interaction dynamics and activation processes but also to deceiver functional dimer formation of physiological relevant GPCRs. Hence, FRET contributed significantly to the current knowledge about GPCR function and signaling.

In addition, new applications like the fragment based screening approach was highlighted. Modern drug development shows high ambitions to develop clinically applicable bivalent ligands that show more than one functional group and very complex binding modes. This complex molecular structure lead to difficulties in pharmacological characterization and this new approach can provide a powerful and structured way of investigating the effect of molecular fragments towards GPCR activation. In a future perspective this way of investigating ligands can also help to understand allosteric modulation or metastable ligand binding.

Dedicated to research groups that are currently planning to apply the FRET technique a whole section was used to discuss advantages and disadvantages of FRET in comparison to the related BRET application. Here not only the necessary instrumentation was discussed but also the flexibility of the methods. For both techniques diverse innovations are mentioned and examples are provided for a preferential FRET or BRET application.

In summary, this review article aims to open again controversially discussed topics in FRET GPCR research and to provoke fair debates in different sections. Additionally, it directs open questions to the research community concerning future directions in research and developments.

4 Discussion

Dualsteric ligands form a modern class of drugs that start to exhibit a high value for pharmacological research. Currently used as a versatile tool to understand GPCR pharmacology they show diverse promising properties for future clinical research like selective receptor binding or biased agonism. This class of ligands consists of three different moieties. The orthosteric moiety binds to the orthosteric (endogenous) binding region and mediates agonism, antagonism or inverse agonism. The allosteric moiety interacts with a second distinct binding region at the receptor and shows allosteric modulation and selective binding properties. The linker moiety serves as the covalent connection between orthosteric and allosteric pharmacophores and represents the least investigated moiety of dualsteric ligands. Dualsteric ligands show a complex binding mode and can bind either dualsteric or allosteric to a GPCR (Antony et al., 2009; Bock et al., 2014). mAChRs represents one model system for investigating dualsteric ligands, a receptor family that is already now of clinical relevance.

The design and characterization of dualsteric ligands is a complex topic that combines medicinal chemistry and pharmacology and moreover requires detailed comprehension in biochemistry to finally link structure activity relations. The overall aim of this thesis was to understand the molecular mechanism of dualsteric ligands and to systematically investigate and improve their design and pharmacology. Rationally designed for the M_1 the here studied hybrids are promising to generate subtype selectivity. Subtype selective M_1 activation is thought to be a promising therapeutic approach against diseases like Alzheimer or Parkinson. Acetylcholinesterase inhibitors represents the current treatment of Alzheimer symptoms because Alzheimer patients show a reduced activity of cholinergic neurons characterized by a reduced ACh release. Thus, the esterase inhibition leads to an increased neurotransmitter concentration in the synaptic cleft. By using subtype selective dualsteric ligands it would be possible to directly target neuronal receptors, which would show benefits compared to the indirect affection via acetylcholine esterase inhibition.

In this respect, dualsteric ligands consisting of iperoxo as the orthosteric moiety and a BQCA derivative as the allosteric moiety were characterized in this thesis with a newly established M1-I3N-CFP FRET receptor sensor. The hybrids clearly showed M_1 preference when compared to the M_3 subtype (3.1). Although the here investigated hybrids cannot be seen as future therapeutics, their interaction properties are promising. A theoretical therapeutic against Alzheimer would need to cross the blood-brain-barrier and therefore the permanent charge and the moderate lipophilicity of the tested compounds are disadvantageous. The here tested hybrids can be seen as a proof of principle for future ligand design and thus as a first step towards more applicable ligands. Furthermore, these ligands can serve as important pharmacological tools for future research. The described hybrids are a very promising contribution for future drug design of cholinergic therapeutics and represents an important finding for muscarinic pharmacology. Drugs with these properties would enable for the first time a direct and selective activation of the M_1 receptors in postsynaptic regions

and new strategies for treating neurodegenerative diseases could be developed. A precise medication results in an effective treatment and would exhibit a reduction of side effects. Based on this knowledge, the future design of subtype selective M_1 antagonist seems possible as well. It was described before that dualsteric ligands interacting with the M_2 subtype show biased signaling properties (Bock et al., 2012). Despite this was not investigated here, it will be subject of future projects with the perspective to systematically design ligands that show preference for one signaling pathway over the other. Comparable knowledge is already now applied in the clinic for other GPCRs to further reduce side effects.
Based on the ligand design and characterization presented in 3.1 and discussed above the concept of iperoxo/dBQCA hybrids was further developed into the photo-pharmacological research field. Photo-switchable ligands are a very promising class of modern therapeutics due to a remotely controllable activation by light. The in 3.2 reported BQCAAI molecule was characterized as the first photo-switchable dualsteric ligand and thus belong to a new and undescribed class of potential therapeutics. BQCAAI was described as a photo-inactivatable ligand and its value can primarily be seen as a new tool for pharmacological research. It could enable to study more detailed the crosstalk between different receptor species not only in single cells but also in tissues or even in organisms. Photo-switching could inactivate the receptor of interest with a high spatial and temporal resolution and thus the importance of e.g. heterodimers in neurons could be investigated. Assuming that the BQCAAI hybrid also shows subtype selective activation properties compared to the hybrids discussed above, future applications of this ligand class can be even more powerful when compared to conventional dualsteric ligands. In theory, the photo-inactivatable properties of BQCAAI could be helpful for the titration of anticholinergic effects, the initial adjustment of a medication or for treating an overdose. Here, especially the tunable characteristics of BQCAAI can be of importance. In 3.2 it was shown, that the conformational equilibrium is dependent on the applied light dose. This means in theory, that for photo-switchable ligands more than one concentration needs to be considered. First, the drug concentration that was administered and second the concentration equilibrium between agonistic and antagonist ligand. This second level of tunable efficacy could rule out beneficial in a therapeutic approach.
Next to the results presented here it would be interesting to further evaluate the design of BQCAAI. First, it could be possible to obtain a photo-activatable dualsteric ligand by varying the substitution of the azobenzene group. Second, it would be beneficial to shift the excitation spectra of the hybrid from ultra violet (UV) to infrared (IR) wavelengths. The high energy UV light is known as cytotoxic and can cause gene damage. In addition, IR light would show a higher tissue penetration depth due to the longer wavelength. In theory this can be obtained by an altered substitution of the photo-responsive moiety. Next, an altered substitution of the photo-responsive group could decrease to spontaneous isomerization process by altering the free energy of the different isoforms. This would be beneficial for future experimental procedures, because until now the spontaneous isomerization and thus a basal activity always needs to be considered when evaluating results. Also for a theoretical

application of these ligands in the clinic, ligands that can rest longer in a distinct conformation could be desirable for triggering different physiological responses.
There is a ligand design that already now exhibits a stable and almost complete ligand activation upon light illumination. The uncaging method can be seen as an alternative attempt to photo-switching. Here, a pharmacologically active molecule is rendered inactive by a light-removable protecting group (caging group) (Deiters, 2010). Thus, the irradiation with UV light can uncage the pharmacophore and trigger a physiological response with high temporal and spatial resolution. In comparison to the here presented photo-switchable ligand technology, the uncaging technology shows some advantages. First, the technology is easier to handle and the pharmacophore design is more straight forward due to the accessible uncaging molecules. Secondly, uncaging does not show an equilibrium of ligand states and thus does not potentially show a basal activity in regions except of the irradiated tissue. In contrast to that photo-switching shows diverse advantages over uncaging. In uncaging the activation can be performed only once and only from the inactive to the active state which shows a distinct inflexibility. Photo-switching can be performed in theory unlimited times. In addition, it is dependent on the molecule design whether the more stable *trans* isoform shows agonistic or antagonistic properties. This means a photo-activatable and a photo-inactivatable ligand design is possible. Furthermore, it was shown here that photo-switchable ligands show tunable agonistic properties dependent on the used amount of light. Thus, also the physiological response of a theoretical therapeutic should be tunable, a property that was never reported for uncaging. This leads to the conclusion that both methods show distinct advantages and disadvantages. Uncaging can be regarded as an approach that should be easier established but photo-switching can be seen as the method that shows much more flexibility and more potential applications.
Both studies 3.1 and 3.2 show that not only the orthoster/alloster combination is of high importance for the functionality of dualsteric ligands but also to linker. The linker moiety represents the least investigated part of dualsteric ligands and its design and the resulting influence on receptor binding and activation is often barely discussed. Considering that the linker also needs to enter the core of a receptor and thus very likely interacts with the receptor structure, like the aromatic lid structure at muscarinic receptors, this circumstance represents a huge lack of knowledge. In both studies valuable information can be found concerning the importance of the linker design for receptor activation.
In 3.1 it was shown, that the receptor activation of dualsteric ligands is highly dependent on the linker length and flexibility. The reported bell-shaped activation pattern of the iperoxo/dBQCA hybrids showed the biggest conformational change for a hexamethylene linker. Shorter and longer linker (tetra- and octamethylene) showed a reduced receptor activation suggesting that the hexamethylene linker connects best the orthosteric and allosteric binding region. From this it can be concluded that the efficacy of a dualsteric ligand can be fine-tuned to some extend by varying the linker length. Until yet, it is not entirely clear what kind of information is encoded in this bell-shaped correlation and there are different explanations possible. First, the signals can be interpreted as indication for the

dynamic ligand binding of the hybrids, because dualsteric ligands can either interact in a purely allosteric (inactive) mode with the receptor or in a dualsteric (active) mode. Thus, the resulting receptor activation can always be seen as a compromise of both binding modes. This can also be seen as the reason why the majority of dualsteric ligands have been described as partial agonists. Thus, the resulting signal intensity can be interpreted as the ratio of both interaction modes and indicate that the hexamethylene linker containing hybrid favors the dualsteric binding mode over the allosteric binding mode in a different extend when compared to the tetra- and octamethylene linker derivatives. A second interpretation of the signals could be that the obtained signal intensity correlates with the extend of the conformational change of the receptor. The energy transfer in FRET shows distance dependence with a factor r^6 and for GPCRs it is known that they can adopt various conformations in a highly dynamic manner. Hence, a ligand that induces a small conformational change would induce a smaller FRET signal when compared to a second ligand that induces a big conformational change. This argumentative connection would lead to the conclusion that the investigated derivatives lead to different conformational changes of the receptor. Investigating this circumstance in detail is planned for future project and could be performed by measuring the downstream signaling characteristics of the hybrids and by investigating the biased signaling characteristics of the corresponding compounds.
Related to that, the hybrid containing a decamethylene linker showed an inverse signal that was discussed as a new and undescribed conformational change induced by dualsteric ligands (3.1). For the M_2 is was reported that dualsteric ligands can show distinct secondary structures dependent on the linker length resulting in a preferential purely allosteric binding mode (Bock et al., 2016). Here, it is more likely that the inverse signal is not due to a purely allosteric binding because the alloster itself does not induce a stable FRET signal. Additionally, the investigated ACh/dBQCA hybrids and derivatives only consisting of alloster and linker moiety with a terminal tertiary ammonium group induced comparable inverse signals. The positively charged ammonium group on orthosteric ligands is known to form charged interactions with the orthosteric binding pocket for aminergic GPCRs. In this respect, it is more likely that the inverse signal represents a new conformational change that is mainly a result of sterical interactions in the orthosteric binding pocket. Until now this statement represents a theory that needs to be validated in future projects by mutational analysis. Furthermore, it would be interesting to analyze the signaling behavior of the corresponding ligand and compare the antiparallel signal with conventional agonism.
In 3.2 results were presented that clearly show, that the chemical characteristics of the linker moiety can influence the receptor activation. BQCAAI showed a very slow activation kinetic when compared to the iperoxo/dBQCA hybrids described in 3.1. The major difference between the two hybrids was that the polymethylene linker was replaced by an azobenzene group. Hence, the reduced activation kinetic of *trans*-BQCAAI can be attributed to the sterically more demanding, more hydrophobic and more rigid azobenzene group. To verify this a second hybrid with a different linker design was synthesized, showing a comparable rigidity, hydrophobicity and size. Indeed, also this RM405 derivative showed a significant

slower and with BQCAAI nearly superimposable activation kinetic when compared to the original iperoxo/dBQCA hybrid (3.2). This finding confirmed the theory that the linker design can have huge impacts on the receptor activation kinetic and is of interest because a reduced activation kinetic could lead to an incomplete activation process and thus to a different downstream signaling. Thus, the linker design can have a huge impact on a signaling bias. This knowledge could be applied for rational ligand design.

A different central question that was investigated in 3.1 was the effect that can be introduced by an attached carbon chain to an orthosteric ligand towards the receptor activation properties. The results clearly showed that a stepwise elongation of a polymethylene chain results in a stepwise reduction of receptor activation at the M_1 receptor. The addition of a single methylene group to iperoxo (iperoxo-C2) resulted in a loss of 30 % receptor activation and the addition of an ethylene group (iperoxo-C3) resulted in a more than 80 % decrease of receptor activation. All following derivatives up to iperoxo-C10 showed no receptor activation properties and in a competition experiment it was shown, that iperoxo-C6 behaves as an antagonist. Related to that, it was shown in 3.2 that photo-iperoxo behaves as an antagonist at the M_1. A reason for that can be assumed in the closure of the of the aromatic lid structure (Tyr106, Tyr381 and Tyr 404) upon receptor activation (Thal et al., 2016). It was reported before, that the alanine substitution of one of the tyrosines results in a drastic loss of receptor activation (Hulme et al., 2003a; Hulme et al., 2003b). Related to this it can be assumed that a sterically demanding linker disturb the closure of the aromatic lid resulting in a decreased receptor activation. Here, it would be interesting for future projects whether this is true for all muscarinic subtypes and whether the gradual decrease is of similar extend. In theory, only minor differences in receptor activation should be visible due to the conserved orthosteric binding pocket. This approach could have the potential to unravel different characteristics in shape and binding properties when compared on all muscarinic subtypes. The until yet available crystal structures represent only a snapshot of the otherwise highly dynamic receptor physiology because four of the five structures show the inactive receptor. It could be possible, that the receptor subtypes show differences solely during the activation process or in one of the multiple receptor states (see Figure 1.6). If this is true such differences can never be explored with a static structure determination method like X-ray crystallography. Dynamic structure determination approaches like NMR are suitable but require a high degree of methodological expertise. Here, FRET applications have a huge potential due to dynamic and distance dependent data acquisition and the variability of the sensor design.

Now the question remains open why dualsteric ligands can induce robust receptor activation whereas the corresponding orthosters modified with a carbon chain show antagonistic characteristics? This can be mainly due to two factors. First, in 3.1 the allosteric moiety was a PAM that probably can modulate the antagonism. Second, in dualsteric ligands the linker is attached to the alloster as well and this should reduce significantly the degrees of freedom for the polymethylene chain. As a result of that it is possible that in the hybrid compound the linker adopts a conformation that can pass the aromatic lid. For the orthosteric

derivative it is likely that the linker is highly dynamic due to Brownian motion and this could result in a reduced binding energy.

To generate detailed insights into the complex interaction process between dualsteric ligand and GPCR a new ligand application strategy was introduced in 3.1. The theoretical potential of this application and the necessity of its development was already claimed before but until yet no research group was able to realize this project (Bock and Mohr, 2013). The fragment based ligand approach represents a new way of investigating dualsteric ligands by combining the advantages of a FRET-based screening system and a rapid perfusion system (Kauk and Hoffmann, 2018). In 3.1 it was shown that it is possible to rebuild on a molecular level the receptor activation properties of a dualsteric ligand by using the single building blocks of this ligand. The fragment based signal showed the same efficacy but a significant slower activation kinetic. The method can be seen as an important link between the chemical ligand design and the resulting pharmacological characterization. It gives information about structure activity relationships and can be used not only to investigate binding mechanisms of dualsteric ligands but also to investigate allosteric modulation in detail. In theory, by varying the order of applying the different building blocks or by preincubating with either the orthosteric moiety or the allosteric moiety it could be possible to deceiver which moiety is the first or the second interaction partner. Out of that the dynamic ligand binding model could be further developed. Investigating the resulting kinetics in greater detail could also enable to deceiver the activation cooperativity factor α.

Furthermore, this method also shows high potential towards future screening applications for multivalent ligands. By using this fragment based approach it would be easier to test different alloster/orthoster, orthoster/orthoster or alloster/alloster combinations in a short time and with high accuracy and variability. The resulting efficacy parameter could be used as a first level of discrimination. This would reduce the need to synthesize the corresponding hybrid combinations for which the screening approach already revealed negative results. The establishment of the described screening approach requires advanced ligand application equipment and a detailed validation and thus can be seen as a future perspective, so far. In combination with a ligand database this could be a valuable contribution for drug screening and furthermore diversify the future ligand design. So far, this application suffers from the small signal intensity that is investigated and it would be desirable to increase the FRET efficiency to have an improved readout.

In 3.3 it was reported that using the M1-I3N-CFP FRET sensor on gold coated coverslips can lead to an increase in energy transfer to up to 60 %. This was shown by chemical bleaching and validated by photo bleaching of the acceptor fluorophore and furthermore shown in an *in vivo* assay for dynamic receptor activation. An increase in FRET efficiency does not only mean that the FRET rate or energy transfer rate from donor to acceptor fluorophore was increased but also that the nonradiative decay losses were reduced. This effect can be explained by beneficial orientation of the dipole moments (κ^2) towards each other. This allows in combination with the plasmonic surface, under normal conditions forbidden dipole-dipole interactions. This is enabled by the multiple attachment points between FlAsH

and the receptor resulting in a more rigid positioning of FlAsH. A related study reported that a perpendicular dipole-dipole orientation can lead to an increase in FRET and thus fully agree with the here reported results (de Torres et al., 2016). Furthermore, the plasmonic surface is understood to act like a mirror for photons on an atomic scale and thus the portion of photons that can be absorbed by the acceptor fluorophore is increased. The here reported findings represents the first proof of principle towards increasing the FRET efficiency for dynamic *in vivo* applications and thus will influence future RET applications. First, a higher FRET efficiency indicates that a higher maximal FRET distance can be bypassed now in theory. Until yet the FRET distance limit was around 10 nm and an increase allows now to study for example more effectively GPCR oligomerization or other protein-protein interactions. Homo- and heterodimerization of GPCRs is an exciting field and of high pharmacological relevance. Next, studying the interaction of GPCRs with other membrane receptors like receptor tyrosine kinases can be investigated in greater detail. Second, it could be possible now to measure FRET across cell membranes. This would enable to study ligand binding, receptor activation and downstream signaling like G protein activation at the same time by using multiple FRET pairs. Third, the results presented here are in theory applicable on diverse other RET technologies and thus can provide a more detailed readout for other FRET sensors or BRET applications. An even further improvement of the FRET efficiency could be reached in theory by investigating a receptor sensor dually tagged with arsenical hairpin binder. This could further optimize the κ^2 value. In this regard, it was already shown that an orthogonal labeling procedure with FlAsH and ReAsH is possible (Zürn et al., 2010).
When compared to other approaches that were reported in the past for increasing the FRET efficiency, the usage of plasmonic mirror surfaces show diverse advantages. By using multiple fluorophores as donor molecules and by utilizing antenna effects the energy transfer to an acceptor molecule can be increased (Hemmig et al., 2016; Olejko and Bald, 2017). The major disadvantage of this approach is the size of the fluorophores that can alter for example the receptor physiology. Second, nanoapertures were successfully designed and applied to increase the FRET rates but their usage in combination with cellular systems would show diverse difficulties due to the exact positioning of the cell respectively the fluorophores relative to the aperture (Ghenuche et al., 2014; Ghenuche et al., 2015). Moreover, the generation of nanostructure modified coverslips is more complex than the gold coating. Out of that it can be concluded that the simplicity of using gold coated coverslips that can easily be adopted to diverse experimental settings is one of the biggest advantages of the here reported method.

5 Summary

G protein-coupled receptors (GPCRs) form the biggest receptor family that is encoded in the human genome and represent the most druggable target structure for modern therapeutics respectively future drug development. Belonging to aminergic class A GPCRs muscarinic Acetylcholine receptors (mAChRs) are already now of clinical relevance and are also seen as promising future drug targets for treating neurodegenerative diseases like Alzheimer or Parkinson. The mAChR family consist of five subtypes showing high sequence identity for the endogenous ligand binding region and thus it is challenging until now to selectively activate a single receptor subtype. A well accepted method to study ligand binding, dynamic receptor activation and downstream signaling is the fluorescence resonance energy transfer (FRET) application. Here, there relative distance between two fluorophores in close proximity (<10 nm) can be monitored in a dynamic manner. The perquisite for that is the spectral overlap of the emission spectrum of the first fluorophore with the excitation spectrum of the second fluorophore. By inserting two fluorophores into the molecular receptor structure receptor FRET sensors can serve as a powerful tool to study dynamic receptor pharmacology.

Dualsteric Ligands consist of two different pharmacophoric entities and are regarded as a promising ligand design for future drug development. The orthosteric part interacts with high affinity with the endogenous ligand binding region whereas the allosteric part binds to a different receptor region mostly located in the extracellular vestibule. Both moieties are covalently linked. Dualsteric ligands exhibit a dynamic ligand binding. The dualsteric binding position is characterized by a simultaneous binding of the orthosteric and allosteric moiety to the receptor and thus by receptor activation. In the purely allosteric binding position no receptor activation can be monitored.

In the present work the first receptor FRET sensor for the muscarinic subtype 1 (M_1) was generated and characterized. The M1-I3N-CFP sensor showed an unaltered physiological behavior as well as ligand and concentration dependent responses. The sensor was used to characterize different sets of dualsteric ligands concerning their pharmacological properties like receptor activation. It was shown that the hybrids consisting of the synthetic full agonist iperoxo and the positive allosteric modulator of BQCA type is very promising. Furthermore, it was shown for orthosteric as well as dualsteric ligands that the degree of receptor activation is highly dependent on the length of and the chemical properties of the linker moiety. For dualsteric ligands a bell-shaped activation characteristic was reported for the first time, suggesting that there is an optimal linker length for dualsteric ligands. The gained knowledge about hybrid design was then used to generate and characterize the first photo-switchable dualsteric ligand. The resulting hybrids were characterized with the M1-I3N-CFP sensor and were described as photo-inactivatable and dimmable. In addition to the ligand characterization the ligand application methodology was further developed and improved. Thus, a fragment-based screening approach for dualsteric ligands was reported in this study for the first time. With this approach it is possible to investigate dualsteric ligands in greater

detail by applying either single ligand fragments alone or in a mixture of building blocks. These studies revealed the insights that the effect of dualsteric ligands on a GPCR can be rebuild by applying the single building blocks simultaneously. The fragment-based screening provides high potential for the molecular understanding of dualsteric ligands and for future screening approaches. Next, a further development of the standard procedure for measuring FRET by sensitized emission was performed. Under normal conditions single cell FRET is measured on glass coverslips. After coating the coverslips surface with a 20 nm thick gold layer an increased FRET efficiency up to 60 % could be reported. This finding was validated in different approaches und in different configurations. This FRET enhancement by plasmonic surfaces was until yet unreported in the literature for physiological systems and make FRET for future projects even more powerful.

5.1 Zusammenfassung

G Protein gekoppelte Rezeptoren (GPCRs) bilden die größte Proteinfamilie, die im humanen Genom verschlüsselt ist. Sie sind nicht nur die Zielstruktur für eine Vielzahl von derzeit gebräuchlichen Medikamenten, sondern gehören auch zu den vielversprechendsten Therapieansätzen für die moderne Medikamentenentwicklung. Muskarinerge Acetylcholin Rezeptoren (mAChRs) gehören zu den aminergen Klasse A GPCRs und sind bereits heute von klinischer Relevanz. Die muskarinerge Rezeptorfamilie wird von fünf Subtypen gebildet, die sich besonders durch eine hohe Sequenzidentität in der endogenen Ligandenbindestelle (orthostere Bindestelle) auszeichnen. Aus diesem Grund ist es mit den herkömmlich verwendeten Medikamenten nicht möglich, einen ganz bestimmten Subtyp zu therapieren, ohne auch andere Subtypen zu beeinflussen und so unerwünschte Nebenwirkungen zu erhalten. Eine Möglichkeit Ligandenbindung, dynamische Rezeptoraktivierung oder Signalweiterleitung von GPCRs nach pharmakologischen Gesichtspunkten zu charakterisieren, stellt der Floreszenz Resonanz Energietransfer (FRET) dar. Mit Hilfe dieser Methode kann über kleine Entfernungen (<10 nm) die relative Orientierung von zwei Fluorophoren mit überlappenden Spektralbereichen mit hoher zeitlicher Auflösung verfolgt werden. Integriert man das Fluorophorpaar mit Hilfe gentechnischer Methoden in die Molekülstruktur des Rezeptors, kann man dessen Konformationsänderung bzw. Aktivierung infolge einer Ligandenbindung aufzeichnen.

Dualstere Liganden sind eine Substanzklasse von hohem zukünftigen klinischen Potential und zeichnen sich durch die Verknüpfung mehrerer pharmakologisch aktiver Untereinheiten aus. Der orthostere Molekülteil interagiert mit der endogenen Ligandenbindestelle und der allostere Molekülteil interagiert mit einem zweiten Rezeptorabschnitt, der häufig in den extrazellulären Schlaufen des Rezeptors zu finden ist. Diese allosteren Bindestellen zeichnet sich durch eine vergleichsweise geringe Sequenzidentität aus, weswegen allostere Modulatoren auch selektiv an Subtypen binden können. Aufgrund des Aufbaus können dualstere Liganden auf vielfältige Weise mit dem Rezeptor interagieren und dieser Bindemechanismus wurde als dynamische Ligandenbindung beschrieben. Zum einen können beide Molekülteile gleichzeitig mit dem Rezeptor interagieren und ihn aktivieren (dualsterer

Bindemodus) und zum anderen findet man einen rein allosteren Bindemodus, der den Rezeptor nicht aktiviert. Der orthostere Molekülteil ist vor allem für die Rezeptoraktivierung zuständig, die sich durch eine hohe Affinität auszeichnet und der allostere Molekülteil kann selektive Rezeptorinteraktionen vermitteln. Da dualstere Moleküle immer Eigenschaften beider Untereinheiten besitzen, werden dualstere Liganden als sehr vielversprechend erachtet, zukünftig subtypselektive Medikamente darzustellen.
In dieser Arbeit wurde der erste Rezeptor FRET Sensor für den muskarinergen Subtyp 1 (M_1) beschrieben und es konnte gezeigt werden, dass sich dieser Rezeptorsensor in seiner physiologischen Funktion nicht von dem *wild Typ* unterscheidet. Des Weiteren können mit Hilfe dieses Sensors liganden- und konzentrationsabhängige Rezeptorantworten aufgezeichnet werden. Der M1-I3N-CFP wurde dazu genutzt verschiedene Reihen dualsterer Liganden zu charakterisieren und auf ihre aktivierenden Eigenschaften bezüglich des M_1 zu testen. Es wurde gezeigt, dass die Kombination aus dem synthetischen und hochpotenten Agonisten Iperoxo als Orthoster und dem in der Literatur als M_1 selektiven positiven allosteren Modulator beschriebenen BQCA als Alloster sehr vielversprechend ist. Es konnte gezeigt werden, dass die rezeptoraktivierenden Eigenschaften sowohl von orthosteren wie auch von dualsteren Liganden stark von der Linkerlänge abhängig sind. Für dualstere Liganden konnte so ein glockenförmiger Zusammenhang zwischen Linkerlänge und Rezeptoraktivierung herausgearbeitet werden. Des Weiteren wurde gezeigt, dass bestimmte Hybride, die den M1 aktivieren, an anderen Subtypen keine Effekte hervorrufen und somit als subtypselektiv beschrieben werden können. Im Anschluss wurde mit Hilfe des gewonnenen Wissens über Iperoxo/BQCA Hybride, das Moleküldesign der dualsteren Liganden weiterentwickelt. So wurden in dieser Arbeit die ersten photo-schaltbaren bzw. photo-dimmbaren dualsteren Liganden beschrieben und charakterisiert. Des Weiteren wurde in dieser Arbeit die herkömmliche Charakterisierung von dualsteren Liganden weiterentwickelt. Es konnte zum ersten Mal gezeigt werden, dass es möglich ist, die Aktivierung eines Rezeptors durch einen dualsteren Liganden nachzustellen, indem die einzelnen Fragmente des ursprünglichen Liganden gleichzeitig appliziert werden. Diese auf Fragmenten basierende Charakterisierung ist die erste Anwendung dieser Art und birgt großes Potential für die zukünftige Suche nach neuen Wirkstoffen. Neben der Untersuchung von pharmakologischen Schwerpunkten wurde auch die Weiterentwicklung der Rezeptor FRET Methodik beschrieben. Die herkömmliche Anwendung der Rezeptor FRET Sensoren geschieht auf Objektträgern aus Quarzglas. In dieser Arbeit wurde diese Anwendung dahingehend weiterentwickelt, dass die Objektträger mit einer 20 nm dicken Goldschicht beschichtet wurden, um den Einfluss von Plasmonoberflächen auf physiologisch relevante FRET Messungen zu untersuchen. Es konnte gezeigt werden, dass mit Hilfe der Goldbeschichtung und in Abhängigkeit des Versuchsaufbaus die Energietransfereffizienz um bis zu 60 % gesteigert werden konnte. Diese Entdeckung zeigt Potential zukünftig die FRET-Reichweite zu erhöhen und so bisher nicht charakterisierbare Sachverhalte aufklären zu können.

6 Literature

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8 Affidavit

I hereby confirm that my thesis entitled “Investigating the Molecular Mechanism of Receptor Activation at Muscarinic Receptors by Means of Pathway-Specific Dualsteric Ligands and Partial Agonists” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Wuerzburg, 2018-12-10
Place, Date Signature
(Michael Kauk)

8.1 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Molekularen Grundlagen der Rezeptoraktivierung von muskarinergen Acetylcholin Rezeptoren durch dualsteren Liganden und Partialagonisten“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 10. Dezember 2018
Ort, Datum Unterschrift
(Michael Kauk)

10 Appendix

I. *FRET Studies of Quinolone-Based Bitopic Ligands and Their Structural Analogues at the Muscarinic M1 Receptor*; ACS Chemical Biology; Volume 12, Issue 3, Pages 833-843

II. *A Photoswitchable Dualsteric Ligand Controlling Receptor Efficacy*; Angewandte Chemie International Edition; Volume 56, Issue 25, Pages 7282-7287

III. *Enhanced Fluorescence Resonance Energy Transfer in G-Protein-Coupled Receptor Probes on Nanocoated Microscopy Coverslips*; ACS Photonics;

IV. *Intramolecular and Intermolecular FRET Sensors for GPCRs - Monitoring Conformational Changes and Beyond*; Trends in Pharmacological Science; Volume 39, Issue 2, Pages 123-135

V. Statement of individual author contributions to figures/tables/chapters included in the manuscripts (signed)

VI. Statement of individual author contributions and of legal second publication rights (signed)