Characterization of allosteric mechanisms on the M_2 und M_4 mACh receptor using the FRET-technique

Charakterisierung allosterischer Mechanismen am M_2 und M_4 mACh Rezeptor unter Anwendung der FRET-Technik

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section Life Sciences

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Nürnberg

Würzburg**, 2010**

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Würzburg, 10.04.2010

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1.1.1 Allostery and receptors

Nature has found various ways, to transmit extracellular signalling into living cells. For example membrane proteins, like receptors or channels provide binding sites for different ligands such as neurotransmitters or hormones, which upon binding of the ligand, activate downstream signalling processes in the cell. With about 800 different receptors in the human body, the G-protein coupled receptors (GPCR) represent the largest receptor family (Lagerstrom and Schioth 2008) and thus represent an important drug target. GPCRs are heptahelical transmembrane proteins consisting of seven transmembrane domains, three extracellular and intracellular loops with a N- and C-terminal tail. The mammalian GPCRs were divided into four different subclasses by means of their functional attributes and homology by the International Union of Pharmacology (IUPHAR) (Foord et al. 2005). Class 1 contains the rhodopsin like GPCRs, such as the adrenergic receptor family or the muscarinic acetylcholine receptors (mAChR). The secretory receptors were allocated to Class 2, the metabotropic glutamate receptors represent class 3. The latest class, frizzled family receptors, contains *Frizzled*, an important receptor family for cell differentiation, social behaviour, embryonic development and many other processes (Malbon 2004), and *Smoothened,* which plays a role in embryonic development (Corbit et al. 2005). In non-vertebrates, two further families (fungal mating pheromone receptors and cAMP receptors) have been identified (Attwood and Findlay 1994).

This thesis will concentrate on class A GPCRs, specifically the subtypes of the muscarinic acetylcholine receptor family ($M_{1\text{-}5}$ mAChR). GPCRs transmit their signal via a heterotrimeric G-protein, which is activated by conformational changes of the specific GPCR. To describe the binding sites of the endogenous receptor on the receptor surface, the term “orthosteric site” is used (Ehlert 1985). All ligands binding exclusively to this site are termed “orthosteric ligands”. Among the muscarinic receptor family, the orthosteric binding site seems to be highly conserved (Hulme et al. 2003). Site-directed mutagenesis has identified several conserved amino acids (see Figure 1.1.1 for example for human M_1 mAChR: C98, D105, Y106, P158, W164, R171, T172, C178, Q181, F182, S184, T189, T192, P200, W378, Y381, Y404, Y408, P415) that seem to play a key role in binding of orthosteric ligands

(Goodwin et al. 2007; Heitz et al. 1999; Matsui et al. 1995; Savarese et al. 1992; Wess et al. 1991; Wess et al. 1993).Within the muscarinic receptor family, they are located in regions of high similarity mainly on transmembrane domains (TMD) III, IV, V and VI, as well as on the second extracellular domain (Figure 1.1.1). Besides the orthosteric binding site for the endogenous ligand some GPCRs also provide binding sites for additional ligands. This site, which possibly may not have evolved to provide endogenous ligand binding (Hardy and Wells 2004) is called an „allosteric site“. Conversely, all ligands, which bind to an allosteric site modulating the binding or signaling properties of the orthosteric site are named “allosteric modulators”. Upon binding to the allosteric site, allosteric ligands can modulate orthosteric receptor signalling. Allosteric modulation has become an important therapeutical concept for the pharmaceutical industry. Allosteric ligands, which have only small or no agonist or inverse antagonist activity on their own, but are able to alter the receptor response to endogenous ligands, provide new exciting possibilities in drug development.

Several allosteric ligands are already used in medical treatment:

Benzodiazepines and barbiturates modulate the function of the $GABA_A$ receptor, another example memantine, a modulator of NMDA receptors (Kohl and Dannhardt 2001; Mohler et al. 2002). Very recently two new allosteric drugs entered the market. Cinacalcet (Sensipar®/Mimpara®; Amgen), a positive allosteric modulator for the Calcium sensing receptor, increases the sensitivity of the receptor for extracellular calcium and is therefore used in the treatment of hyperparathyroidism. Maraviroc (Celzentri®/Selzentry®; Pfizer), an antiretroviral drug for HIV treatment, is a negative allosteric ligand of the chemokine receptor CCR5 that stabilizes a receptor conformation with lower affinity for the HIV thus blocking CCR5-dependent entry. Especially promising is the progress that was made in the research for new allosteric drugs against CNS disorders (Conn et al. 2009).

Despite these efforts still little is known about how allosteric ligands act on the target receptors in general and the impact this modulation has on the cell itself.

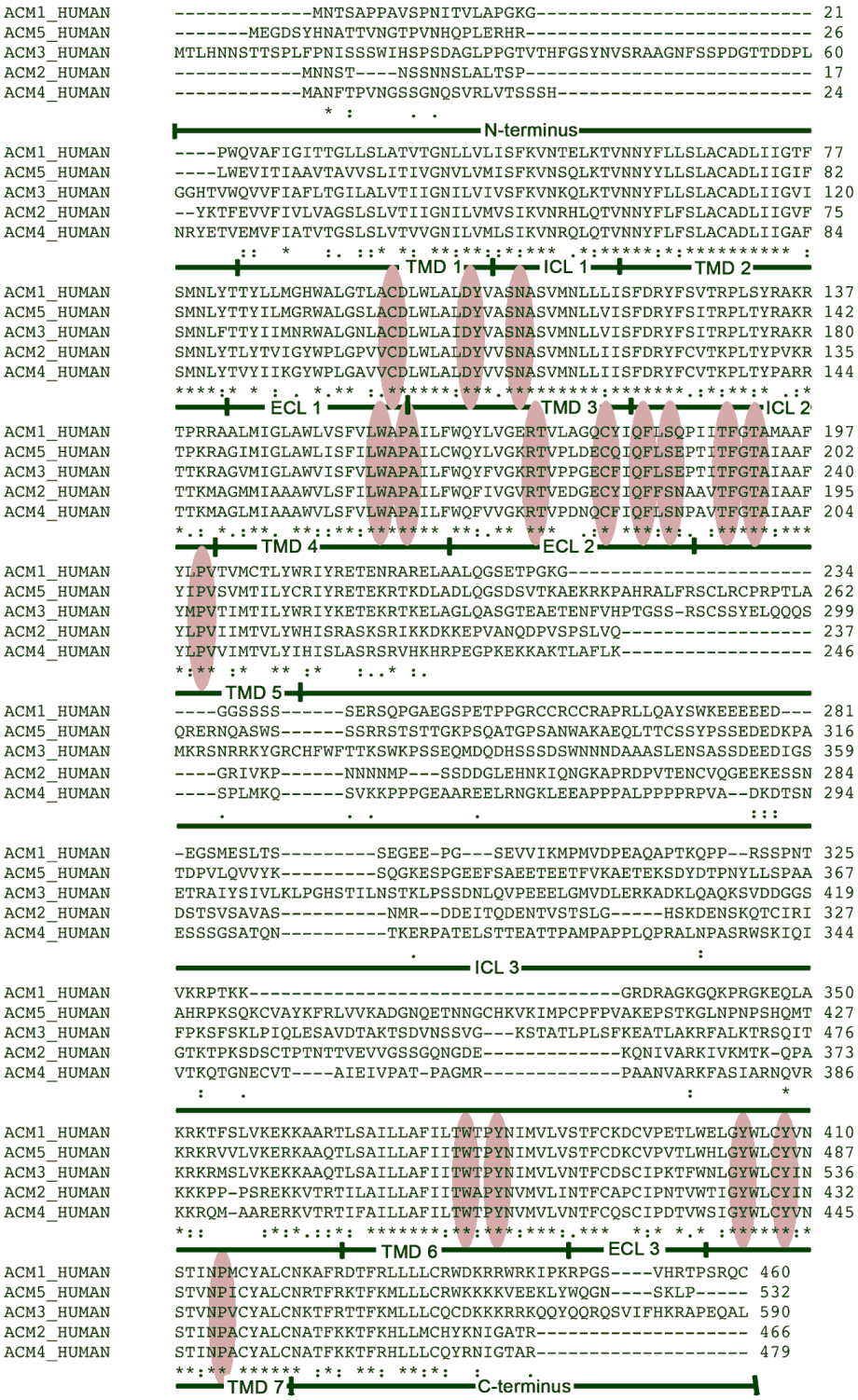


Figure 1.1.1 *Alignment of the human muscarinic acetylcholine receptors M_{1-5}, which displays sequence similarities between the proteins. Site-directed mutagenesis revealed residues within transmembrane domains III, IV, VI and VII and the second extracellular domain important for orthosteric ligand binding (examples are highlighted in red) The fully conserved residues, strong and weak conservation are marked as star, semicolon and colon, respectively (alignment created and modified from www.ebi.ac.uk/clustalw/).* *TMD: Transmembrane domain, ECL: extracellular loop, ICL: intracellular loop*

1.1.2 Definition

The term “allosteric” was first introduced by Monod and Jacob in 1961. During their studies it became evident that some of the used enzyme inhibitors had a structure, which was entirely different from the structure of the substrate. This led to the conclusion that these inhibitors might use another binding site on the enzyme surface.

Later Monod determined that biological activity of “allosteric” enzymes can be altered by ligands which bind to a topographically distinct site from the so called “isosteric” (now termed „orthosteric“) site (Monod et al. 1963). Whereas the isosteric site is the binding site of the substrate, the second binding pocket, which was termed “allosteric” (from the Greek allos (ἄλλός) =different and stereos (στερεός) = place) site, can be occupied by an “allosteric ligand”. This definition was initially limited to enzymatic processes.

In biological processes, allostery has been observed long before the term “allosteric” was introduced. Allosteric modulations were determined using several different concepts:

Probably the most renowned allosteric model is the hemoglobin protein, which was studied by Bohr in the early 20th century. His studies revealed that hemoglobin is able to bind up to four oxygen molecules simultaneously (Bohr et al. 1904; May et al. 2007b) which was termed “cooperativity”. So the first concept of allosterism is, that many proteins posses more than one binding site, which can interact with each other. The equation A.V. Hill described in the early 20th century, nowadays used to fit concentration response curves, was initially used to describe such cooperative binding reactions (Christopoulos and Kenakin 2002; Hill 1910).

Wyman and Allen (1951) described another concept of allostery. They examined the oxidation of the heme to describe the so-called “Bohr-effect”. They sought to explain the oxygen effect of the hemoglobin and the Bohr effect in terms of structural effects. Based on entropy effects they postulated that the Bohr effect, the possibility of heme to bind more than one oxygen molecule at a time, is due to conformational changes of the protein induced by the binding of the oxygen itself So, they were the first to suggest, that ligand binding to distinct ligand binding sites could induce a conformational change that itself increases the affinity for such ligands on other sites.

Monod then introduced three different concepts of ligand interaction:

Class I describes the interaction between two ligands competing for the same binding site. This was regarded the classical interaction between two ligands.

A class II interaction is present when an inhibitor binds to a moiety of the receptor, which is not recognized by the original substrate and simultaneously binds to the substrate specific site. An example for this interaction is salmeterol. Its alkyl side chain attaches to the receptor, which allows its salbutamol-like property to interact with the substrate site (Coleman et al. 1996; Green et al. 1996)

The allosteric, also termed "indirect", interaction (class III) describes the interaction, which results from ligand binding to the allosteric site. This binding results in a conformational change of the enzyme thus altering the binding of the substrate and possibly the activity of the enzyme.

It was assumed that this change in affinity was not brought about directly by the allosteric ligand but resulted from the conformational change induced by the allosteric ligand.

Finally, Monod proposed a formal mechanism for allosteric proteins. It required cooperativity in binding, oligomeric architecture and various conformational changes that were preferentially activated by different ligands (Monod et al. 1965). Nowadays, the concept of allostery also includes multi-site interactions, independently whether they are oligomeric or not (May et al. 2007b). In addition, the recently discovered bitopic ligands (Valant et al. 2008) also broaden the definition of "allostery" and "allosteric ligands".

In conclusion, the strict definition of "allostery" described by Monod et al cannot be fully applied to the contemporary concept of allostery. A new definition of the term "allosteric" has to be found, to take all recent aspects into consideration.

1.1.3 Allosteric mechanisms on G-protein coupled receptors

Allosteric interaction occurs if both orthosteric and allosteric binding site are occupied by ligands. The nature of the allosteric interaction is dependent on experimental conditions and the orthosteric ligand. Also, contingent on the used orthosteric ligand, the same allosteric modulator can act as an enhancer or an inhibitor of the orthosteric receptor modulation (Neubig et al. 2003). Some reports described the possibility of "allostery" on GPCRs already in the 1960's (for example see Lullmann et al. 1969; Monod et al. 1963), but not until the last decade it has become evident that GPCRs are potential targets for allosteric modulation. This discovery has an important impact on understanding structure-function relationships and, of course, provides a vast new field for the pharmaceutical drug development.

Since G-proteins themselves interact with a binding site distinct from the endogenous ligand binding side (Christopoulos and Kenakin 2002; Wess et al. 1997), thereby altering the

receptor conformation, G-proteins can be considered allosteric ligands for G-protein coupled receptors. Thus, possessing more than one binding site, all G-protein coupled receptors can be contemplated as naturally allosteric proteins (Christopoulos and Kenakin 2002; Ehlert 1985). In addition, a variety of different cellular molecules has been described in the last years, that interact with GPCRs in addition or independently to G-proteins (Brady and Limbird 2002). The ubiquitous cell surface distribution of GPCRs and their involvement in almost all biological processes explain their vast role in current medicine and drug targeting. With specific allosteric enhancers, new possibilities could evolve which could offer new therapeutic options for a plethora of diseases.

1.1.3.1 Allosteric sites at GPCRs

To date, little is known about the location of allosteric sites in GPCRs. In contrast to the often highly conserved orthosteric site, for example in the muscarinic GPCR family (M_1-M_5) (Hulme et al. 2003), allosteric sites can vary between different receptor subtypes. The recent determination of the crystal structure of the β_2 adrenergic receptor (Rasmussen et al. 2007) may provide a useful information for further identification of allosteric sites on GPCRs. There is strong evidence, that some GPCR, especially muscarinic receptors, can have more than one allosteric site (Birdsall and Lazareno 2005; Lazareno et al. 2000; Lazareno et al. 2002).

1.1.3.2 Structural mechanisms

Despite the former assumption that receptors can just switch between an “on” and an “off” state, it became apparent in recent years that in fact receptors can take on a variety of conformational states depending on the bound ligand and thus show a far wider range of behaviors (Kenakin 2005).

Being a target for allosteric modulation, GPCRs offer several conformational changes and thus new downstream reactions. Still radioligand binding assays are the common method to analyze allosteric modulation of the GPCRs. However, with the growing interest in new, specific allosteric ligands, some methodologies have been developed to study allosteric interactions at the molecular level.

The NMR-based methods provide a suitable tool to identify conformational changes. Fluctuations in protein structure are measured using a hydrogen/deuterium exchange technique and the resulting data are then being analyzed using a structure based algorithm (COREX) to produce a snapshot of the distribution of states (Freire 2000; Onaran and Costa 2009).

Molecular modeling seems to be another promising technique in developing new and specific ligands for allosteric binding sites of different GPCRs (Holzgrabe et al. 2000). This approach attempts to design allosteric ligands into the allosteric binding pocket. Resulting molecules can then be synthesized for further use and analysis of pharmacological properties and receptor on specificity. Of course, this method is rather time consuming since the necessary residues have to be first identified using mutational experiments.

1.1.3.3 Allosteric modulation of Affinity and Efficacy

Allosteric modulators can affect orthosteric ligand signaling in various ways. One effect is the influence on an orthosteric ligand's affinity, which affects the binding to the orthosteric site but not necessarily the response of the target protein. To achieve a modulation of the response a modulation of orthosteric ligand efficacy is needed. Additionally, some allosteric ligands show an agonistic effect on their own evoking a response in the target structure (Conn et al. 2009).

The affinity of a ligand to its binding site is determined by the ratio of ligand association to dissociation rates. One definition of an allosteric interaction is that allosteric interactions modulate the affinity of the orthosteric ligand.

This leads to further definitions:

Allosteric enhancement is present if the allosteric ligand increases association's rates of the orthosteric ligand and/or decreases the dissociation rates.

Allosteric inhibition occurs if the opposite is the case.

A model to visualize allosteric interactions on GPCRs is the allosteric ternary complex model (ATCM) (Figure 1.1.2). GPCRs are known to exist in various conformational states (receptor activation models), coupled to G-proteins (ternary complex models) (Weiss et al. 1996). In 1995 Paul Leff introduced the "Two-State" Model as the simplest way to describe the change of receptor conformation from the inactive to the active state (Leff 1995). A similar model had already been proposed by Monod, Changeux and Wyman for hemoglobin and oxygen (Monod et al. 1965). With the discovery of the constitutively active state of GPCRs, this model was improved to the contemporary used „Extended Two State Model" (Oberschmid et al.) (Oberschmid et al. ; Samama et al. 1993).

Further "upgrading" of the ETM allows modeling G-protein binding to the cubic ternary receptor model (CTM) (Kenakin 2004) which can easily be adapted for allosteric interactions. Several publications on interactions at A family GPCRs (Christopoulos and Kenakin 2002;

Hall 2000; Tucek and Proska 1995) and C family GPCRs (Parmentier et al. 2002) described how to modify this model to make it suitable for allosteric interactions. The result is the allosteric two state model.

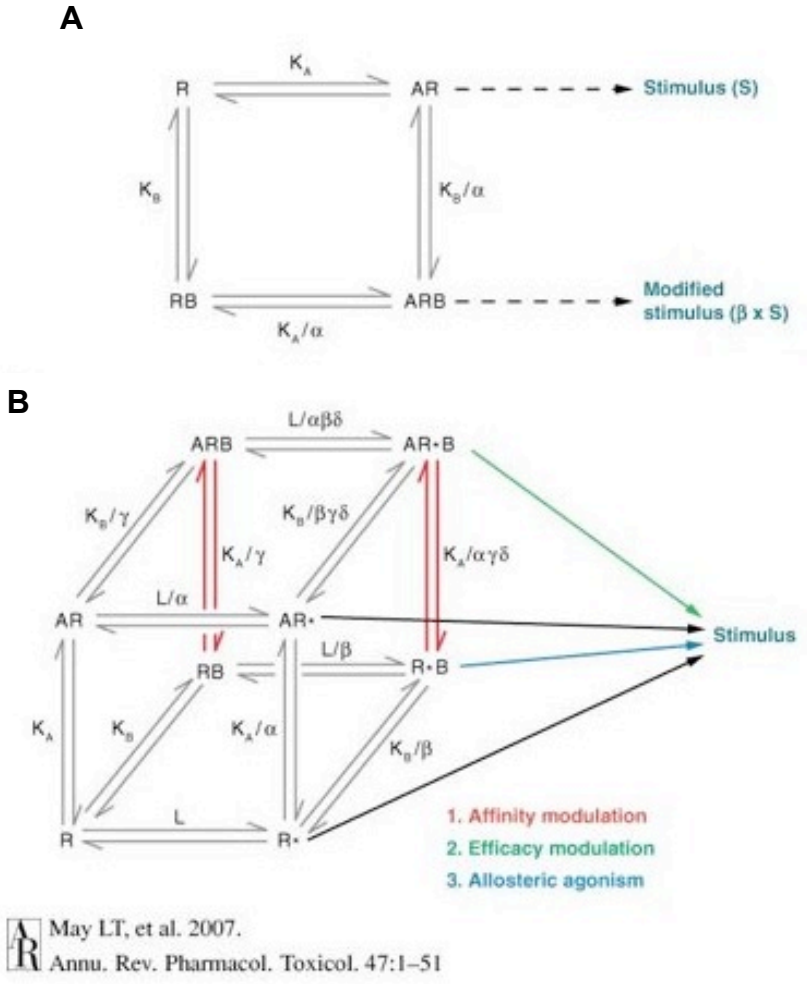


Figure 1.1.2 *Ternary models describing allosteric interactions*

A *the allosteric ternary complex model (ATCM), where A describes the orthosteric and B the allosteric ligand in terms of their equilibrium constants (K_A, K_B), which bind to the receptor R. the cooperativity factor α shows the impact and direction of the allosteric modulator on the orthosteric binding affinity. AR and ARB can induce a stimulus on the cell, β is the proportionality factor for the allosteric interaction on the stimulus*

B *the allosteric two state model, which takes affinity and efficacy into account while describing the allosteric interaction. The active (R*) and inactive (R) receptor states are modulated by different ligands according to the cooperativity factors of the different states. This model also describes three different manifestations of the allosteric effect (May et al. 2007b).*

Figure 1.1.2 A depicts the simple ATCM. Here allosteric and orthosteric ligands bind to the receptor saturably and reversibly. The interaction depends on the ligand concentration and on their equilibrium constants (K_A, K_B) and the parameter α, also termed cooperativity factor (Ehlert 1988). The cooperativity factor describes the impact of the allosteric ligand on the orthosteric ligand affinity, when both ligands are bound to their respective sites. Both ligands are conformationally linked, so that what A does to B, B does to A in return. A value of $\alpha > 1$ describes positive cooperativity (allosteric enhancement), $\alpha < 1$ and > 0 describes negative cooperativity (allosteric inhibition). Hence, $\alpha = 1$ is neutral cooperativity. Assuming, that the effect of AR on the cell is the same as the effect of ARB on the cell, the ATCM describes allosteric effects solely for affinity modulations.

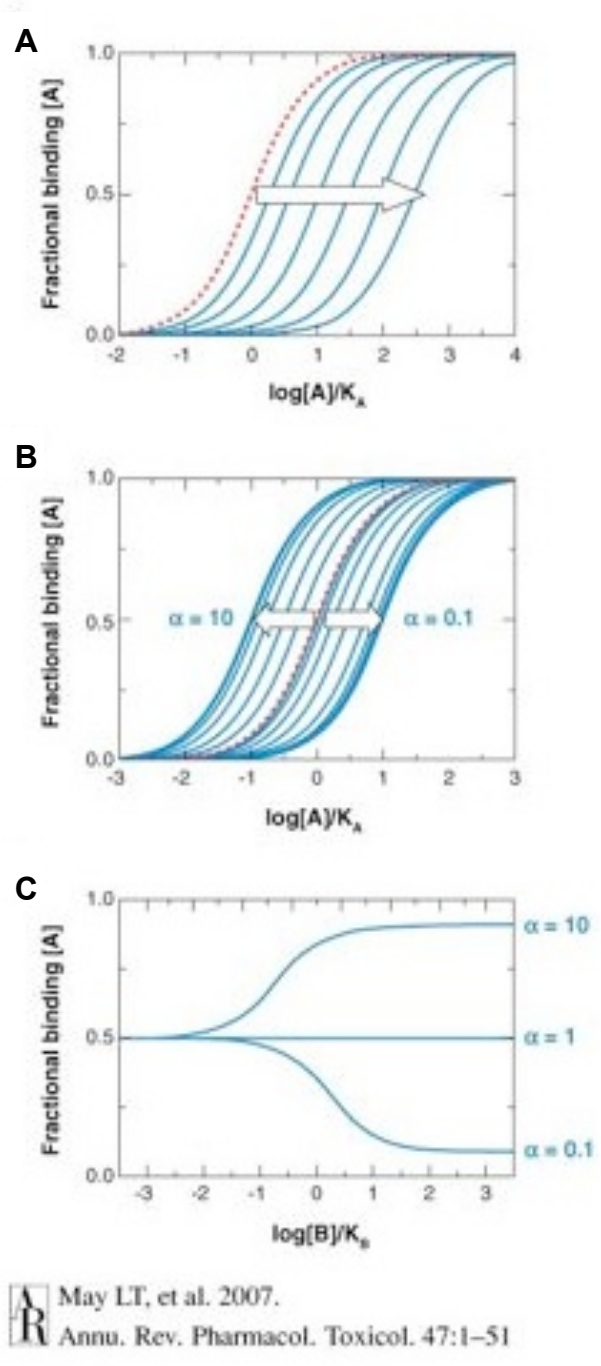


Figure 1.1.3 *Effects of orthosteric antagonist, allosteric enhancer and inhibitor.*
*Whereas competitive interaction via an orthosteric antagonist results in a theoretically limitless rightward shift of the orthosteric ligands concentration-occupancy curve (**A**), allosteric ligands exhibit increasing inability to shift the orthosteric occupancy at maximal modulator concentrations. Arrows indicate increasing concentrations of allosteric ligands (**B**).*
C *Using a fixed concentration of orthosteric ligand (K_A) and increasing concentrations of allosteric ligands, every curve exhibits an asymptotic run for all, positive, negative and neutral allosteric ligands.*
(May et al. 2007b)

The change in receptor conformation brought about by an orthosteric agonist can be influenced via an orthosteric antagonist, an allosteric enhancer or an allosteric inhibitor. These changes can be visualized using sigmoidal curve fitting (Figure 1.1.3). Different concentrations of orthosteric antagonist, allosteric enhancer and inhibitor were used together with an orthosteric ligand.

Whereas increasing concentrations of the orthosteric antagonist, seem to induce an infinite right shift of the agonist curve (Figure 1.1.3 A) allosteric modulation of the curves reaches a clear limit. Addition of allosteric enhancer (α=10) results in a leftward shift, so that the same

effect is reached with lower concentrations of agonist, addition of allosteric antagonist (α=0,1) effects a rightward shift of the curve. Both shifts show a progressive inability to maximally shift the orthosteric ligand occupancy curve despite high concentrations of allosteric modulator (Figure 1.1.3 B).

If a single concentration of orthosteric agonist is used, increasing concentrations of the allosteric modulators result in an asymptotic curve. This elucidates, that allosteric modulations are limited and cannot be forced using higher concentrations of allosteric ligands (Figure 1.1.3 C).

The conclusion one can draw from the described observations is that the ATCM emphasizes that for allosteric drug development one needs at least to determine two different thermodynamic parameters - α and K_B, contrary to orthosteric ligands, where just the K_B is necessary. As α and K_B are independent, both parameters can be manipulated individually facilitating the development of pharmacologically selective allosteric ligands (May et al. 2007b).

Some allosteric ligands do not alter the interaction with the orthosteric ligand but change downstream signals. For example 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), a metabotropic glutamate mGluR1 antagonist, does not alter binding of [^{3}H]glutamate but changes the outcome of inositol signalling. This is an example for classical non-competitive antagonism (Litschig et al. 1999).

The ATCM is a very good model to be applied to binding data, giving quantitative estimates of modulator affinity and binding cooperativity. When applied to functional data it requires the assumption that the modulator does not perturb the signaling capacity of the receptor. As a consequence from the examples mentioned above, the ATCM is not sufficient to describe allosteric modulation of the GPCRs. Taking the permissive nature of allosteric modulation into account, additional parameters are needed, to describe the different variations of allosteric modulations (Leach et al. 2007). The already mentioned factor β (Figure 1.1.2A) is used to assess the influence of the allosteric ligand on the intrinsic efficacy (ε) of the orthosteric ligand. This factor modifies the effect of the cell stimulus triggered by the [AR] complex when the allosteric ligand joins the complex resulting in a ternary complex [ARB].

It is expected that allosteric modulators bias the conformational equilibrium of GPCRs to favor specific conformational sets over others. In 2000 Hall described a model that accommodates such properties by extending the ATCM to the allosteric two state model (ATSM) (Figure 1.1.2B) (Hall 2000). This new model uses different cooperativity factors to

describe the abilities of orthosteric and allosteric ligands to cause an active receptor state (cooperativity factors α and β), to describe the effect of allosteric and orthosteric ligand on each other (χ) and to describe the ability of either ligand to modify the transition to the active state of the ternary complex (δ).

Still, not every allosteric modulation discovered experimentally can be explained with the ATCM and the ATSM, but needs further extension of those models. Discoveries like multiple allosteric sites (Lazareno et al. 2000), bitopic ligands (Valant et al. 2008) or the addition of G-protein coupling to the receptor (Christopoulos and Kenakin 2002; Durroux 2005) require these. In addition, allosteric effects on receptor dimers (Durroux) have to be considered. But still these models can be of use tool if additional experiments have to be considered.

1.1.3.4 Therapeutic advantages of allosteric modulations

1.1.3.4.1 Allosteric receptor response

Orthosteric receptor response is dependent on affinity and concentration of the ligand. Orthosteric agonists activate and orthosteric antagonists inactivate the receptor, regardless of the fluctuating concentrations of endogenous ligand, thus leading to possible side effects, toxicity, desensitization and, especially under long-term treatment, can cause receptor up or down regulation (May et al. 2004).

Allosteric ligands have little or no intrinsic effect that modulate receptor function resulting in an attenuation or enhancement of the receptor signal and they can only modulate the receptor, if the endogenous ligand is present. One major advantage is that allosteric effects, as mentioned above, are saturable which reduces the risk of overdosage. In addition, allosteric modulation is closely linked to the presence of an orthosteric ligand. In terms of medical treatment this means, that allosteric ligands can only modulate the receptor function if the endogenous ligand is present. The spatial and temporal character of endogenous receptor activation thus remains unharmed. This may be an advantage for yet insufficient treatment of certain diseases, for example Alzheimer's disease. Whether this also is an advantage for receptor desensitization is still unclear since the studies by Klaasse et al on the adenosine A_1 receptor (Klaasse et al. 2005) and May et al on the muscarinic M_2 receptor (May et al. 2005) revealed, that allosteric modulators can have an effect on receptor desensitization.

1.1.3.4.2 Selectivity

Orthosteric sites, in most cases, are highly conserved among receptor families because their structure was developed through evolutionary adjustment. So it is rather difficult to manufacture subtype selective ligands. Especially for the muscarinic GPCR family, due to

their high structural similarities within the orthosteric site (Figure 1.1.1), this has proven to be rather difficult. Allosteric sites seem to be receptor residues that can have an influence on receptor conformation upon ligand binding, but without being structured for this sole purpose. These sites are rather non-conserved through a certain receptor family, so that it is even possible that an allosteric ligand is an enhancer on some subtypes and neutral or an inhibitor on the others. This behavior was termed “absolute selectivity”(Lazareno et al. 2004; Lazareno et al. 1998).

1.1.3.4.3 Signaling

A possible, but still insufficiently studied advantage is the permissive receptor action resulting from allosteric modulations. GPCRs have been shown to activate different signaling pathways with distinct conformations (Kenakin 2003; Palanche et al. 2001). So it is possible that upon stimulation with a specific allosteric ligand a distinct receptor conformation is stabilized which evokes only certain cell signaling pathways. Hence, it may be possible to further reduce side effects by just applying the specific allosteric ligands for the specific intracellular signal.

1.1.4 Allosteric ligands of GPCRs

A plethora of allosteric ligands have been designed to study the effects of allosteric modulations and to find new therapeutics. Here, allosteric ligands of the mAChR subfamily are described in some more detail.

The muscarinic receptor family was the first GPCR family to provide evidence for allosteric modulation. With a highly conserved orthosteric site among the subtypes M_1-M_5, it was difficult to design orthosteric drugs for a specific receptor subtype. With allosteric ligands, this became a realistic possibility.

1.1.4.1 “Typical” allosteric ligands

Whole tissue studies with alkane-bis-ammonium compounds (Lüllmann H 1969) and gallamine in the heart (Clark 1976) suggested, that the described observations were not a result of competitive but of allosteric effects. Both concluded this from concentration-response data, which, unlike a competitive effect, reached a maximum at higher concentrations. To date, those groups, the alkane-bis-ammonium compounds and the neuromuscular-blocking agents such as gallamine, represent the most extensively studied allosteric ligands. In the following years several studies also witnessed the distinct effects of gallamine and other neuromuscular-blocking agents in radioactive binding assays with the antagonist [^{3}H]N-methylscopolamine ([^{3}H]NMS) and the agonist [^{3}H]3-Quinuclidinyl

benzilate ([^{3}H]QNB), which differentiated them from sole muscarinic antagonists (Dunlap and Brown 1983; Stockton et al. 1983).

To date, numerous studies with different agents of those modulator groups have been performed. All of them affirm typical allosteric attributes, such as incomplete inhibition of specific ligand binding or retardation of ligand kinetics. Furthermore these studies revealed a certain preference of the used substances for the M_2 mAChR. Although no subtype-specific substance was found, all allosteric ligands showed rather low affinity for the M_5- mAChR subtype and the highest affinity for the M_2 mAChR subtype (Lanzafame et al. 1996; Lanzafame et al. 1997; Maass et al. 1995; Maass and Mohr 1996). Thiochrome was the first substance, for which subtype-selectivity was found. Although thiochrome binds to all five mAChR subtypes its selectivity derives from the positive interaction between acetylcholine and thiochrome on the M_4 mAChR subtype (Lazareno et al. 2004).

With a few exceptions, the described substances are mostly allosteric inhibitors. The effect of alcuronium, a neuromuscular-blocking compound, though, is dependent on the orthosteric ligand and the muscarinic receptor subtype. Whereas a positive allosteric effect was described with the orthosteric antagonist [^{3}H]NMS, both for the M_2 as well as the M_4 mAChR subtype, it exhibits a negative effect on the M_1, M_3 and M_5 receptor subtypes. In addition, alcuronium has a negative allosteric effect on the orthosteric agonists [^{3}H]QNB on all subtypes and, for the M_2 mAChR subtype, on the partial agonist pilocarpine (Hejnova et al. 1995; Jakubik et al. 1995; Zahn et al. 2002).

The alkaloid-like structure of alcuronium was used as a lead for various new allosteric compounds, such as strychnine or vincamine, and also for the first sole allosteric enhancers like brucine, which increases the effects of orthosteric agonists (Jakubik et al. 1997). Taking into account the plethora of structure/activity, today two categories of allosteric modulators can be distinguished:

- neuromuscular blockers and bis-onium modulators and
- mono-quaternaries and tertiary amines related to alkaloids (Birdsall and Lazareno 2005; Mohr et al. 2003).

In search of promising drugs a large number of allosteric modulators have been synthesized by the pharmaceutical industry. Norclozapine (N-desmethylclozapine), the major metabolite of the antipsychotic drug clozapine, was regarded as a possible adjunct in treatment of schizophrenia as it stimulates not only dopamine D_2-receptors but also serves as an allosteric agonist for the M_1 mAChR subtype. Unfortunately, the outcome of a recent controlled

randomized trial was that its effect is at placebo level (for review see Bishara and Taylor 2008). Eli Lilly was successful in synthesizing a specific allosteric enhancer for the M_4 mAChR subtype, LY2033298. Applied with the agonist oxotremorine, this substance already showed promising results in a preclinical animal model for the treatment of schizophrenia (Chan et al. 2008), indicating its potential use as an allosteric muscarinic antipsychotic agent.

1.1.5 Atypical allosteric modulators

Quite recently Birdsall et al discovered the existence of at least one more allosteric binding site, when they used the protein kinase inhibitor KT5720, an indolocarbazole derivative of staurosporine, in addition to the allosteric compounds gallamine and brucine on the muscarinic M_1 mAChR subtype. Both compounds did not affect the effect of KT5720 on [^{3}H]NMS binding (Birdsall et al. 2001). These findings suggest that muscarinic receptors could bind more than one allosteric compound, which increases the possibilities for new substances and therefore new therapeutic approaches. KT5720 and a number of other indolocarbazole derivatives of staurosporine can be called atypical allosteric ligand as they lack the "typical" positively charged nitrogen. In addition the "atypical" compounds exhibit higher affinity for the muscarinic M_1 than for the M_2 mAChR subtype, and have little or no effect on [^{3}H]NMS dissociation rate (Gregory et al. 2007). Also, the neurokinin receptor antagonists WIN 62,577 and WIN 51,708 have been described to bind at an additional allosteric binding site. Both substances exhibited competitive behavior with KT5720 but affected substances like gallamine, strychnine C7/3-phth and alcuronium in a non-competitive manner even with an unoccupied orthosteric site (Lanzafame et al. 2006; Lazareno et al. 2000).

Another class of atypical allosteric compounds like tacrine, the bispyridinium 4,4'-bis-[(2,6-dichloro-benzyloxy-imino)-methyl]-1,1'propane-1,3-diyl-bis-pyridinium dibromide (Duo 3) and a group of pentacyclic carbazolones are substances, whose behaviors are not in line with the ATCM (Gharagozloo et al. 2002; Potter et al. 1989; Surig et al. 2006; Trankle et al. 2005).

1.1.6 Novel developments

Taking into account the vast possibilities of allosteric modulation and its importance for drug development, a lot of efforts are made to identify relevant structures (Trankle et al. 1996) in order to synthesize allosteric modulators with specific qualities. A very recent development was the synthesis of a gallamine-tacrine hybrid which can apparently occupy two different allosteric sites. This hybrid exceeds the allosteric potency of the basic molecules (Elsinghorst et al. 2007).

A similar approach could be the use of so-called “bitopic” (or “dualsteric”) compounds, which bind not only to an allosteric site but also to the orthosteric-binding pocket. Interestingly for the substance McN-A-343 (4-(m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride) indications for this behavior can be found in many studies, some of them even dating back to the 1980’s (Birdsall et al. 1983; Waelbroeck et al. 1988). Very recently Valant et al found convincing data that McN-A-343 exhibits „bitopic“ behavior on the M_2 mAChR (Valant et al. 2008).

In February 2009 two hybrids were introduced which are composed of moieties of an oxotremorine-like agonist and of the alkane-bis-ammonium-like allosteric inhibitors. These two hybrids show binding specificity with preference for the M_2 mAChR subtype and in addition activate a specific signaling pathway via the $G_{i/0}$-protein, which was verified using PTX to block $G_{i/0}$-related signaling. Despite these achievements, no additive gain of affinity could be achieved due to the use of compounds that stabilize functionally different receptor conformations (Antony et al. 2009).

1.2 Muscarinic receptors

The first one to report that physiological effects evoked by acetylcholine (ACh) were mediated by different receptors was Henry Hallett Dale at the beginning of the 20th century. His Nobel prize awarded studies revealed that acetylcholine produces similar effect on smooth muscle, glands and parasympathetic fibers (Dale 1914).

These findings lead to the first distinction into the ionotropic nicotinic and the metabotropic muscarinic receptors (Figure 1.2.1), and the first definition, whereby muscarinic receptors can be activated by muscarine and blocked by atropine and accordingly nicotine receptors are stimulated by nicotine and blocked by curare (Hulme et al. 1990).

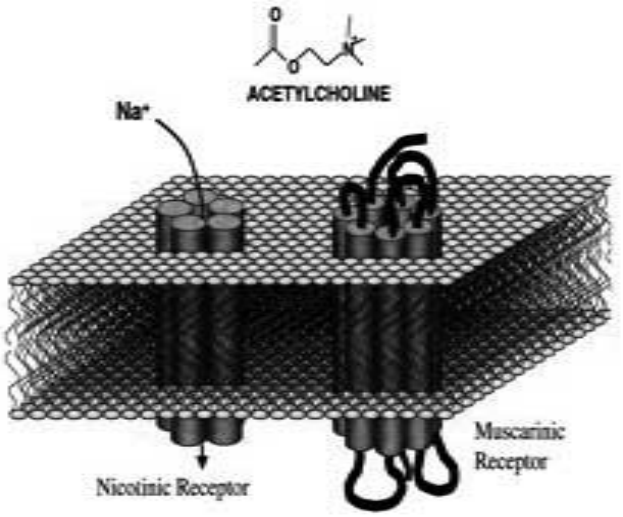


Figure 1.2.1 *Scheme of a nicotinic and muscarinic receptor (Felder et al. 2000).*

While nicotinic receptors quite early were identified as members of the family of ligand-gated ion-channels (Paterson and Nordberg 2000), the identification of the more complex coherencies regarding muscarinic receptors took almost 70 years.

In the early 1950's Wescoe and Riker during their tests with the compound gallamine on cat heart tissues, found evidence for heterogeneity of muscarinic effects (Riker and Wescoe 1951) but it took another 30 years until, with the discovery of the substance pirenzepine (Hammer et al. 1980) that the first two muscarinic receptor subtypes, M_1 and M_2, were identified (Hammer and Giachetti 1982). As cloning techniques became a tool in laboratory practice, five different muscarinic receptor species were successively identified (M_1- M_5). Currently there is no evidence for the existence of further subtypes.

Kubo et al managed to clone the porcine M_1 and M_2 mAChR followed by Bonner et al, who identified the human M_2 - M_5 mAChR species (Bonner et al. 1987; Bonner et al. 1988; Kubo et al. 1986a; Kubo et al. 1986b). Pharmacologically, the M_1 – M_4 mAChR subtypes can be clearly discriminated, which was also stated by the Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) (Caulfield and Birdsall 1998). At the amino acid level, muscarinic receptors are very similar among the mammalian species and also the receptor pharmacology seems to be comparable (Hannan and Hall 1993). Their gene products can be related to a functional property in both, humans and animals.

For the hM_2 and hM_3 mAChR the entire genes have already been mapped (Forsythe et al. 2002; Zhou et al. 2001).

Despite characterization through binding studies in CHO cells expressing the cloned M_5 mAChR (Buckley et al. 1989) and ongoing efforts to clearly identify the receptor in tissue samples (Ryberg et al. 2008) , further studies regarding the M_5 receptor subtype are needed to elucidate its function, receptor distribution and pharmacology.

1.2.1 Receptor distribution

1.2.1.1 Peripheral tissues

The following paragraphs will give a short overview of the most important features of muscarinic receptors and their function in peripheral tissues of mammalians.

1.2.1.1.1 M_1 mACh receptors

The M_1 mAChR are present in the eye, specifically in the iris, sclera and lens epithelial cells, mediating cytosolic calcium release (Collison et al. 2000; Gil et al. 1997; Ishizaka et al. 1998). This subtype was also detected, along with the M_3 subtype, in salivary glands (Culp et al. 1996), where it seems to play a role in the control of high-velocity lubrication (Gillberg et al. 1998). In the heart M_1 mAChRs were reported in mouse cardiac tissue and human atrial myocytes (Islam et al. 1998; Wang et al. 2004), where they affect heart rate presumably trough activation by catecholamines released by sympathetic ganglia (Hardouin et al. 2002).

In gut smooth muscle cells (So et al. 2003) this subtype can induce an NO driven relaxation. These findings could be observed in M_3–KO mice, as this function of the M_1 subtype is normally masked by M_3-mediated contractility (Stengel and Cohen 2003). One of the first tissues to be identified expressing M_1 mAChR were sympathetic and parasympathetic ganglia. Here the M_1 subtype plays a role in ganglionic neurotransmission (Hammer and Giachetti 1982).

1.2.1.1.2 M_2 mACh receptors

This muscarinic receptor subtype can be found in a plethora of peripheral tissues. It is the most important subtype in heart tissues, where it induces negative chronotropic, dromotropic and inotropic effects (Caulfield 1993). Its role in these effects was appreciated by using M_2-KO mice, which showed no bradycardic effect upon stimulation with carbamylcholine. Smooth muscle preparations from the same mice displayed reduced excitability towards muscarinic agonists (Stengel et al. 2000). In fact, the M_2 mAChR is the most predominant receptor in smooth muscles, even outnumbering the M_3 (for review see Abrams et al. 2006; Eglen et al. 1994). The receptors are located pre- and postjunctionally, however their postjunctional function is not yet fully determined (Caulfield 1993).

1.2.1.1.3 M_3 mACh receptors

In the skin M_3 mAChR inhibit lateral keratinocyte migration (Chernyavsky et al. 2004) and affects cell-cell adhesion (Nguyen et al. 2004). As mentioned before, the M_3 receptors, along with the M_2 receptor subtype, are identified as an important subtype in smooth muscle cell function. Their major role seems to be the contraction of this cell type as studies with M_3-KO mice displayed major contractile impairment in smooth muscle preparations of the gastrointestinal tract and urinary bladder (Matsui et al. 2002; Stengel and Cohen 2002). Accordingly, these mice had enlarged pupils confirming the role of the M_3 in the pupil sphincter muscle (Matsui et al. 2002). In the gastrointestinal tract and the bladder, the M_3 subtype is outnumbered by the M_2 subtype, nevertheless according to studies in rodents and dogs, it seems to be more important for gastrointestinal motility (Chiba et al. 2002; Li et al. 2002) and also it is the subtype mediating detrusor function (Fetscher et al. 2002; Matsui et al. 2000). Salivation is predominantly mediated by M_3 mAChR (Bymaster et al. 2003; Gillberg et al. 1998) and it increases the insulin secretion from pancreatic β-cells (Duttaroy et al. 2004). In the heart, however, this subtype seems to play only a minor role (Stengel et al. 2002).

1.2.1.1.4 M_4 mACh receptors

Despite being identified in a vast number of tissues, for example the eye or the gastrointestinal tract the role of the M_4 receptor subtype often remains unclear (Abrams et al. 2006).

It is suggested that the M_4 mAChR mediates autoinhibition of acetylcholine release in guinea pig trachea (Kilbinger et al. 1995) and also plays a minor role in salivation (Bymaster et al. 2003). D'Agostino et al indicated that the M_4 subtype is the autoreceptor inhibiting the ACh

release in the human detrusor. Also in the mouse heart atria, along with other non-M_4 receptors, the M_4 mAChR acts as an autoceptor (Zhou et al. 2002).

The receptor subtype is abundant in limbal and conjunctival epithelial cells (Liu et al. 2007). It is also regarded as the receptor mediating non-adrenergic non-cholinergic relaxation in the rabbit anococcygeus muscle (RAM). Preparations of the RAM are therefore used as a functional model (Gross et al. 1997).

1.2.1.1.5 M_5 mACh receptors

In the last years many efforts have been made in identifying the M_5 mAChR in tissues throughout mammalian tissues. Unfortunately, in many tissues its function still has to be uncovered. It has been found in the human heart tissue (Wang et al. 2001a) and in the human iris sphincter (Ishizaka et al. 1998). Blood vessels in brain and periphery also express this receptor subtype (Phillips et al. 1997) and quite recently a M_5- knockout mice model supported the suggestion that the M_5 mAChR is responsible for relaxation of cerebral, but not peripheral arterioles and arteries (Yamada et al. 2001a).

1.2.1.2 Central nervous system

Several different experimental approaches such as radioligand binding studies (Ferrari-Dileo et al. 1994) or immunological studies (Flynn et al. 1995) revealed that all five muscarinic receptor subtypes are present in the CNS. Another very important approach to determine localization and function of the specific subtype is the generation of knockout mice (KO), which has been successful for all five muscarinic receptor subtypes (for review see Wess 2004). Results of these KO-studies will be described in detail for the specific receptor in the following paragraphs. In summary, the M_1 mAChR seems to be the most predominant receptor in the CNS, the M_2 receptor subtype is dominant in the thalamus and the brainstem. The M_4 mAChR is most prominent in the striatum where it's suggested to play a role in dopamine release and locomotor control. Compared to the other subtypes, expression of M_3 and M_5 mAChR are much lower (Langmead et al. 2008).

1.2.1.2.1 M_1 mACh receptors

The M_1 mAChR is most abundant in the neostriatum, neocortex and the hippocampus (Levey 1993). In animal models for Alzheimer's disease antagonists of the M_1 receptor subtype reverse impairment of learning and spatial memory (Fisher et al. 2003) and also mice lacking the M_1 receptor display cognitive dysfunction (Anagnostaras et al. 2003). Thus both studies support the assumption, that this subtype plays a major role in higher cognitive functions such as learning and memory.

In addition M_1-KO mice showed an increase in locomotor activity and it is suggested, that this increase is referred to elevated dopamine levels in the striatum (Gerber et al. 2001; Miyakawa et al. 2001).

In pyramidal cells of the hippocampus, the M_1 mAChR induces neuronal differentiation (VanDeMark et al. 2009). Studies in rats showed involvement in the circadian rhythm via M_1 receptors located in the hypothalamic suprachiasmatic nucleus (SCN) (Gillette et al. 2001).

1.2.1.2.2 M_2 mACh receptors

In M_2-KO mice oxotremorine fails to induce akinesia and whole-body tremor, which suggests the involvement of striatal mAChR. Also oxotremorine-mediated hypothermia in these mice was reduced, but not abolished indicating that also non-M_2 mAChR are involved in hypothalamic thermoregulation (Gomeza et al. 1999a).

Like the M_1 mAChR, the M_2 receptor subtype seems to play a major role in cognitive function. In passive avoidance tests, mice lacking the M_2 receptor subtype displayed significant performance deficits (Tzavara et al. 2003). These results strengthen previous findings from pharmacological studies, implicating a major role of the M_2 mAChR in learning and memory processes (Carey et al. 2001; Quirion et al. 1995).

Another important feature of the M_2 mAChR in the CNS is mediation of an analgesic effect. Experiments with mice lacking either the M_2 receptor or the M_4 receptor proved that the M_2 receptor plays the major part in mediating this effect (Gomeza et al. 1999a; Gomeza et al. 2001). Nevertheless only in mice lacking both receptor subtypes oxotremorine-mediated analgesia was completely abolished (Duttaroy et al. 2002).

Systemic administration of a partial muscarinic agonist led to elevated serum corticosterone levels in WT mice, but not in M_2-KO mice, probably through stimulation of the hypothalamic-pituitary-adrenocortical-axis and the following release of corticotropine releasing hormone (Hemrick-Luecke et al. 2002)

Another important physiological function of the M_2 mAChR in the CNS is its participation in ACh mediated signaling. Studies in rodents and in human brain tissues revealed that it acts as an autoreceptor inhibiting neurotransmitter release, but predominantly in cortex and hippocampus, whereas in striatum the M_4 mAChR plays the major role (Kitaichi et al. 1999; Starke et al. 1989; Zhang et al. 2002a).

1.2.1.2.3 M_3 mACh receptor

Despite its presence in different regions of the brain (Levey et al. 1994) only very little is known about the physiological function of the M_3 mAChR. Lack of the M_3 receptor subtype in

mice results in a hypophagic and lean phenotype with decreased serum levels of leptine and insulin levels and a reduced food intake compared to the WT mice (Yamada et al. 2001b). These findings indicate a role for the M_3 mAChR in food intake and body weight control. In the striatum this receptor subtype has inhibitory effects on the dopamine release (Zhang et al. 2002b).

1.2.1.2.4 M_4 mACh receptor

As mentioned above, the M_4 receptor together with the M_2 mAChR subtype plays a role in analgesia (Duttaroy et al. 2002). In the striatum it controls autoinhibitory ACh release and facilitates dopamine release (Zhang et al. 2002a; Zhang et al. 2002b).

M_4-KO mice show an increased basal locomotor activity supporting the concept that striatal M_4 mAChR inhibits D1-receptor mediated locomotor activity (Gomeza et al. 1999b). The prepulse inhibition (PPI) of the startle reflex, a measure of attention, is also modulated in mice lacking the M_4 receptor subtype (Felder et al. 2001). These findings might present this subtype as a worthwhile target in the treatment of schizophrenia.

1.2.1.2.5 M_5 mACh receptor

It took almost ten years after the discovery of this receptor subtype before studies on M_5-KO mice revealed a role in dopamine transmission in the striatum and nucleus accumbens (Forster et al. 2002; Yamada et al. 2001a; Zhang et al. 2002b). As the nucleus accumbens is hypothesized to play a role in the rewarding effect of opioids (Koob 2006), it was tested whether mice lacking the M_5 receptor subtype exhibit changes in drug-seeking behavior (Basile et al. 2002). Interestingly, rewarding effects and withdrawal symptoms after application of morphine or cocaine were diminished compared to the WT mice but the analgesic effect of morphine remained the same in both phenotypes (Basile et al. 2002; Fink-Jensen et al. 2003; Thomsen et al. 2005). As mentioned above, this subtype is involved in the dilation of cerebral blood vessels (Yamada et al. 2001a). Finally, this receptor subtype may play a role in the regulation of fluid intake (Takeuchi et al. 2002).

1.2.2 Signal transduction

In contrast to the nicotinic acetylcholine receptors, which belong to the ligand-gated ion channels, the mACh receptor family belongs to the super family of G-protein coupled receptors. The genes encoding the muscarinic receptors are intronless (Felder et al. 2000), and hydrophobicity studies showed that the receptor topology upon folding is similar to the well-characterized protein rhodopsin (Lu et al. 2002) which is composed of seven transmembrane domains spanning through the cell membrane, connected by hydrophilic intracellular and

extracellular loops, with an extracellular N- and an intracellular C-terminus (Palczewski et al. 2000; Schertler et al. 1993).

The receptor domains involved in G-protein coupling seem to be located in the intracellular loop 2 (IL2), intracellular loop 3 (IL3) and the C-Terminus (Brann et al. 1993; Burstein et al. 1998). Accordingly, these regions are quite similar between the receptor subtypes sharing the same signaling pathways, as would be the odd-numbered and even numbered muscarinic receptor subgroups. Heterogeneity between $M_{1\text{-}5}$ can be found in the rather long IL3, which consists of over 200 amino acids (Hulme et al. 1990).

The muscarinic receptor family shows a plethora of possible signaling mechanisms (Nathanson 2000) (Figure 1.2.2). M_1, M_3 and M_5 receptors preferentially couple to a specific subtype of the Gα-subunit, $G\alpha_q$. Activation of $G\alpha_q$ leads to an activation of phospholipase Cβ (PLC Cβ), which then triggers the breakdown of phosphatidyinositol-4,5-bisphosphate (PIP_2) to the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG).

IP_3 via the IP_3 ion receptor releases Ca^{2+} from intracellular compartments causing a transient calcium increase. DAG activates protein kinase C which can induce a variety of cellular effects via phosphorylation of target proteins.

The M_2 and M_4 receptor subtypes dominantely activate G_i–proteins leading to the inhibition of adenylate cyclase (AC) and thus decreasing intracellular cAMP levels which results in reduced PKA activity.

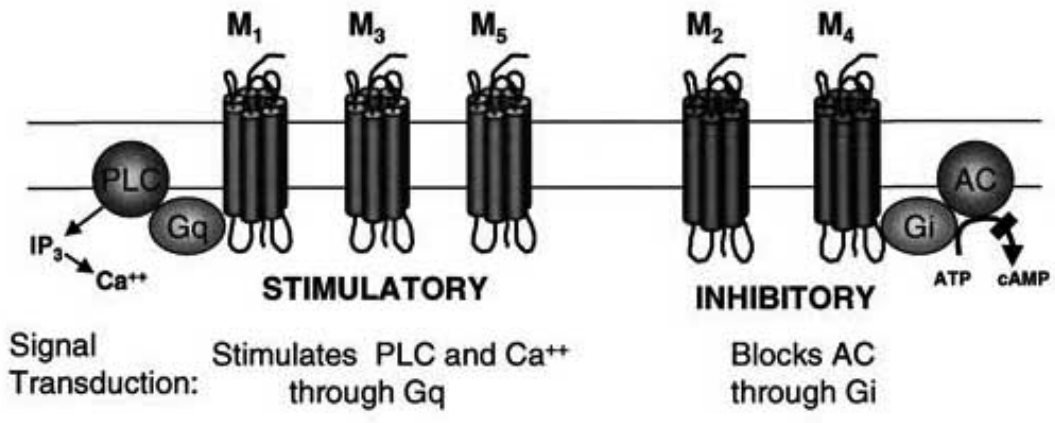


Figure 1.2.2 *Preferential signaling pathways of the muscarinic receptor family. (axxora ; Felder et al. 2000)*

The Gβγ subunit also activates a variety of cell responses. Activation of the Gβγ subunit via the M_2 and M_4 mAChR in the heart leads to direct opening of G-protein coupled inward rectifying potassium channels (GIRKs) resulting in a decrease of the heart rate (Nathanson 1987; Wickman et al. 1999). Presynaptical direct binding of the Gβγ subunit to Ca^{2+} channels reduces the Ca^{2+} influx resulting in a decreasing sensitivity to changes in the membrane potential (Brown and Sihra 2008).

Due to the vast numbers of G-protein subtypes it is quite difficult to delineate the precise signaling pathways. It has also to be considered that multiplicity was not only detected among the G-proteins (currently 17α, 5β and 12γ-have been cloned to date) but also 11 isoforms of PLC and 9 of AC have been identified (Hur and Kim 2002; Ulloa-Aguirre et al. 1999).

Another complication in defining signaling cascades for specific receptors is receptor oligomerization (Dean et al. 2001; Rios et al. 2001), which was also shown for muscarinic receptors (Maggio et al. 1999).

Interestingly for all five receptor subtypes constitutive activity has been shown. Amino acids within the TM3, TM6 and the IL2 seem to beresponsible for this activity (Spalding and Burstein 2001).

1.2.3 Desensitization and sequestration

Upon prolonged agonist exposure mAChRs undergo desensitization. First, the activated receptor is phosphorylated by either a homologous desensitization via phosphorylation by G-protein dependent receptor kinases (GRK) or a heterologous phosphorylation, which is agonist-independent, via second messenger-activated protein kinases such as diacylglycerol-activated protein kinase C (PKC) or the cAMP-dependent protein kinase. Internalization of the mAChRs is mediated by β-arrestins, which bind to the phosphorylated receptor thereby inhibiting further G-protein coupling. Dynamin regulated internalization via clathrin-coated vesicles then leads to intracellular sequestration of the receptor. For mAChR sequestration two possibilities have been described. The receptor can be resensitized and transported back to the cell-membrane or it is down-regulated and degraded (Haga et al. 1996; van Koppen 2001). Fast desensitization and internalization has been discovered for $M_{1\text{-}5}$ mAChR, but the extent and rate of sequestration seems to depend on cell type and receptor subtype (Bunemann et al. 1999; Koenig and Edwardson 1996).

For the M_2 receptor, desensitization and uncoupling from the G-protein appear to be mediated by β-arrestins and G-protein receptor kinases (GRK). GRK mediated agonist-depended

phosphorylation takes place at the residues within the intracellular loop 3 (IL3) (Hosey et al. 1999).

Agonist- and Gβγ independent phosphorylation by diacylglycerol-activated protein kinase C (PKC) has been described for the M_1 and M_3 receptor subtypes (Haga et al. 1990; Haga et al. 1996).

Regarding internalization β-arrestins play an important role for the M_1,M_3 and M_4 receptor subtype, but their role for receptor trafficking of the M_2 mAChR is yet unclear. For the M_2 mAChR β-arrestin-mediated internalization could be confirmed in JEG-3 cell experiments (Schlador and Nathanson 1997). However, experiments in HEK-293 cells using β-arrestins with impaired GPCR-interaction showed an effect on internalization of the M_1,M_3 and M_4 mAChR, but not for the M_2 mAChR (Vogler et al. 1999). In HEK293 cells M_2 mAChR are supposed to be internalized by an adaptor protein distinct from β-arrestin (Werbonat et al. 2000).

1.2.4 Muscarinic ligands

Due to similar structure of the orthosteric site, muscarinic ligands, primarily agonists, lack subtype specificity. Especially in drug therapy, this is a major disadvantage as non-selective ligands can lead to various side effects by activation of several receptor subtypes. In the last years efforts have been made to develop new selective muscarinic ligands. In addition, the allosteric sites are promising targets in achieving subtype selectivity with designed allosteric ligands. This paragraph will give a short summary of known and new compounds.

1.2.4.1 Muscarinic agonists

The endogenous ligand acetylcholine (ACh) is the model substance for several non-selective agonists like carbachol. Pilocarpine, another non-selective agonist, is used in treatment of glaucoma (Lee and Higginbotham 2005).

The assumed importance of the M_1-subtype in the development of Alzheimer's disease and schizophrenia led to several attempts to synthesize selective agonists. Unfortunately, the developed substances did not show sufficient selectivity and bioavailability in clinical studies. Substances like xanomeline, milameline and others were found promising, but complex pharmacology and severe side effects resulted in discontinuation of trials (Langmead et al. 2008). Although cevimeline, which was approved by the Food and Drug Administration (FDA) in January 2000 for the treatment of xerostomia in patients with Sjögren's syndrome (Fox 2007), was regarded as "M_1-selective", comparisons to new compounds revealed only

slight specificity. But also the new compounds, such as 77-LH-28-1, AC-260584 or LY-593039, display activity on several other receptors, including 5-HT_{2B} and dopamine D_2-receptors (Heinrich et al. 2009). Thus, more information regarding pharmacology and side effects is needed to evaluate the selectivity and the therapeutic benefit of these substances.

1.2.4.2 Muscarinic antagonists

Besides the commonly known non–selective antagonists, a lot of new, rather specific compounds for blocking of muscarinic effects have been identified. In addition, snake toxins display high subtype selectivity, thus representing a new starting point for drug development (Karlsson et al. 2000; Servent and Fruchart-Gaillard 2009).

1.2.4.2.1 Non-selective antagonists

Non-selective muscarinic antagonists are phytochemicals derived from plants of the poisonous nightshadow family (*Solanaceae*). Atropine, occurring in *Atropa belladonna* has very high affinity for all muscarinic receptor subtypes (Eglen et al. 2001) and [^{3}H]N-methylscopolamine is frequently used in muscarinic radioligand binding assays (Mohr et al. 1992; Schroter et al. 2000; Surig et al. 2006).

Furthermore quaternary ammonium salts derived from tropane alkaloids, for example tiotropium bromide, are used in the therapy of pulmonary diseases (Grynkiewicz and Gadzikowska 2008). Fesoterodine is a recently published non-selective compound for the treatment of overactive bladder (Ney et al. 2008) and has already been approved by the European medicines agency (EMEA).

1.2.4.2.2 M_1-selective antagonists

The first substance for which subtype specificity was reported was pirenzepine (Hammer et al. 1980). Telenzepine has a similar structure and displays about 10-20 fold increased selectivity for the M_1 mAChR, which is comparable to ipratropium bromide. Revatropate (Pfizer laboratories, UK) is a dual-selective compound for the M_1 and M_3 mAChR (Lee et al. 2001). Another highly subtype selective compound is the polyamine spirotramine (Melchiorre et al. 1995). Also, two toxins, MT-1 and MT-2, have been identified in the venom of the green mamba, both display high selectivity for the M_1 mAChR (Harvey et al. 2002).

Very recently, with the discovery of VU255035 with 75 fold higher selectivity for M_1 compared to the other subtypes, a possible new treatment for CNS disorders has been reported. In mice, VU255035 reduced pilocarpine-induced seizures without impairing hippocampal-dependent learning (Sheffler et al. 2009), which is a common side effect of non-selective muscarinic drugs.

1.2.4.2.3 M_2-selective antagonists

The M_2 mAChR plays an important role for the treatment of overactive bladder. Recently ,the compound TD-6301 was described, which in radioligand binding assays exhibits 30- to 128-fold higher affinity for the M_2-subtype than for the G_q-coupled mAChR. Unfortunately, compared to the M_4 mAChR, selectivity is only increased 2 fold, which is due to the similarity of the orthosteric sites. Therefore, TD-6301 is called a $M_{2/4}$ –selective substance (McNamara et al. 2009). Also for the formerly regarded M_2- selective compounds AF-DX116 and AF-DX384, this dual selectivity was reported (Vilaro et al. 1992).

Methoctramine was the first substance to display slight selectivity for the M_2 mAChR (Melchiorre 1988), but its "successor" tripitramine shows even better results with 24 fold higher selectivity for M_2 than the M_4 mAChR; however, its M_2- affinity is only 6-fold higher than for M_1 mAChR (Maggio et al. 1994). BIBN-99 exhibits subtype selectivity and showed improvement of spatial memory performance in rats (Rowe et al. 2003).

The Schering-Plough research institute published a range of different substances, mostly diphenyl sulfoxides, which all displayed preference for the M_2 over the other mAChR subtypes (Billard et al. 2000; Boyle et al. 2000; Clader et al. 2004; Greenlee et al. 2001; Kozlowski et al. 2000; Kozlowski et al. 2002; Lachowicz et al. 1999; McCombie et al. 2002; Wang et al. 2001b; Wang et al. 2000; Wang et al. 2002a; Wang et al. 2002b).

1.2.4.2.4 M_3-selective antagonists

Darifenacin (Emselex®, Bayer), a drug used in the treatment of overactive bladder, is a known selective antagonist of the M_3 mAChR. Its selectivity has been reported in various publications in vitro and in vivo (Kay and Ebinger 2008; Olshansky et al. 2008; Zinner 2007).

Imidafenacin (Kyorin Pharmaceuticals) and SVT-40776 are two recently introduced new compounds with possible benefit for patients with overactive bladder especially regarding side effects like xerostomia and bradycardia (Kobayashi et al. 2007; Salcedo et al. 2009).

Also, Banyu Pharmaceuticals identified a series of M_3 selective ligands, but yet without further suggestion of a possible application (Sagara et al. 2003; Sagara et al. 2005; Sagara et al. 2002; Sagara et al. 2006).

1.2.4.2.5 M_4-selective antagonists

The structure of orthosteric site of the M_2 and the M_4 mAChR is quite similar, thus creating a selective antagonist was a difficult task. Several highly selective ligands for the M_4 mAChR were synthesized using the first identified highly M_4 selective compound PD 102807 with increased affinities and selectivity values up to 13 183-fold, 339-fold, 151-fold, and 11,220-

fold versus M_1, M_2, M_3 and M_5 respectively (Augelli-Szafran et al. 1998; Bohme et al. 2002; Olianas and Onali 1999). Another quite recent approach took diphenidol (Vontrol®; Smith Kline Beckham), a FDA-approved antiemetic that is regarded as M_2/ M_3-selective, as template to create new M_4 selective compounds. Those 1-substituted-2-carbonyl derivatives exhibit up to 60-fold higher selectivity for the M_4 mAChR (Varoli et al. 2008).

As mentioned above, some toxins derived from green mamba venom exhibit selectivity for certain muscarinic receptors. Thus, the MT-3 toxin was identified as highly specific for the M_4 mAChR (Olianas et al. 1996).

1.2.4.2.6 M_5-selective antagonists

To date, no antagonist with M_5-subtype selectivity has been reported.

1.2.4.3 Allosteric ligands

For information about these compounds see chapter 1.1.4.

1.2.5 Therapeutic opportunities

The possibilities for therapeutic modulation of muscarinic receptor function in the body are vast. A summary of the most important options and already established treatments will be given in the next paragraphs (Eglen et al. 2001; Wess et al. 2007). The major obstacle in utilizing muscarinic receptors as drug targets is the conserved structure of the orthosteric site throughout all five subtypes. Thus, application of an agonist or antagonist can evoke side effects by activating several mAChR subtypes. At present, major efforts are taken, to design subtype specific ligands for the orthosteric or allosteric site(s) to reduce these effects.

1.2.5.1 Peripheral tissues

Muscarinic receptors in smooth muscle cells have been longstanding targets for pharmaceutical treatment. N-butyl-scopolamine has been used as a spasmolytic agent for gastrointestinal disorders for quite a while. The M_3-selective compound darifenacin may be a promising new drug for this application but further studies are needed to determine its potencial (Eglen et al. 2001).

For the treatment of asthma and chronic obstructive pulmonary disease (COPD), which is known to be associated with increased vagal activity, the muscarinic antagonists ipratropium and tiotropium have proven to be very useful (Eglen 2005; Scullion 2007). Studies with M_2 and M_3-KO mice revealed that both receptors influence vagally induced bronchoconstriction. But whereas the bronchoconstriction response was almost abolished in M_3-KO mice, mice lacking the M_2 subtype showed increased pulmonary constriction upon vagal stimulation

(Coulson and Fryer 2003; Fisher et al. 2004). According to that, specific ligands for the M_3 mAChR, preferably applicable by inhalation, with small or no activity at the M_2 receptor, might prove advantageous in the treatment of obstructive pulmonary diseases.

In the therapy of "unstable bladder", anticholinergic drugs such as oxybutynin, tolterodine and trospium can relieve urge incontinence, enuresis and nocturia. Typical side effects of anticholinergic treatment in overactive bladder include dry mouth, constipation, sedation, impaired cognitive function, tachycardia and blurred vision. Trospium shows poor CNS penetration, thus its central nervous side effects are reduced compared to oxybutynin and tolterodine,

With the discovery of the rather M_3-selective, and thus rather bladder-selective, compounds solifenacin and darifenacin, the side effect of dry mouth could be reduced but being substrates of CYP3A4 their potential for drug interactions is rather high. Compared to tolterodine and oxybutynin, respectively, both substances demonstrated higher efficacy (Lam and Hilas 2007). On the other hand, xerostomia (dry mouth), a typical symptom of Sjögren's syndrome (Karaiskos et al. 2009), which is mediated via M_1/ M_3 receptors, can be treated with the non-selective agonists pilocarpine and cevimeline (Exovac®; Daiichi Sankyo).

Pilocarpine is also used in reducing the intraocular pressure in glaucoma but with common side effects such as blurred vision and night blindness, both M_3-mediated, compounds with decreased M_3-receptor affinity seem advantageous (Gil et al. 2001).

Other well-known compounds such as pirenzepine are still in use in the treatment of peptic ulcer, but with the development of more potent drugs like the H_2-receptor antagonists and the proton pump inhibitors, pirenzepine has lost its significance. Its potential in reducing the development of myopia is under investigation (Collison et al. 2000; Le et al. 2005).

Studies with M_3 –KO mice revealed the importance of this subtype in glucose-dependent insulin-secretion, it might thus provide a new target for treatment of glucose intolerance or type 2 diabetes (Gautam et al. 2006b).

The involvement of the M_3 and M_4 receptor subtypes in keratinocyte migration might provide new therapies for skin diseases characterized by abnormal cell adhesion -for example pemphigus- or in the promotion of wound healing (Wess et al. 2007).

1.2.5.2 Central nervous system

Muscarinic receptors can be found in the entire central nervous system (CNS) and are involved in a lot of critical brain functions, thus rendering them potential targets for several CNS related diseases (Langmead et al. 2008). Studies with mice lacking different muscarinic receptor subtypes also introduced various new options for treatment (Wess et al. 2007).

The analgesic potential of the M_2- and M_4-receptor was already discussed and by preferring M_4-selective compound over M_2-selective compounds, fewer side effects can be expected. Two novel ligands, CMI-936 and CMI-1145, both derived from epibatidine, displayed higher affinity for the M_4-receptor and produced significant analgesic activity in M_2-KO mice (Duttaroy et al. 2002; Ellis et al. 1999).

With over 25 million diagnosed people worldwide, Alzheimer's disease (AD) is the most common neurodegenerative disorder at present. The symptoms of this illness are profound memory loss and cognitive dysfunction (Fillit et al. 2002) accompanied by an increase of amyloidal plaques, largely composed of amyloid-β peptide (Aβ) and neurofibrillary tangles formed by hyperphosphorylated τ-proteins (Selkoe 2001). As the accumulation of Aβ is the major hallmark of AD, drug development is presently focusing on inhibition of Aβ production (Hardy and Selkoe 2002).

Early therapies focused on the cholinergic response in the brain, as this is diminished by a loss of cholinergic neurons in the basal forebrain upon increasing Aβ-concentration (Kar and Quirion 2004; Muir 1997). This approach is strengthened by results from M_1-KO mice which displayed impaired learning and working memory (Anagnostaras et al. 2003; Gautam et al. 2006a; Wess 2004). Indeed, besides memantine, all current FDA-approved active agents against AD are acetylcholine esterase inhibitors (AChEIs), which only provide a symptomatic therapy with little efficacy. In addition, these compounds, due to activation of all central and peripheral muscarinic subtypes, exhibit major side effects such as gastrointestinal disorders, nausea and salivation (Ibach and Haen 2004).

Despite reduced cholinergic cortical activity, M_1-receptor density in the cortex seems to be unaltered in AD patients but the numbers of high affinity- or G-protein coupled receptors appear to be decreased (Ladner and Lee 1999; Tsang et al. 2006). Furthermore, a solid body of evidence states the possibility that mAChR stimulation promotes degradation of amyloid precursor protein (APP) into increased amounts of soluble PPAα via a non-amyloid pathway. (Buxbaum et al. 1992; DeLapp et al. 1998; Pittel et al. 1996) whereby activation of M_1- and M_3- mAChR appears to play an important role (Nitsch et al. 1992).

Although a lot of efforts have been made in the development of M_1-selective agonists, until now all compounds failed the expectations due to their lack of selectivity (Clader and Wang 2005; Fisher et al. 2003). As the development of subtype-specific substances appeared to be difficult, a superior approach could be the use of the M_1-allosteric site (Spalding et al. 2002). Substances like AC-42 display specificity for the allosteric site of the M_1-AChR thus renewing hope for a novel therapy with decreased side effects (Langmead et al. 2006). Another possible approach is the blockage of presynaptic M_2-autoceptors, which increases ACh release (Clader and Wang 2005). Regretfully, M_2-mAChR expression in the frontal cortex seems impaired in patients with AD and this may limit the beneficial effect of selective M_2-antagonists (Lai et al. 2001). Furthermore, the clinical side effects may be precarious, as blockage of M_2-receptors in the heart can lead to tachycardia and blood pressure responses. These side effects would become increasingly dangerous in elder patients, who represent the majority of AD patients. Whether the development of highly selective compounds or allosteric agents can be of advantage still remains unclear.

Parkinson's disease (PD) comes along with a progressive loss of dopaminergic neurons in the substantia nigra compacta leading to impaired innervations to the striatum. For locomotor activity a proper balance between dopaminergic and striatal muscarinic cholinergic neurotransmission is required (Di Chiara et al. 1994). Prior to the development of L-DOPA, PD was treated with anticholinergic compounds of synthetic or natural origin (Duvoisin 1967). Despite the success of Levodopa (L-DOPA), anticholinergic activity can still be beneficial because it can restore the balance between striatal dopamine and acetylcholine. With Biperiden (Akineton®, Bayer/Knoll Pharma) an anticholinergic drug with effects on central M_1 mAChR is used for the treatment of Parkinson's disease. It seems especially useful in improving parkinsonian symptoms during antipsychotic therapy. However, biperiden is not M_1-subtype selective and is distributed throughout the body. Therefore it it can show dose-dependent side effects in the central nervous system, like dizziness, headaches and anxiety, and peripheral tissues, such as dry mouth, tachycardia and obstipation (Biperiden-neuraxpharm® 2008; Eltze and Figala 1988). Unfortunately to date, the lack of receptor-specific substances and the ubiquitous distribution throughout the body resulting in severe side effects such as urinary retention, nausea and constipation, restricts application of those drugs (Lees 2005).

Studies with KO-mice revealed, that a loss of M_1-receptors can lead to increased dopamine levels and in M_4-KO mice a significant increase in locomotor activity upon stimulation with a dopamine D_1 -receptor agonist was observed. These results suggest that the development of a

M_1/ M_4 selective antagonist might improve locomotor activity but unfortunately M_1-antagonism seems to induce senile plaque and neurofibrillary tangle formation (Perry et al. 2003). Although accruement of these effects is smaller than in patients with AD, these side effects should be avoided. Therefore development of a specific antagonist of the M_4-receptor seems to be the preferable option, and indeed specific compounds have already been synthesized (Bohme et al. 2002). Whether they can convince in the clinic still has to be elucidated.

To date it is considered that the positive and negative symptoms of schizophrenia are initiated by dysfunction of dopaminergic pathways (Kapur et al. 2005). But there is growing evidence that forebrain cholinergic neurons play an important role in schizophrenia, as mAChR density in several regions of the brain is decreased (Dean et al. 2002), and that AChEIs might have beneficial effects on cognitive dysfunction in schizophrenic patients (Guillem et al. 2006). Results from several studies, including M_1-KO mice and tests with compounds displaying functional selectivity for this mAChR subtype, display the possibility of reducing cognitive symptoms by administration of M_1-selective agonists (Schwarz et al. 1999; Shannon et al. 2000; Wess 2004). Also, the existence of a specific allosteric site on the M_1-mAChR may be of advantage by developing receptor-selective allosteric ligands or even agonists (Spalding et al. 2002). In addition, the M_4-receptor subtype seems to be involved in mid-brain dopamine hyperactivity, which is speculated to be a factor in psychosis (Tzavara et al. 2004). Taken together, the data indicate that a M_1/M_4-selective agonist might have a positive effect on schizophrenic symptoms, but it is doubtful, that such a ligand can be created regarding the structural similarities of the orthosteric site among the mAChR's. Under this aspect, synthesis of a positive allosteric ligand with selectivity for the M_1-receptor seems the most promising strategy.

In the last years, a role of the M_5-mAChR in drug dependence has been discussed. The M_5-receptor subtype localization is discrete in the ventral tegmental area (VTA), and there is evidence that activation of the receptor is involved in reward (Koob et al. 1998; Yeomans et al. 2000). As mentioned before, M_5-KO mice display changed behavior in cocaine and morphine self-administration (Basile et al. 2002; Thomsen et al. 2005). With the development of specific substances, the possibilities deriving from the M_5-recepor in the therapy of drug-addicted patients might be further elucidated.

A very recent discovery is the involvement of the M_3-receptor in obesity. M_3-KO mice have significantly improved insulin sensitivity as well as glucose tolerance and showed pronounced decrease in body fat compared to WT mice (Gautam et al. 2006b; Yamada et al. 2001b).

These data may lead to new therapies for obesity utilizing the positive effects of M_3 antagonism on energy expenditure and food intake.

1.3 FRET Imaging

FRET imaging is a sophisticated tool to determine intermolecular distances and conformational changes in living cells and thus is not easily matched by other techniques. In addition, this method enables to develop life-imaging techniques to measure protein-protein-interactions in real time with high sensitivity and reliability. As FRET takes place in the range of the size of many biological macromolecules, it has become a useful tool for microbiological studies (Hoffmann et al. 2005; Miyawaki 2003; Rizzo et al. 2009).

At present the FRET technique is continuously being refined and the development of new FRET dyes and appliances is going to further push the limits of this innovative tool.

1.3.1 Definition of FRET

FRET (Förster or Fluorescence resonance energy transfer) was discovered by Theodor Förster, a German physicochemist, in 1946. The FRET method enables to measure distances between a donor and an acceptor molecule. This physical process, by which energy is transferred non-radiatively via a long-range dipol-dipol coupling (Figure 1.3.1), is useful for detecting inter- and intramolecular changes at distances of roughly 1-10nm (Clegg 1992; Selvin 1995). The energy is transferred via a *virtual photon*, a messenger particle that cannot be detected as it is subsequently annihilated upon its creation (Andrews and Bradshaw 2004). The energy transfer can be monitored using any type of fluorescence microscope or fluorometer.

Whereas the donor has to be a fluorescent molecule, the acceptor can also be a non-fluorescent compound that just accepts the energy released by the donor with its chromophore (Jares-Erijman and Jovin 2003).

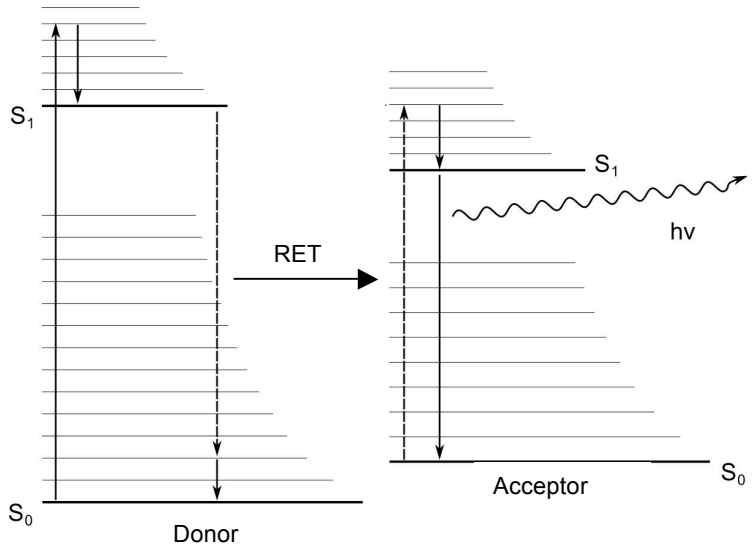


Figure 1.3.1 *Jablonski energy diagram explaining the FRET principle:*
The energy level of the donor electrons is increased from an energy ground-state S_0 to a higher energy level (S_1). When the energy level of the donor returns to the S_0, the nascent photon, due to the close proximity, is absorbed by the acceptor during the process of its formation (virtual photon). This energy transfer is termed a non-radiative resonance energy transfer (RET). The acceptor then, upon RET, also reaches its higher energy state S_1. When the acceptor electrons return to the S_0 fluorescence ($h\nu$) is emitted (scheme modified from www.wikipedia.de; Rasmussen et al.).

To transfer the energy, the donor fluorophore has to be excited with a certain wavelength that depends on the structure of the donor chromophore. In addition, three key parameters to allow the Förster mechanism are

- the chromophore of the donor and acceptor have to overlap in their the emission/excitation spectra (Figure 1.3.2),
- the distance between donor and acceptor is typically shorter than 100 Å and
- the relative orientation of the dipole moment of donor and acceptor should be parallel.

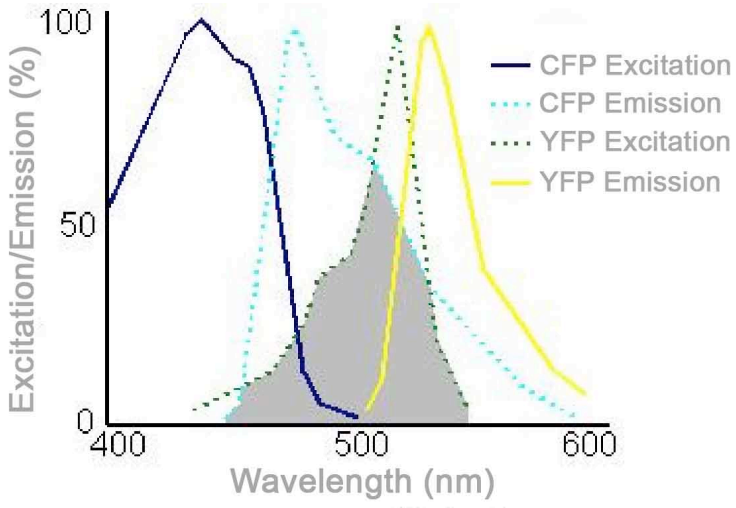


Figure 1.3.2 *Excitation and emission spectra of the FRET pair CFP/YFP The spectral overlap integral (J) between CFP and YFP is indicated by the grey area (Scheme modified from www.wikipedia.de).*

The efficiency of the energy transfer (E) is determined by the inverse power of the donor-acceptor distance (r^6).

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

Where R_0 equals the Förster radius the distance between the two molecules at which the FRET efficiency is half-maximal (50%) and it can be determined using with the formula.

$$R_0{}^6 = \frac{9\,Q_0\,(\ln 10)\kappa^2\,J}{128\,\pi^5\,n^4\,N_A}$$

where

Q_0 is the fluorescence quantum yield of the donor in absence of the acceptor,

κ^2 is the dipole orientation factor,

n is the refractive index of the medium,

N_A is Avogadro's number and

J is the spectral overlap integral between the donor emission and the acceptor absorption.

Since the proximity of the donor and the acceptor molecules is crucial for the energy transfer, FRET can also be used as "molecular ruler" (dos Remedios and Moens 1995; Stryer 1978).

1.3.2 Measuring ratiometric FRET

Ratiometric FRET, which measures changes in donor and acceptor fluorescence upon resonance energy transfer between them, can be monitored using various experimental setups. One possibility is to display changes in acceptor emission upon donor excitation or to measure the decrease in donor fluorescence life time due to presence of the acceptor. Another approach is to monitor donor dequenching, which means the recovery of donor fluorescence upon photobleaching of the acceptors fluorophore (Majoul et al. 2002).

1.3.3 Potential problems using FRET technique

1.3.3.1 Cross-talk

As mentioned above, it is essential for FRET that the donor emission spectrum and the acceptor excitation spectrum overlap. On the other hand, there should be no overlap of the excitation and emission spectra, because this might impair the measurement. In practice, this can be hardly avoided. As a result, the excitation of the donor can also result in an excitation of the acceptor, which is termed "cross talk" or direct excitation of the receptor (Takanishi et al. 2006). The direct excitation of the acceptor is determined by the extinction coefficient of the acceptor chromophore at the excitation wavelength and the excitation light intensity.

1.3.3.2 Bleed through

While "cross talk" refers to signal contamination due to overlap of the excitation spectra, "bleed through" occurs when the emission spectra of the FRET pair are overlapping. This "donor bleed through" contaminates the acceptor emission signal. Bleed through can be estimated by examining the donor/acceptor emission/absorption spectra or by using acceptor photobleaching (Takanishi et al. 2006; Wallrabe and Periasamy 2005)

1.3.3.3 Non-specific FRET

To increase the intensity of the FRET signal it can be necessary to increase the intensity of the excitation light or to enhance the expression of the labeled proteins. Since the increase of the light is restricted by the used or by increased photobleaching, this option is limited. Boosting the expression levels of the tagged protein, on the other hand, may often result in non-specific FRET between non-associated donor acceptor pairs of unrelated proteins ("by-stander-FRET") due to decreased distance between the tagged proteins (Takanishi et al. 2006; Vogel et al. 2006).

1.3.4 Fluorophores for FRET

Before FRET experiments can be performed successfully, it is crucial to choose the right fluorescent markers. Depending on the biological problem, the choice of the right fluorescent dye can enhance or decrease feasibility of experiments (Shaner et al. 2005). In the last years, a plethora of different fluorescent materials for biological studies have been developed.

1.3.4.1 Fluorescent Proteins

The green fluorescent protein (GFP), the first identified fluorescent protein (FP), was found in different coelanterate species like *Aequorea*, *Renilla* or *Obelia* (Tsien 1998).

As expression of GFP leads to fluorescence independent of co-factors such as aequorin (Inouye and Tsuji 1994). The fluorophore of the GFP consists of the amino acids Ser/Thr65-Tyr66-Gly67, which are surrounded by 11 β-strands and an α-helix (Figure 1.3.3) (Ormo et al. 1996; Yang et al. 1996).

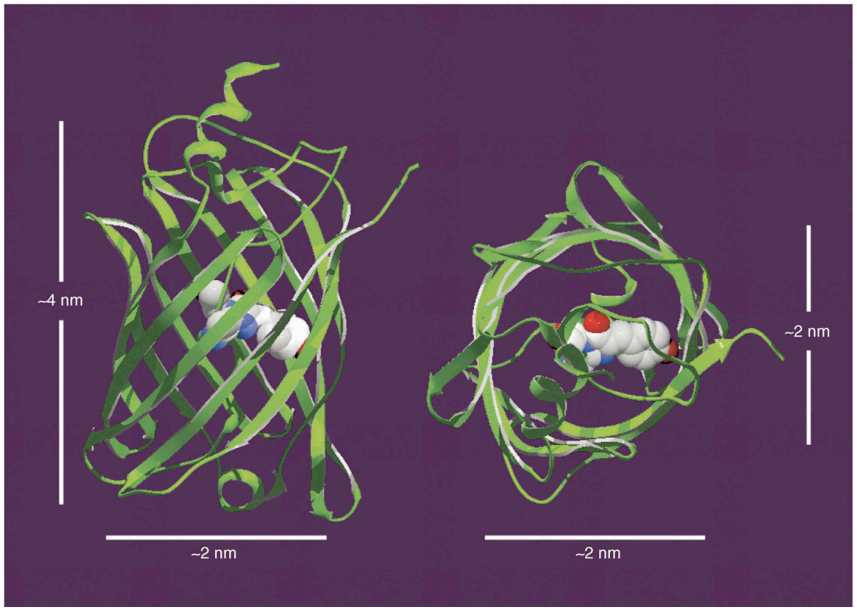


Figure 1.3.3 *Crystal structure of the wild type GFP*
(Piston and Kremers 2007)

Mutations of the fluorescent properties of GFP from jellyfish lead to a vast variety of different FPs with different qualities, which have been used for the past two decades to study a plethora of biological questions (Figure 1.3.4)(Wang et al. 2008).

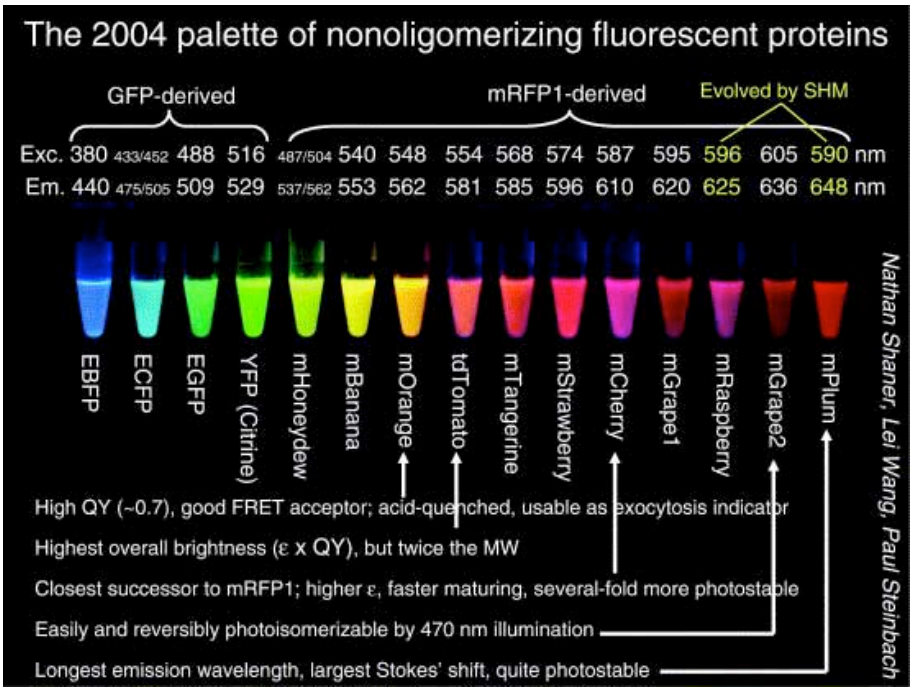


Figure 1.3.4 *Different variations of the GFP and the DsRed (Tsien 2006)*

In addition, mutating other amino acids of GFP allowed changing its chemical properties (for example pH-stability), brightness, photobleaching stability and the disposition for oligomerization (Griesbeck et al. 2001; Shaner et al. 2005; Zapata-Hommer and Griesbeck 2003).

For the excitation wavelength range between 380 and 520, which includes the blue, green, cyan and yellow fluorescent proteins, a lot of different FPs have been developed, each having its strengths and weaknesses (Table 1).

Name	Excitation (nm) #	Emission (nm) #	Brightness	Photostability	O.M.
Wild-type GFP	395/475	508/503	+	+	w.d.
T-Sapphire	399	511	++	+	w.d.
EBFP	383	447	++	+	w.d.
EBFP2	383	448	+++	++++	w.d.
Azurite	384	450	+++	++++	w.d.
mECFP	433/452	475/505	++	+++	m.m.
Cerulean	433/451	475/503	+++	++	w.d.
CyPet	435	477	++	+++	w.d.
mTFP1	462	492	++++	++++	m.m.
Emerald	487	509	+++	+	w.d.
EGFP	488	507	+++	++++	w.d.
EYFP	514	527	++++	++	w.d.
mCitrine	516	529	++++	++	m.m.
Venus	515	528	++++	+	w.d.
YPet	517	530	++++	+++	w.d.

Table 1 *Mutations of GFP (Wang et al. 2008);*

#: maximal wavelengths of excitation and emission spectrum
O.M.: Oligomerization, m.m.: mostly monomeric, w.d.: weak dimers

Many efforts have been made to mutate DsRed, a red-fluorescent protein initially isolated from *Discosoma sp*., which have rather high excitation wavelength, ranging between 540 and 600nm (exception dsHoneydew, excitation wavelength under 500nm) (Table 2).

Name	Excitation (nm) #	Emission (nm) #	Brightness	Photostability	O.M.
DsRed	558	583	++++	++++	t.m.
mRFP1	584	607	++	+	m.m.
tdTomato	554	581	++++	+++	t.d.
mHoneydew	487/504	537/562	+	+	m.m.
mBanana	540	553	+	+	m.m.
mOrange	548	562	++++	+	m.m.
mKO	548	559	+++	+++	m.m.
mTangerine	568	585	++	+	m.m.
TagRFP	555	584	++++	++	m.m.
mStrawberry	574	596	+++	+	m.m.
mCherry	587	610	++	+++	m.m.
mRaspberry	598	625	++	+	m.m.
mKate	588	635	+++	++++	m.m.
mPlum	590	649	+	++	m.m.

Table 2 *DsRed and mutations (Wang et al. 2008)*

#: Maximal wavelengths of excitation and emission spectrum
O.M.: Oligomerization, m.m.: mostly monomeric, w.d.: weak dimers, t.m.: tetramer, t.d.: tandem dimers

DsRed itself has a high quantum yield, pH-resistance and a high extinction coefficient but tends to form tetramers and has a very long maturation time to develop its red fluorescence (Baird et al. 2000). Thus, its applications are limited.

Which FP or FP pair is most suitable for the experiment in question is dependent on experimental conditions like medium, setup, structure of the molecule/proteine and further more. The most commonly used FRET-donor acceptor pair for inter- and intramolecular FRET-studies nowadays is enhanced CFP and enhanced YFP (ECFP/EYFP), where ECFP is the donor and EYFP the acceptor fluorophore. Cyan fluorescent protein (CFP) is derived from GFP via mutation of the Tyr 66 into a Trp, so that an indole is introduced into the chromophore, but further mutations were needed to prevent premature folding, which resulted in ECFP (Heim et al. 1994; Heim and Tsien 1996). Reducing the excited-state energy of the chromophore created YFP. This can be achieved placing a molecule with an aromatic ring system, like tyrosine, next to the anionic chromophore thus leading to higher wavelengths. As EYFP is sensitive to chloride concentrations and its maturation is rather slow at 37°C, further mutations have been introduced to produce Citrine, YPet or Venus. Citrine has improved brightness and photo stability as well as lower sensitivity to chloride. YPet has superior efficiency when paired with a cyan FP and Venus shows faster maturation (Griesbeck et al. 2001; Nagai et al. 2002; Nguyen and Daugherty 2005). With the pair CyPet/YPet, described by Nguyen and Daugherty, a new enhanced CFP/YFP based donor acceptor pair has been introduced that shows enhanced sensitivity and dynamic range (Nguyen and Daugherty 2005), but on the other hand needs to dimerize to show its high efficiency (Ohashi et al. 2007).

FPs are mostly used for in vivo imaging of cells, and can be placed in the center, the N-or the C-Terminus of a protein. In one of the first attempts to show intermolecular FRET, Tsien et al used a donor (BFP or CFP) and an acceptor (GFP or YFP) to flank calmodulin and the calmodulin-binding peptide M13. Upon binding of Ca^{2+}, calmodulin wraps around the M13 peptide decreasing the distance between the FPs, which results in an increase in FRET (Miyawaki et al. 1997).

A similar approach was used by Nikolaev et al to construct a sensor for cAMP, using ECFP and EYFP as donor/acceptor pair fused to a single cAMP binding domain of the Epac protein. This sensor allows to visualize changes in the cAMP levels in living cells via a decrease in FRET (Nikolaev et al. 2004).

1.3.4.2 Natural fluorophores

Phenylalanine, tryptophan and tyrosine are fluorophores that can also be used for FRET measurements, especially in a range <5nm (Wu and Brand 1994). This makes them suitable markers for measuring intramolecular FRET. Their biggest advantage is their ubiquitous appearance in proteins and peptides so they can easily be introduced into protein structures. On the other hand, these modifications can also influence the proper function of the modified protein.

Natural fluorophores have been used in various different studies, for example for measuring the distance between the helices of the M13 transmembrane procoat protein with tyrosine as donor and tryptophan as acceptor (Eisenhawer et al. 2001) or to show conformational changes upon binding of calmodulin-binding protein via introducing modified amino acids, which emit fluorescence over 500nm (Kajihara et al. 2006).

1.3.4.3 Synthetic fluorophores

The variety of organic fluorophores used for FRET is enormous.

1.3.4.3.1 "Traditional" FRET Dyes

Organic dyes, which emit light between UV to IR spectrum, are the most common FRET materials (Sapsford et al. 2006). The UV dyes are typically naphthaline-, coumarin- or pyrene-based structures, whereas the VIS/IR dyes are modified structures of fluorescein, rhodamine and cyanine (Figure 1.3.7).

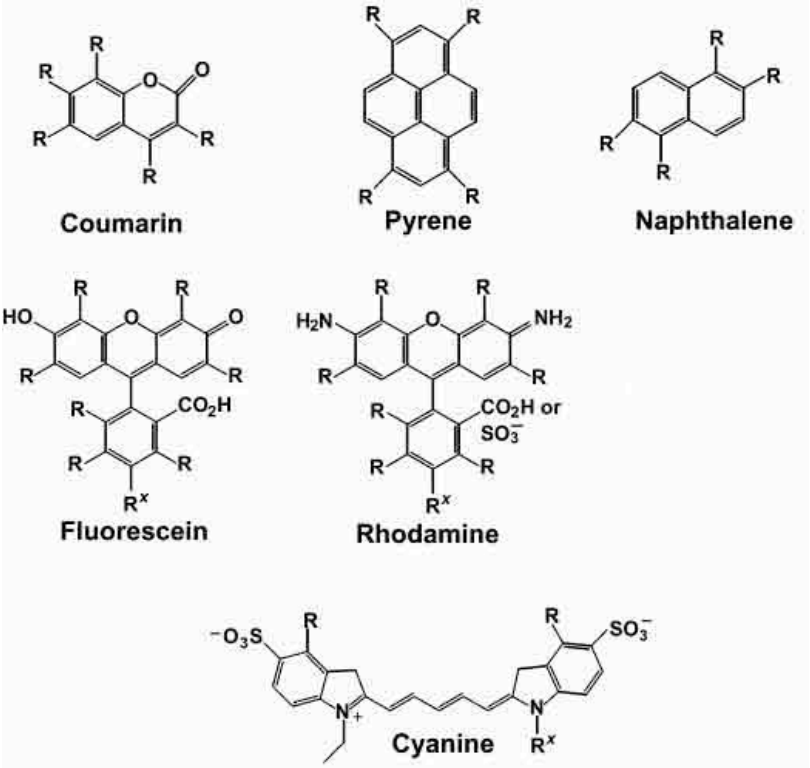


Figure 1.3.7 *Structures of common organic FRET dyes (Sapsford et al. 2006)*

These dyes usually have a small Stokes shift and their strengths and weaknesses have to be considered when chosen for FRET experiments. Fluorescein dyes, for example, can easily be bio-conjugated, have a high quantum yield and solubility and can be excited with wavelengths around 480nm. On the other hand they show fast photobleaching, can be self-quenching and are pH-sensitive (which in some cases can also be of advantage).

Recently introduced fluorescein-based dyes are the hairpin binder molecules FlAsH (4,5–bis(1,3,2-dithiarsonal-2-yl) fluorescein) and ReAsH (4,5–bis (1,3,2-dithiarsonal-2-yl) resorufin). Both molecules are membrane-permeable and their fluorescence is activated by their binding to a specific amino acid motif (Figure 1.3.8) (Adams et al. 2002). This motif consists of the amino acids Cys-Cys-Xaa-Xaa-Cys-Cys, with Xaa being a non-cysteine amino acid. The two non-cysteine amino acids Xaa can enhance binding and fluorescence of the fluorescein-based dyes. For FlAsH-binding the hairpin-forming motif Cys-Cys-Pro-Gly-Cys-Cys (CCPGCC) shows increased affinity and contrast. The specificity for FlAsH or ReAsH can even be increased if this six amino acid motif is flanked by three certain amino acids on either side (HRWCCPGCCKTF and FLNCCPGCCMEP) (Madani et al. 2009; Martin et al. 2005).

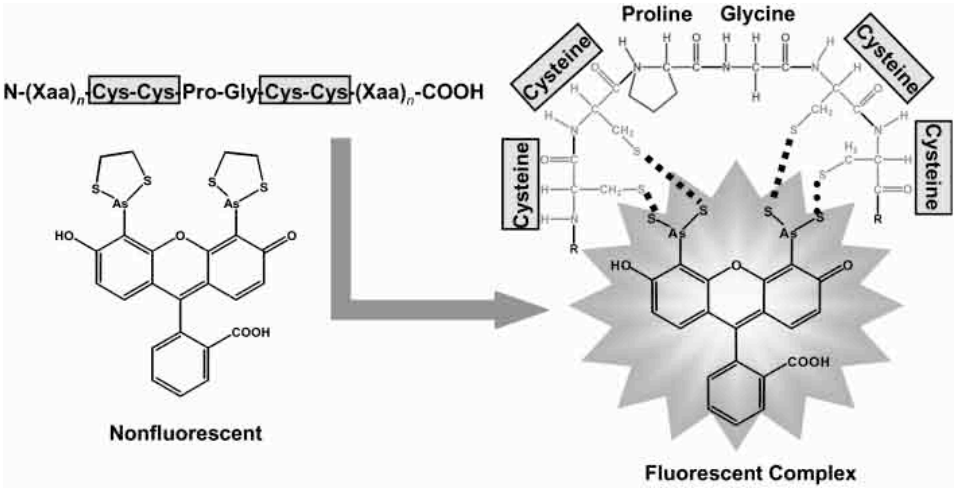


Figure 1.3.8 *Structure of the fluorescein based hairpin binder FlAsH before and after binding to its specific amino acid motif. Fluorescence of the molecule is achieved upon binding (Sapsford et al. 2006)*

ReAsH and FlAsH as donor or acceptor fluorophore can be paired with a fluorescent protein for FRET measurements.

Hofmann at al succeeded in showing the advantages of a FlAsH/CFP-based approach compared to the commonly used CFP/YFP pair. Their FRET-measurements on a tagged adenosine A_{2A} receptor and mouse α_{2A}-adrenergic receptor exhibited a five-fold higher FRET-signal for the FlAsH/CFP pair as well as comparable kinetics and, most importantly, normal downstream signaling (Hoffmann et al. 2005).

Very recently, Zürn et al used the FlAsH/CFP-pair , to determine different behaviors of the third intracellular loop (ICL3) of the α_{2A}-adrenergic receptor upon ligand binding. For this purpose, they inserted the CCPGCC motif on to different locations of the ICL3. They discovered that the intensity and kinetics of the ICL3 movement depends on the activating ligand (Zurn et al. 2008).

1.3.4.3.2 Quencher molecules

An interesting approach is the use of quencher molecules as acceptors. The obvious advantage of those molecules is the elimination of background fluorescence. Quencher molecules can be organic materials but also metallic compounds like gold. Dapsyl and dabcyl are two commonly used quencher molecules, their absorption maxima are at 466 and 485 nm.

Quenchers are often used in DNA analysis, mostly paired with an organic donor molecule (Tan et al. 2005).

1.3.4.3.3 Photochromic dyes

Photochromic dyes are a group of molecules that can undergo a light-driven, structural transformation, resulting in different structural forms with different absorption spectra (Giordano et al. 2002). Molecules that are used for this approach often have a spiropyran-structure or spiropyran-related structure. The closed spiro-form of these molecules, with an absorbance under 400 nm, rearranges into an open merocyanine form, with an absorbance between 500 and 700 nm, in response to illumination (Bahr et al. 2001).

1.3.4.4 Inorganic fluorophores

Inorganic materials used for FRET analysis are mostly metal or chelat complexes, most of them using Lanthanides as central atoms. Compared to organic dyes Lanthanides provide longer fluorescence lifetime (organic dyes: nanoseconds, Lanthanide-complexes: milliseconds). In addition, Lanthanides display sharp emission spectra, thus decreasing background fluorescence (Selvin 2002).

The most commonly used metal for FRET studies are gold nanoparticles, as gold has a very good quenching ability and, like all noble metals, is very resistant to photobleaching (Daniel and Astruc 2004). Furthermore, gold can be used as a surface cover, to enable assays with attached molecules, that are linked to an donor-dye (Seidel et al. 2004).

1.3.5 Other FRET-applications

In addition to intensity-based methods (ratiometric FRET) the principle of fluorescence energy transfer can also be used for other applications, which will be mentioned in a short overview.

1.3.5.1 Fluorescence lifetime imaging microscopy (FLIM)

FLIM measures the fluorescence lifetime of the donor dye so it is less prone to cross-talk artifacts (van Munster and Gadella 2005). Similar to measuring donor dequenching upon acceptor photobleaching (see 1.3.2), FLIM concentrates on the reduction of the donor fluorescence lifetime upon FRET, which results in an exponential decrease of the donor fluorescence thus giving a value for the FRET efficiency. As FLIM-FRET does not study changes in acceptor fluorescence it is less sensitive but therefore non-fluorescent acceptors can be used, which also decreases interferences (van Munster and Gadella 2005). As the FLIM measurements have to take place in nanoseconds, a specific and costly instrumentation

is needed. FLIM measurements can take minutes, as the decrease in fluorescence lifetime has to be monitored by many repetitive determinations. This also is a problem, when fluorescent proteins are used,which can have multi-exponential lifetimes and which bleach rapidly. (Piston and Kremers 2007).

2 Aims of the study

2.1 General considerations

As mentioned above, allosteric receptor modulation is gaining more and more interest in the scientific community and the pharmaceutical industry, since allosteric ligands are a promising new target for drug development.

To date, the majority of the studies on allosteric modulations have been performed using *in vitro* methods such as radioligand binding assays. Thus, an approach that can visualize conformational changes of receptors in living cells seems to be an interesting new method, which would allow to examine allosteric receptor interactions. For visualizing conformational changes in receptors, the FRET-technique has proven to be a useful tool as it enables not only to analyze these changes in living cells but also to display these measurements in real time with high temporal resolution. Therefore, this method can be used to measure activation and deactivation kinetics, which can help to dissect the influence of different ligands on the receptor.

The muscarinic acetylcholine receptors are the best yet characterized GPCR receptor family regarding allosteric modulation. These receptors regulate a plethora of crucial functions and are important targets for drug development. In addition, numerous allosteric ligands have been identified and characterized using "classical" methods. Therefore developing a mACh-receptor FRET-construct to examine the behaviour of identified allosteric ligands seems to be a very interesting approach. Understanding the conformational changes brought about by allosteric ligands as well as further characterization of different ligands with regard to their kinetics in intact cells may lead to a deeper insight into allosteric modulation and therefore facilitate exploring new therapeutic options.

2.1.1 Manufacturing of mACh-receptor constructs

Most of the identified allosteric ligands show some selectivity for the M_2 mACh-receptor subtype (Gregory et al. 2007). To avoid insufficient receptor responses due to the lack of allosteric ligand affinity, this subtype was therefore chosen to generate a FRET based sensor. Due to its critical role in a plethora of neuronal diseases, such as schizophrenia, the M_4 mAChR poses a critical protein for the development of new allosteric treatments. With the

substance LY2033298, a possible new allosteric drug for the treatment of schizophrenia, could be tested using a M_4 mAChR FRET sensor.

To achieve reliable results, the obtained receptor construct had to be optimized to conform with critical parameters such as receptor expression, functionality or FRET efficiency. The foremost aim was thus to create a FRET-construct which exhibits sufficient FRET response while maintaining receptor functionality. Furthermore this construct should express well in the selected cell lines.

The general aims of receptor sensor generation therefore were:

1. Generating a mAChR construct containing a pair of donor/acceptor fluorophores to monitor the effects of ligands on the receptor conformation.

2. The construct had to express sufficiently at the surface of the chosen cell lines.

3. Despite genetic modifications introduced intro the receptor sequence, the mAChR had to remain functional. This had to be ensured using methods determining the integrity of ligand binding and downstream signaling.

2.1.2 Analysis of allosteric receptor modulation

Since allosteric interactions have previously not been studied using the FRET technique, the potential of this method in elucidating allosteric modulation of GPCRs had to be determined.

The general aims of allosteric FRET measurements were:

1. To determine whether FRET is a suitable method for the analysis of allosteric interactions with GPCRs.

2. To study the effects of different allosteric ligands on receptor conformation and to compare them with already published results obtained in classical assays.

3. To measure the kinetics of the conformational switch induced by allosteric ligands and to compare the results with the kinetics of orthosteric ligands. These data should elucidate the nature of allosteric interactions and reveal new mechanisms of allosteric ligand binding.

3 Materials and Methods

3.1 Materials

3.1.1 Bacteria strains

Escherichia coli JM109	Stratagene
Escherichia coli XL10 gold	Stratagene

3.1.2 Plasmid vectors

pcDNA3.1	Invitrogen
pcDNA3-Epac2-camps	V.Nikolaev, Würzburg
pIres-GIRK1/GIRK4	L.Hommers, Würzburg

3.1.3 Template cDNA

M_2mAChR in pcDNA3.1+	Missouri University of Sci. & Tech.
M_4mAChR in pcDNA3.1+	Missouri University of Sci. & Tech.

3.1.4 Oligonucleotides

list see appendix	Eurofins MWG

3.1.5 Cell lines

Flp-In™-293	Invitrogen
Flp-In™-CHO	Invitrogen
TSA 201	Invitrogen

CHO M_2 wt	Dr. Katie Leach, Monash University, Melbourne

3.1.6 Chemicals

Agar	GIBCO BRL
Agarose	PeqLab
Ampicilline	Applichem
Atropine	Sigma-Aldrich
Bromphenol blue	Sigma-Aldrich
$CaCl_2$ dihydrate	Merck
Carbachol	Alfa Aesar
Dimethyl-W84	Alexis Biochemicals
DNA ladders	New England Biolabs
EDTA	Merck
Ethanol	Sigma-Aldrich
Ethidium bromide	Sigma-Aldrich
FlAsH (Lumino Green^{TM})	Invitrogen/Dr. Eberhard Heller, Würzburg
Gallamine	Sigma-Aldrich
Geniticin (G-418)	Calbiochem
Glucose monohydrate	Applichem
Glycerol	Sigma-Aldrich
Hank's buffered salt solution (HBSS)	Invitrogen
HCl	Merck
HEPES	Applichem
Isopropanol	Merck
KCl	Applichem

LY2033298	Gift from Eli Lilly laboratories/ Prof. A. Christopoulos, Monash University
McN-A 343	Sigma-Aldrich
Methoctramine	Sigma-Aldrich
$MgCl_2$	Applichem
[^{3}H]-NMS	Amersham
Natrium acetate	Applichem
NaCl	Applichem
NaOH	Merck
dNTP	PEQLab
Oxotremorine-M	Sigma-Aldrich
Pilocarpine	Sigma-Aldrich
[^{3}H]-QNB	Amersham
1,2,3,4-tetrahydroacridine-9-amine (Tacrine)	Merck
Tris	Applichem
Trypton	Applichem
Yeast extracts	Applichem

3.1.7 Kits

AlphaScreen® Protein A Kit	Perkin Elmer/TGR Biosciences
AlphaScreen® *SureFire*® Kit	Perkin Elmer/TGR Biosciences
Effectene Transfection Reagent	Quiagen
Lipofectamine™ 2000	Invitrogen
Millipore DNA Gel Extraction Kit	Millipore
Qiagen Gel Extraction Kit	Qiagen
Qiagen HiSpeed Maxi Kit	Qiagen

Qiagen Midi Kit	Qiagen

3.1.8 Enzymes and specified buffers

Pfu DNA-polymerase	Promega
Restriction enzymes	New England Biolabs
T7-ligase	Promega

3.1.9 Cell culture

Dulbecco's modified Eagle's medium with 4,5% Glucose (DMEM)	PAN Biotech
DMEM/F12	PAN Biotech
Fetal calf serum (FCS)	Invitrogen
Glutamin	PAN Biotech
Opti-MEM®	Invitrogen
Phosphate buffered saline (PBS)-Buffer	PAN Biotech
Penicillin/Streptomycin	PAN Biotech
Poly-L-Lysine	Sigma-Aldrich
Trypsin	PAN Biotech

3.1.10 Other materials

384-well proxiplate opaque	Nunc
96-well plates	Nunc
Cell culture dishes, flasks and multi-well plates	Nunc
Cryotubes	Nunc
Glass coverslips 24mm	Hartenstein

GF/B glass fibre filters	Millipore
GF/B glass fibre filter paper	Sigma-Aldrich
Falcon Tubes 15ml and 50ml	BD Biosciences
Patch clamp capillaries	Harvard Apparatus
Reaction tubes 1,5 and 2ml	Eppendorf

3.2 Methods

All solutions were prepared using desalinated and pyrogen-free water from a Barnsteadt-Reversed-Osmosis System, if not recommended otherwise by the supplier. All used chemicals were at least of "pro analysi" (p.a.) grade.

3.2.1 Molecular biology

3.2.1.1 Polymerase chain reaction (PCR)

M_2-and M_4-mAChR wt-cDNA was amplified and mutated using PCR-method, and then the constructs were built together in apcDNA3-based vector containing the C-terminal CFP sequence after digestion with HindIII and XbaI endonucleases.

A PCR-reaction tube was filled with a solution consisting of 0,5µM of each Primer, 200µM dNTP's each, 1/10 of 10x Pfu-buffer, 0,5µg Template-DNA and water ad 100µl. The tubes were then placed into a PCR-Cycler (GeneAmp® 2450, Perkin Elmer) and preheated by 95°C/5min. To start the reaction, 0.1µl of Pfu polymerase (5U/µl) were added. Amplification was accomplished by denaturation of DNA fragments (95°C/30sec), annealing of primers to the DNA (50°C-60°C dependent on primer length/30sec) and elongation of the DNA strains (72°C/ 2min per 1000bp) during 25-30 cycles. The last elongation period was extended by 10min to ensure reparation of incorrect PCR-products. The PCR-tubes were then cooled down to 4°C.

3.2.1.2 Purification of PCR-products

To purify the PCR products, a 1% agarose gel was prepared by dissolving 1 g of agarose and 7 µl ethidiumbromide solution (10 mg/ml) in 100 ml TAE-Buffer with low EDTA (Millipore). The PCR product was then mixed with 1/5 volume of a 5x DNA Buffer (0,125% bromphenol blue, 70mM EDTA, 2,5 ml Glycerol 85%, 2,5 ml water) and loaded into the gel pockets. A DNA-ladder was loaded as a reference. Identification of the DNA bands was achieved via excitation of the intercalating ethidiumbromide at 254 nm using a UV-lamp. The DNA fragments were cut out carefully and isolated from the gel. To prepare the DNA-fragment for restriction or ligation, the QIAquick Gel Extraction Kit was used to ensure sufficient yields of DNA. The gel fragment was treated according to instruction manual by first dissolving the fragment in the supplied buffer. The solution was then applied onto a filter resin by centrifugation (1min/12000 *g*). After the cleaning steps, the DNA was eluted with water or the supplied elution buffer into a 1.5 ml reaction tube.

Nested PCR

Gel fragments were dissolved in a Millipore Ultrafree-DA spin column (10 min/12000 *g*). 3 µl of each fragment were then added to the PCR mix instead of template DNA to enable fusion of both fragments during the next PCR amplification process.

3.2.1.3 DNA restriction

Restriction of DNA served two possible purposes. On the one hand, it was used to identify or control the cloned plasmids and, on the other hand, it was needed to prepare DNA fragments for the following ligation step.

DNA was restricted using 2-10 units of enzyme per µg DNA for 45-90 min. Buffer and temperature were chosen according to supplier's instructions. The processed DNA was then loaded on an agarose gel (see 3.2.1.2) to control the restricted product and to purify it from residual enzymes.

3.2.1.4 DNA ligation

To ligate the insert DNA with a vector plasmid, 5 units of T4-DNA-ligase and 1/10 of supplied buffer were used. Sufficient yields can be expected by choosing a molecular ratio insert DNA : vector DNA of 2-10:1. Total volume of the solution was 15 µl, and ligation took place either under room temperature for 30 min or at 16°C overnight. The solution was then transformed into bacteria.

3.2.1.5 Transformation of bacteria strains

Competent bacteria were manufactured according to Chung et al (Chung et al. 1989). The bacteria were defrosted on ice and 100 µl suspension were added to 100 µl mixture of 15 µl ligation solution and 1/5 5x KCM-buffer consisting of 500 mM KCl_2, 150 mM $CaCl_2$ and 250 mM $MgCl_2$. The suspension was kept on ice for 20 min and at room temperature for additional 10 min.

LB medium, produced from (m/v) 1% Trypton, 0,5% yeast extract and 1% NaCl, was autoclaved and 1 ml was added to the transformation suspension. The Suspension was then mixed at 37°C for 60 min to ensure expression of the antibiotic resistance. After this, the suspension was centrifuged at 3000 *g* for 1 min to concentrate the solids.

To produce selection agar 1.5% agarose and 100 µg/ml ampicillin were added to LB medium and this was spread on sterile 10cm cell culture dishes. After the selection agar has set, 20 µl

of the concentrated bacteria were plated with a sterile spatula and incubated at 37°C for at least 12 h to allow colony growth.

3.2.1.6 Control of plasmid DNA

To identify the cloned plasmid DNA, the colonies were suspended in 1-2 ml LB medium containing 100 µg/ml ampicillin and incubated at 37°C for at least 8 h. 1 ml of suspension was transferred in a 1,5 ml reaction tube and centrifuged at 5000 *g* for 1 min. The pellets were resuspended in buffer P1 from Qiagen Midi Extraction Kit and lysed with the alkaline lysis buffer P2 (200 mM NaOH and 1% SDS (w/v)). To neutralize and precipitate the proteins, buffer P3 (3.0 M potassium acetate, pH 5.5) was added and precipitated proteins were separated by centrifugation. Plasmid DNA was precipitated by adding 1 ml 70% Ethanol to the supernatant and subsequent centrifugation at 12000 *g*/4°C for 30 min. After the pellet has dried, it was dissolved in 100 µl water and further analyzed using DNA restriction (3.2.1.3).

3.2.1.7 Preparation of plasmid DNA

The transformed bacteria were suspended in LB Medium mixed with 100 µg/ml ampicillin and incubated at 180 *g*/min and 37°C over night. To purify the plasmid DNA from bacteria, the Qiagen Plasmid Midi Kit or the Qiagen HiSpeed Maxi Kit was used. Both kits consist of several buffer solutions and a special anion-exchange resin, which binds the plasmid DNA.

The bacterial culture was treated according to the instruction manual. Resin size was determined by culture volume, plasmid type and expected DNA amount. After bacterial lysis, the obtained plasmid solution was applied onto the resin by gravity flow and washed using supplied solutions. For the HiSpeed Kit no centrifugation steps were necessary. The plasmid DNA was then eluted with 5 ml solution and precipitated by using 11.5 ml of isopropanol followed by a centrifugation step (45 min/4500 *g*/4°C). The obtained pellet was then precipitated with 1ml of 70% ethanol, to purify the plasmid from remaining proteins. After another centrifugation step (12000 *g*/15 min), the pellet was left to dry for about 30 min and was then diluted in water.

Yield and purity of plasmid DNA was determined using a spectrophotometer with 1cm path length, and the DNA concentration was calculated according to Beer's law. Absorption of 1 at 260 nm indicated a DNA concentration of 50 µg/µl.

The purity was calculated using the coefficient of the absorptions at 260 and 280 nm wavelength, whereas at 280 nm residual protein absorption was detected. A coefficient

between 1,6 and 1,8 was regarded sufficient for further usage. Plasmid DNA solutions were then diluted to a standard concentration of 1 mg DNA/ml.

3.2.2 Cell culture and transfection methods

All procedures were carried out at a sterile workbench using sterile pipettes and tubes.

3.2.2.1 Cultivation of mammalian cell lines

HEK cell lines were incubated in Dulbecco's Modified Eagle Medium (DMEM) containing 4,5 g/l glucose, 2 mM L-glutamine, 100 µg/ml penicillin and streptomycin each and 10% fetal calf serum (FCS) at 37°C and 7% CO_2. For culturing CHO cells, 5% of FCS was added and cells were incubated at 37°C and 5% CO_2.

At 80% confluence, which was reached after about three days of incubation, cells were passaged. The best cell quality for following experiments was achieved by washing the cells carefully with 5ml of phosphate buffered saline (PBS) buffer and application of 1 ml trypsin. After 1-2 min of incubation time, cells were gently shaken off the cell culture dish or flask and pipetted into a Falcon tube filled with 5 ml of room temperature medium. The cell suspension was then centrifuged at 1500 *g*/3min and carefully taken in 30 ml of DMEM. 5 ml of the cell suspension were then transferred into a cell culture dish or flask filled with 5 ml DMEM. Alternatively, 100 µl of suspension were added to a 6-well plate prepared with poly-L-Lysine treated glass cover slips (3.2.2.3) in 2 ml DMEM per well for transfection purposes.

3.2.2.2 Freezing of cell lines

Best transfection efficiency with the construct was achieved between cell passage 5 and 20. To ensure availability cell lines were frozen in a medium consisting of DMEM with 20%FCS and 10% DMSO. Cells were treated as described under 3.2.2.1. After centrifugation, cells were taken into the medium and dispensed in cryotubes and cooled down for 3 h at -20°C. Subsequent the tubes were kept at -80°C over night to prepare for long-term storage in liquid nitrogen.

To defrost stored cell lines, cryotubes were taken from storage tanks, immediately followed by gentlerapid thawing with room temperature DMEM medium. The suspension was applied to a 10cm culture dish already filled with DMEM. After the cell adhesion took place (about 4h), medium containing DMSO was removed and replaced with fresh DMEM.

3.2.2.3 Coating of glass cover slips and 96-well plates

For FRET experiments, HEK cells were grown on poly-L-lysine (0.1mg/ml) coated glass cover slips or 96-well plates. Cover slips were kept in 70% ethanol to assure sterility.

Before coating, cover slips were washed in PBS buffer to remove residual ethanol and then placed into 6-well plates. 1 ml of poly-L-lysine was dropped onto each cover slip and left for 30min. The solution was then removed, and coverslips were washed with 1 ml PBS buffer. The cells were prepared as described under 3.2.2.1. Cells were incubated for another 4 h to ensure adhesion before transfection.

96-well plates were coated by adding of 100 µl of poly-L-lysine for 30 min. After flicking and washing off residuals with PBS buffer, 100 µl of cell suspension was placed into each well.

3.2.2.4 Transfection procedures

Best results were obtained using lipofection with slightly modified protocols. Thus, only these procedures will be described.

3.2.2.4.1 Effectene

For FRET experiments, HEK cells were transfected using the Qiagen Effectene Kit. For one 6-well plate, 300 µl of transfection buffer were mixed with 2 µg of DNA. 16 µl of enhancer solution were added and gently pipetted up and down and then left for 5 min. Next 32 µl of Effectene were added and the mixture was gently shaken to thoroughly mix the lipofectant. To ensure forming of liposomes the solution was left for another 15 min. 300 µl of DMEM were carefully mixed with the transfection solution, and 100 µl were dropped into each well.

The cells were incubated at 37°C for at least 36 h to allow the expression of the construct. Effectene solution was not removed during this process.

3.2.2.4.2 Lipofectamine 2000

For radioligand binding and ERK assays, CHO and HEK cells grown in 25 cm^2 cell culture flasks were transfected using Lipofectamine 2000. For one flask 0.5 ml of Opti-MEM were mixed with 2 µg of DNA and 20µl of lipofectamine by short pulses on a vortex. The mixture was left for 45 min to allow complexes formation. Medium was removed from the flask and replaced with serum-free DMEM. Lipofectamine mixture was added to the flask, and the cells were incubated at 37°C for 4 h. After this step 5 ml of complete medium was added without removing the DNA complexes and incubated for at least 36 h.

Transfected cells were then removed from flask as described under 3.2.2.1 and dispensed into a 96-well plate with 100 µl each. The plate was then incubated for another 4 h to allow cell adhesion.

3.2.2.5 Atropine treatment to enhance transfection efficiency

In an attempt to enhance receptor expression, cells were incubated with different atropine concentrations. After transfection 1 nM, 5 nM and 10 nM of atropine were added to the cell-medium and cells were incubated at 37°C overnight.

3.2.2.6 Physostigmine pretreatment to inhibit esterases

Cellular esterases may hydrolyze alkane-bis-ammonium compounds, such as dimethyl-W84. To test whether this effect plays a significant role for FRET experiments, transfected coverslips were incubated with 10 µM physostigmine for 2 h at 37°C prior to the FRET experiments. Results were then compared to non-physostigmin treated cells.

3.2.2.7 FlAsH labeling

The specificity of FlAsH-binding has already been shown *in vitro* and *in vivo* (Adams et al. 2002). Cell-growth medium was removed from the transfected cells and they were washed twice with Phenol Red-free Hank's balanced salt solution (HBSS). 1000 µl dimethylsulfoxide (DMSO) and 2,1 µl ethandithiol (EDT) were mixed (Solution 1). 0,5 µl of Solution 1 and 0,5µl FlAsH per well were then mixed in 1 ml of HBSS and added to each well resulting in 500 nM of FlAsH and 12.5 µM ethandithiol per well. The well was then incubated by 37°C for 1h to allow FlAsH binding to take place. After incubation the solution was then carefully washed off with HBSS. For Solution 2 1000 µl DMSO and 42 µl EDT were mixed. To remove unspecific binding cells were incubated with 0,5 µl Solution 2 in 1ml HBSS per well (250 nM EDT per well) for another 10 min at 37°C. The cells were washed thoroughly with

HBSS and 2 ml of DMEM with 10% FCS were added. Cells were kept in the incubator at 37°C prior to experiments.

3.2.3 Assays to determine receptor functionality

3.2.3.1 ERK 1/2 phosphorylation-assay

For the ERK 1/2 Phosphorylation Assays, the AlphaScreen® *SureFire* Phospho-ERK Assay Kit and the AlphaScreen® Protein A Kit were used. This non-radioactive assay method detects ERK 1/2 using fluorescent beads. After cell lysis with the AlphaScreen® *SureFire* Kit, IgG, a streptavidin- and a protein A-coated fluorescent bead (all ingredients of AlphaScreen® Protein A Kit) are added to the lysate. The beads are then forming a sandwich-complex around the biotynlyated protein of interest (here: ERK 1/2). Upon excitation with 680 nm the donor bead produces singlet oxygen, which leads to a chemiluminescent reaction in the acceptor bead. The acceptor then emits light, which can be detected at 520-620 nm.

Prior to the assay, cells were washed twice with PBS buffer and then serum-starved in a 96-well plate in serum-free DMEM at 37°C for 4 h. All data were expressed as a percentage of ERK 1/2 phosphorylation after 6 min of stimulation with 3% FCS. To obtain the basal phosphorylation values, cells were treated with serum free medium. To determine the peak response of each ligand, time course and dose response curve experiments with a stable CHO M_2 wt cell line were performed in parallel. The ligands oxotremorine-M, McN-A 343, LY2033298, xanomeline, carbachol and acetylcholine were applied to the cells for 0, 3, 5, 10, 20 and 30 min at 37°C in a total volume of 200 µl/well. For concentration-response curves the same ligands were applied in various concentrations according to their peak response measured in the time course experiments.

Interaction experiments were performed with oxotremorine-M, acetylcholine and carbachol, all applied for optimal time (5 min) and concentrations (10^{-4} to 10^{-9} M), mixed with either atropine (30 min) or gallamine (6 min) in a concentration range of 10^{-4} to 10^{-8}M for atropine and 10^{-2} to 10^{-6} M/gallamine. Reaction was stopped by removing the ligand solution and application of 100 µl lysis buffer and the plate was agitated for 1-2 min. 40 µl of lysate and 10 µl of reaction buffer were then mixed in a fresh 96-well plate and 5 µl of the mixture were applied to a white opaque 384-well sterile Proxiplate.

The following steps were performed under diminished light conditions. For one 384-well plate, 11 µl of AlphaScreen® beads each were mixed with 2.6 ml of reaction buffer and then

6 µl were added to the lysate mixture in the Proxiplate. The plate was shaken to ensure mixture of the solutions and kept in the dark at 37°C for 1 h. The fluorescence signal was measured in a Fusion-α plate reader (Perkin Elmer) using standard AlphaScreen® settings. The obtained data were analyzed using the Prism 4.03 software (GraphPad, San Diego, CA).

3.2.3.2 Radioligand binding assay

3.2.3.2.1 FLAG-tagged receptor sensor

Flp-In-293 cells were transfected with 6 µg receptor construct DNA in 75 cm^2 flasks using lipofectamine 2000 as described under 3.2.2.4.2. 48 hours later the cells were harvested, counted and then resuspended at a density of $1x10^6$ cells/ml in HEPES buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM glucose, 20 mM HEPES and 58 mM sucrose; pH 7.4, adjusted with NaOH) in a final volume of 1 ml. Cells were incubated with increasing concentrations of [^{3}H]-NMS or [^{3}H]-QNB for 3 hours at 4°C in HEPES buffer. Non-specific binding was determined in the presence of 10 µM atropine. Radioactivity bound to the cells was separated from unbound using rapid vacuum filtration onto GF/B glass fibre filter paper. Filters were soaked in 4 ml Ultima Gold scintillation cocktail for at least 4 hours before radioactivity was determined by liquid scintillation counting. Flp-In CHO M2 cells were used as a control to measure the receptor numbers at the cell surface.

Data sets of specific and non-specific binding were analyzed with Prism 4.03 (Graph Pad Software, San Diego, CA) using the formula

$$Y=\frac{B_{max}\,x\,[A]}{[A]+K_A}+NS\,x\,[A]$$

The factor Y denotes radioligand binding, B_{max} depicts maximal density of binding sites, [A] represents the concentration of the radioligand, K_A is the radioligand equilibrium dissociation constant and NS is the fraction of non-specific binding.

3.2.3.2.2 HA-tagged receptor sensor

CHO cells were maintained at 37°C, 5% CO_2 in DMEM/F12 medium supplemented with 10% fetal calf serum. Transfections were performed using the Lipofectamine 2000 reagent (3.2.2.4.2).

The binding of [^{3}H]NMS to the the M_2 receptor sensor and wildtype M_2 receptor was analyzed in membranes prepared from transiently transfected CHO cells and from the CHO M_2 cell line. Membranes (containing 10 µg membrane protein) were incubated for 2 h at room temperature in the assay buffer (25 mM phosphate buffer saline, 5 mM $MgCl_2$, pH=7.4) with 0.1 to 10 nM of [^{3}H]NMS. Nonspecific binding was determined in the presence of 10 µM atropine. The reactions were terminated by vacuum filtration through GF/B glass fibre filters.

3.2.3.3 Patch clamp experiments

For the Patch Clamp experiments, TSA 201 cells were transfected with GIRK1/GIRK4-IRES plasmid and the cloned M_2 mAChR-construct according to 3.2.2.4.1. For the measurement of GIRK-currents whole cell measurements were performed in an extracellular solution of the following composition (mM): NaCl, 120; KCl, 20; $CaCl_2$, 2; $MgCl_2$, 1; Hepes-NaOH, 10, pH 7.3. The internal (pipette) solution contained (mM): potassium aspartate, 100; KCl, 40; MgATP, 5; Hepes-KOH, 10; NaCl, 5; EGTA, 2; $MgCl_2$, 1; GTP, 0.01; pH 7.3. Membrane currents were recorded under voltage-clamp conditions, using conventional whole cell patch clamp techniques (Bunemann et al. 1995). Patch-pipettes were fabricated from borosilicate glass capillaries, (GF-150-10, Warner Instrument Corp.) using a horizontal puller (P-95, Fleming & Poulsen). The DC resistance of the filled pipettes ranged from 3-6 MΩ. The coverslips were mounted on an Attofluor holder, the reference electrode was placed into the solution and the patch pipette was placed manually above the specified cell. With a micromanipulator, the patch pipette was then lowered onto the cell surface and contact was made by gentle suction. Upon contact with the cell membrane, pipette resistance displayed a sudden increase. The experimental set-up has to remain in the “cell-attached” mode, as a rupture of the cell membrane would result in a loss of metabolic functions of the cell. Various concentrations of acetylcholine were applied using a computer-assisted solenoid valve-controlled rapid superfusion device ALA-VM8 (ALA Scientific Instruments; solution exchange in 5-10 ms). Membrane currents were recorded using either a patch-clamp amplifier (Axopatch 200, Axon Instruments) or an EPC 9 (HEKA Instruments). Signals were analog-filtered using a lowpass Bessel filter (1-3 kHz corner frequency). Data were digitally stored using either a Mac (Centrion 640 with pulse software) or an IBM compatible PC equipped with a hardware/software package (ISO2 by MFK, Frankfurt/Main, Germany) for voltage control, data acquisition, and data evaluation. IK_{ACh} was measured as an inward current using a holding potential of -90 mV. Voltage ramps (from -120 mV to +60 mV in 500 ms, every 10

s) were used to determine current-voltage (I-V) relationships. All measurements were performed at room temperature.

3.2.4 Fluorescence measurements

For all fluorescence measurements, TSA 201 cells were transfected using Effectene as described under 3.2.2.4.1. After FlAsH labeling the cells were placed into an Attofluor holder (Molecular Probes, Leiden, The Netherlands) and then maintained in a FRET-buffer consisting of the following ingredients (mM): NaCl 144, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 20, pH 7.4.

3.2.4.1 Confocal imaging

Confocal microscopy was performed using a Leica TCS SP2 system. FlAsH and CFP were excited with the 514 nm line of an Argon ion laser and a 430 nm frequency-doubled diode laser, respectively. Images were obtained with a 63x objective using the standard Leica software (version 2.5) and stored on a personal computer.

3.2.4.2 FRET detection measurements

Measurements were performed at room temperature using a Zeiss Axiovert 200 inverted microscope equipped with an oil immersion 100x objective, a dual emission photometric system and a Polychrome IV light source (Till Photonics, Gräfelfing. Germany). The cycle time was chosen at 50 ms for kinetic measurements of gallamine and 100 ms for kinetic measurements of dimethyl-W84, both antagonists and non-kinetic measurements. Illumination time was set to 40 (gallamine) or 80 ms at 436 ± 10 nm (beam splitter DCLP 460 nm), applied with a frequency between 10 Hz for kinetic measurements of dimethyl-W84, both antagonists and non-kinetic measurements and 20 Hz for kinetic measurements of gallamine. Fluorescence was recorded from entire single cells in FlAsH and CFP emission channels using 535 ± 15 nm and 480 ± 20 nm emission filters and a DCLP 505 nm beam splitter. To study agonist-induced changes in FRET, cells were continuously superfused with FRET buffer supplemented with various ligands as indicated, applied using a computer-assisted solenoid valve-controlled rapid superfusion device ALA-VM8 (ALA Scientific Instruments; solution exchange in 5-10 ms). Signals detected by avalanche photodiodes were digitalized using an AD converter (Digidata 1322A, Axon Instruments) and computer-stored using Clampex (Version 8.1, Axon Instruments).

3.2.4.2.1 cAMP-measurements

To measure M_2 receptor-induced inhibition of cAMP signaling, CHO cells expressing the wild type receptor or the M_2-receptor sensor were transfected with the FRET-based cAMP sensor Epac1-camps, and the cAMP levels were measured by FRET imaging as described previously (3.2.4.2). After stimulation of cAMP production by 2 µM of forskolin, increasing concentrations of carbachol were added to the cells in presence or absence of 100 µM gallamine.

3.2.4.2.2 Data processing

FRET was monitored as the emission ratio FlAsH/CFP, which was corrected offline for the spillover of CFP into the FlAsH channel, direct FlAsH excitation and photobleaching using Origin software (version 6, OriginLab corporation). Figures displayed in "Results" show corrected values for F_{535}, F_{480} and F_{535}/F_{480}.

4 Results

4.1 M_2 mACh Receptor

The M_2 mAChR is the GPCR with the most published data regarding the mechanisms of allosteric regulation. Many different allosteric ligands have been identified and developed for this receptor. Therefore the M_2 mAChR seemed to be the most suited receptor candidate for this project.

4.1.1 Cloning of the M_2 mAChR FRET-sensor construct

Until now, no muscarinic receptor FRET sensors for monitoring of ligand-induced receptor activation have been published. The first aim was to design a sensor, which would demonstrate sufficient cell surface expression and an agonist-dependent change in FRET above 5% to ensure reproducibility of the measurements.

The ICL3 within the M_2 mAChR's, which is crucial for G-protein coupling, desensitization and conformational changes after receptor-ligand interaction, is very long (Figure 4.1.1).

First, this loop was shortened to increase the basal FRET signal between FlAsH introduced into this loop and C-terminally located CFP. A similar strategy had already been used for the α_{2A}-adrenergic receptor sensor (Hoffmann et al. 2005).

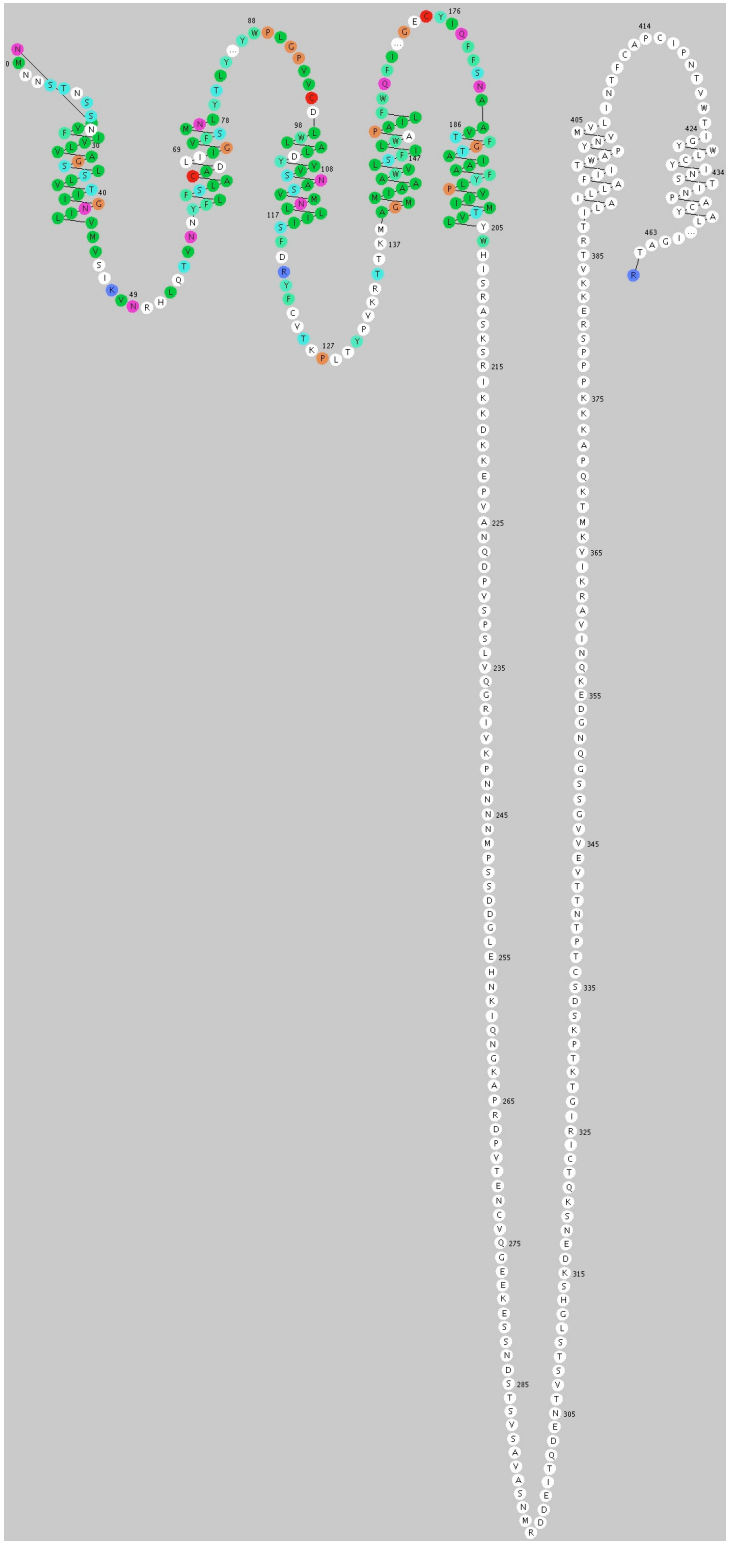


Figure 4.1.1 *Two-dimensional representation of the wild type M_2 mAChR*

1-22 N-terminus, extra cellular; 23-45 TMD1; 46–59 ICL1, cytosolic; 60–80 TMD2; 81–97 ECL1, extra cellular; 98–119, TMD3; 120 –139 ICL2, cytosolic; 140–162 TMD4; 163–184 ECL2, extra cellular; 185–207 TMD5; 208–388 ICL3, cytosolic; 389–409 TMD6; 410 – 423 ECL3, extra cellular; 424–443 TMD7; 444-466 C-Terminus, cytosolic;

(www.gpcr.org, www.uniprot.org/uniprot/P08172)

TMD: Transmembrane domain, ECL: extracellular loop, ICL: intracellular loop

4.1.1.1 First construct

Previously published mutational data (Kostenis et al. 1999) suggested that no important sites for receptor - G-protein interaction could be found between amino acids P230 and T369. This part of the ICL3 was deleted and the six amino acid FlAsH-binding motif CCPGCC was inserted instead using standard cloning procedures. In addition, CFP was fused directly to the end of the C-terminus after R465. Unfortunately, after transient transfection this construct was expressed in only a very low number of cells, showed weak fluorescence and some intracellular staining. However it did exhibit a slight decrease of FRET upon stimulation with carbachol (1-2%). This, however, was not sufficient to be used as a FRET construct in this study.

4.1.1.2 Improving the expression levels

To improve the cell surface expression of the receptor sensor, different modifications were tried. Since the C-terminus of the M_2 mAChR is relatively short (24 amino acids) and contains a palmitoylation site close to the extreme C-terminus, it was decided to elongate the C-terminus with a flexible amino acid linker GSGEG that was inserted between R465 and the CFP sequence. Similar linkers have already been used to interconnect ECFP and EYFP in intramolecular FRET constructs (Evers et al. 2006). The resulting construct M_2-sl3-FlAsH-GSGEG-CFP indeed exhibited much better levels of overall expression in cells but still could not be sufficiently well trafficked to the cell membrane of the TsA201 cells.

4.1.1.3 Final constructs

The next improvement was to fuse the FLAG motif to the N-terminus in front of the amino acid D2 (Figure 4.1.2). This motif has already been used in our laboratory to improve the membrane expression of the β_2-adrenergic receptor (personal communication, Ulrike Zabel)

This construct FLAG-M_2-sl3-FlAsH-GSGEG-CFP exhibited much more reliable transfection efficiencies and sufficient membrane expression. First experiments with the ligand acetylcholine showed FRET changes of about 5-8 %, which was sufficient for further studies (Figure 4.1.8). Receptor expression, but not signal amplitudes could then be further improved by addition of a HA signal peptide to the N-terminal FLAG-tag.

XXX	Cell surface expression	FRET-Signal
FLAG	+	+
HA-FLAG	++	+

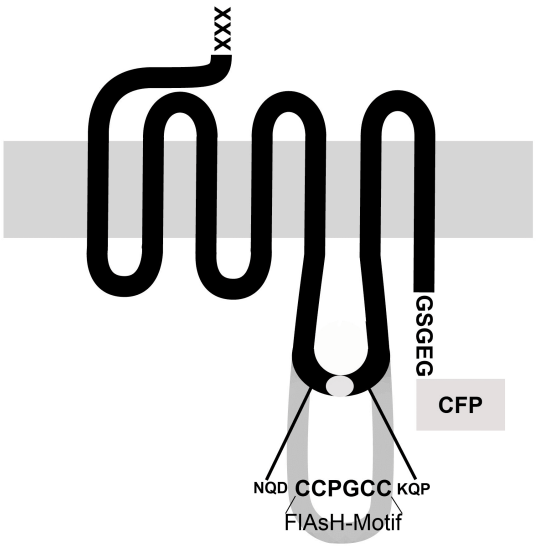


Figure 4.1.2

Schematic structure of the M_2 mAChR sensor constructs used in this study. CFP was fused to the C-terminus of the human muscarinic M_2 receptor via a 5 amino acid linker GSGEG and the FlAsH motif CCPGCC was introduced into the shortened ICL 3 with the indicated linkers. N-terminal tagging with FLAG or HA-FLAG resulted in different cell surface expression.

Both constructs were then used to study the mechanism of allosteric regulation of the receptor.

4.1.1.4 Further improvements of receptor expression

One more attempt to improve the expression of the receptor sensor has been undertaken. As described in „Methods“, incubation with different concentrations of the antagonist atropine was used to try to enhance expression of the receptor after transfection. Unfortunately, the cells did not tolerate atropine and underwent apoptosis. Few remaining cells could be labeled but showed no FRET-change upon addition of the agonist acetylcholine. Probably atropine, with its high affinity to the receptor, might have occupied the orthosteric site and prevented further reactions to other ligands.

4.1.2 Confocal microscopy

To visualize membrane localization of the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor, confocal microscopy was performed. This method allows to prove that the receptor still maintains its plasma membrane localization after labeling with the FlAsH dye .

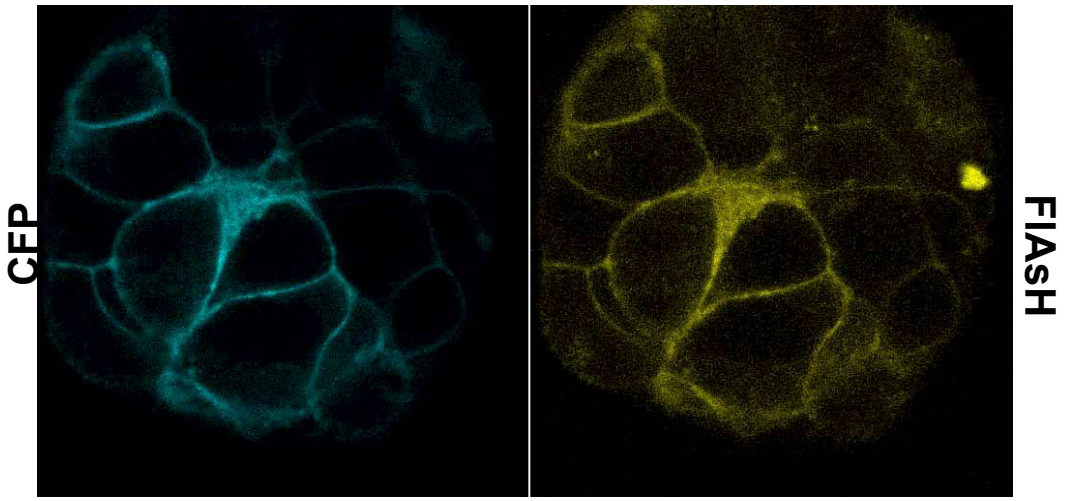


Figure 4.1.3 *Representative confocal pictures of TsA201 cells transiently transfected with the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor construct after labeling with FlAsH. The CFP and FlAsH images show predominant cell surface staining of the construct*

Directly after labeling the sensor construct was predominantly localized at the plasma membranes of the transfected cells. However, a substantial amount of fluorescence was still observed in intracellular compartments (Figure 4.1.3).

4.1.3 Receptor functionality

4.1.3.1 Flag-tagged receptor sensor construct

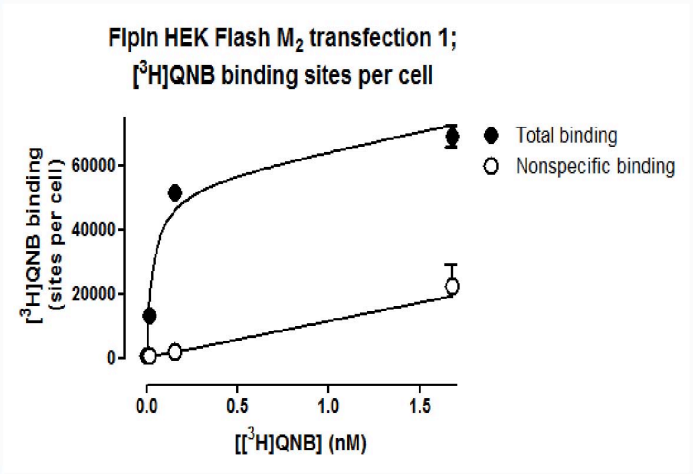
4.1.3.1.1 Radioligand binding assay

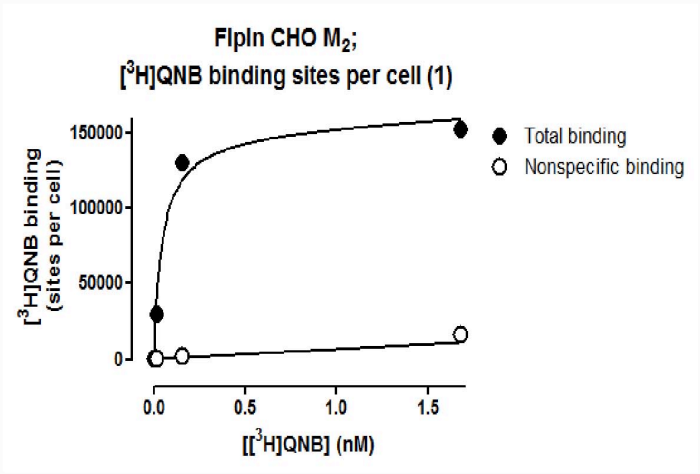
Radioligand binding studies were performed to determine whether the used transfection protocol is optimal and to assess the percentage of membrane bound receptor construct in more detail.

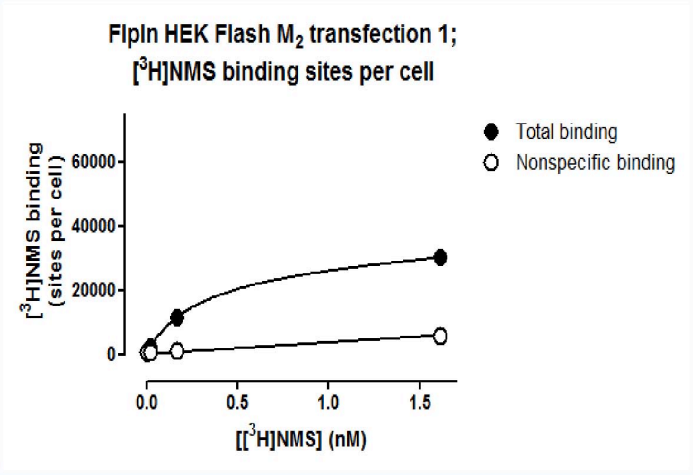
Assays with the membrane permeable radioactively labeled antagonist [^{3}H]QNB revealed that the receptor expression in HEK293 cells transiently transfected with the FLAG-tagged sensor construct was similar to that of CHO M_2 WT cells stably expressing the wild type M2 receptor. With the FLAG-M2-sl3-FlAsH-GSGEG-CFP a B_{max}= 100,333 was reached, the maximal value for CHO M_2 WT was B_{max}=159,388. The calculated average $pK_{d,}$ of the FLAG-M2-sl3-FlAsH-GSGEG-CFP receptor and [^{3}H]QNB was 10.1 which was comparable to the $pK_{d,\ FlplnCHO\ M2WT}$=10.3.

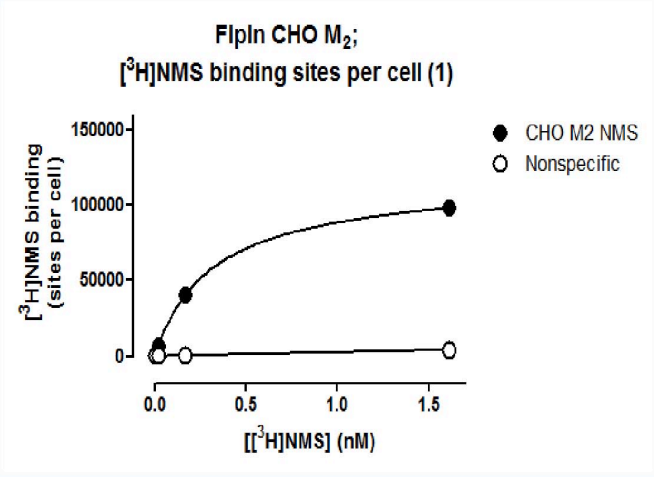
The non-membrane permeable antagonist [^{3}H]NMS showed that, as expected, the cell surface staining was less effective. Compared to the previously used CHO M_2 WT cell line, which exhibits an average rate of 80 % of [^{3}H]NMS to [^{3}H]QNB binding sites, the transfected HEK cells only showed an average rate of 48% of [^{3}H]NMS to [^{3}H]QNB binding sites (Figure 4.1.4).

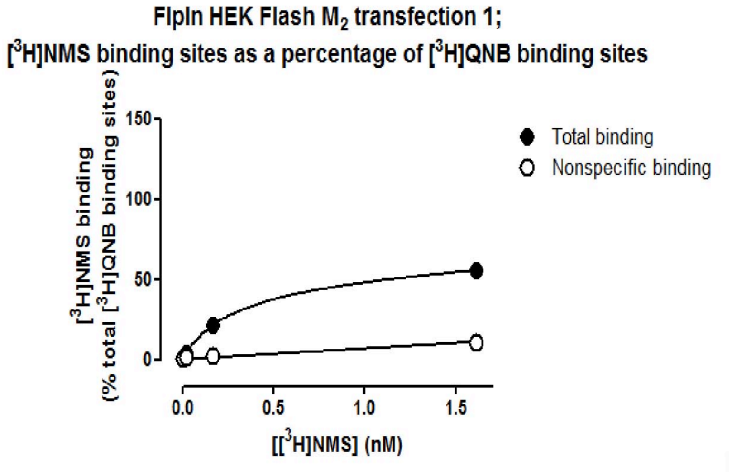
The results from the radioactive binding data indicate that expression levels are acceptable, but that the cell surface expression of the receptor is reduced compared to stably expressing cells.











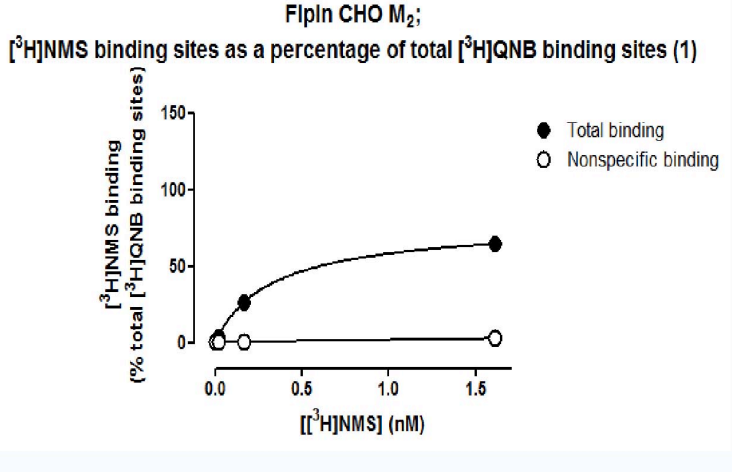


Figure 4.1.4 *Representative saturation curves for the HEK 293 cell line transiently transfected with the FLAG-M_2-sl3-FlAsH-GSGEG-CFP construct and for the Flpln CHO cell line stably expressing the M_2 WT receptor. Cell lines were incubated with different concentrations of [^{3}H]NMS and [^{3}H]QNB to determine differences in saturation data (n=2).*

4.1.3.1.2 ERK Alpha Assay

The ERK assay was performed to analyze the downstream signaling of the receptor sensor via G-proteins in transfected HEK293 cells. Unfortunately we were not able to measure increase in ERK activity, which might be due to the low amounts of cell surface bound receptors, as simultaneous control assays with CHO M_2 WT cells worked fine.

4.1.3.1.3 GIRK measurements

GIRK channels are directly activated by the Gβγ-subunit of the G-protein (Peleg et al. 2002). This approach was used to verify that the construct was able to efficiently couple to and activate Gi-proteins. The path clamp experiments showed acetylcholine-induced GIRK currents, which revealed an $EC_{50} \approx 100 \pm 7$nM (Figure 4.1.5). This value is in good agreement to previously published data (Ben-Chaim et al. 2003) which show an $EC_{50} \approx$ 40 nM for acetylcholine at the wild type receptor under similar conditions. The receptor sensor can effectively couple to G_i-proteins and induce downstream signaling such as GIRK-currents.

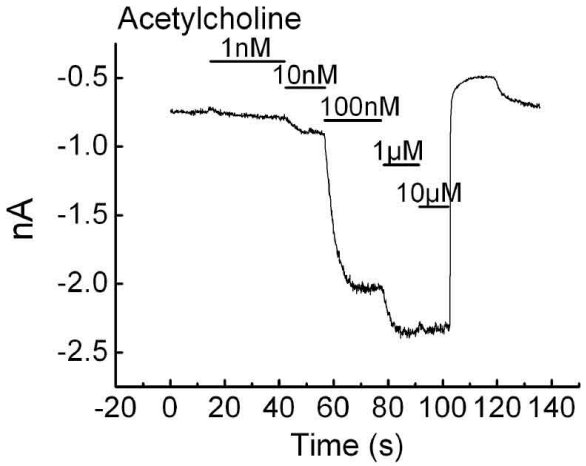


Figure 4.1.5 *Representative GIRK current trace. TsA201 cells transfected with FLAG-M_2-sl3-FlAsH-GSGEG-CFP were stimulated with different concentrations of acetylcholine and the change in the GIRK current was measured (n=3).*

4.1.3.2 HA-tagged sensor construct

4.1.3.2.1 Radioligand binding assay

To assess whether the affinity for carbachol and the allosteric ligands gallamine and dimethyl-W84 are similar for the receptor sensor construct and the wildtype receptor further radioligand binding assays were performed. The competitive displacement curves (Figure 4.1.6) show comparable affinities for the ligands on both receptors.

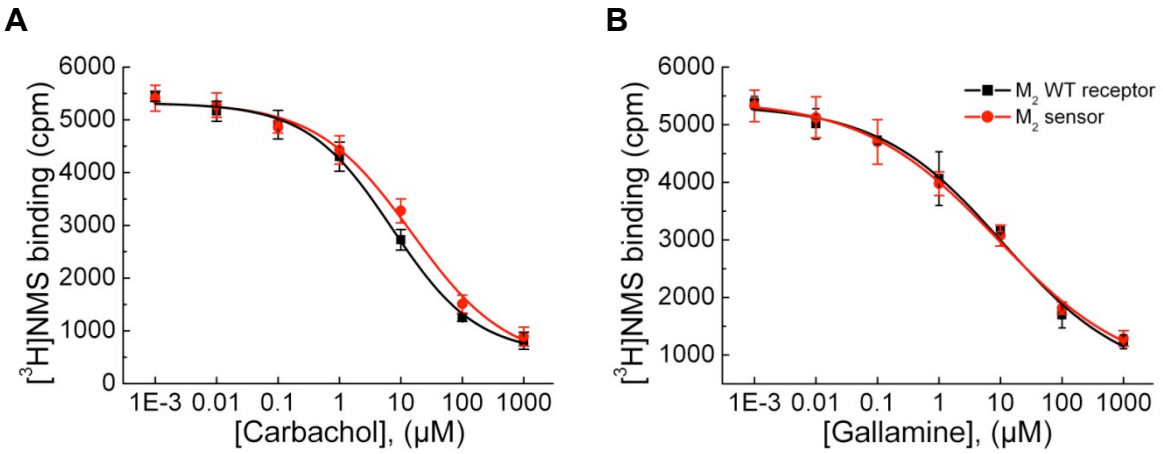


Figure 4.1.6

*Radioligand binding assay for **A** Carbachol and B Gallamin, both with CHO cells expressing the HA-tagged sensor construct and the M_2 mAchR wt. Both receptors display similar affinities for the tested ligands (data are shown as means ± SEM, n=3).*

IC_{50} values were 7.3 ± 2.2 and 10.0 ± 1.7 µM for carbachol, 11.5 ± 1.4 and 11.5 ± 3.9 µM for gallamine, wildtype and receptor sensor, respectively.

4.1.3.2.2 cAMP-Inhibition

CHO cells expressing the M_2 mAChR wt or the HA-tagged construct were transfected with the FRET-based cAMP sensor Epac1-camps and inhibition of forskolin-stimulated cAMP production in the cells by carbachol in absence and presence of the negative allosteric ligand gallamine was measured. Upon carbachol stimulation, the cAMP signal was blocked. The obtained concentration-response curves for the carbachol-mediated inhibition of cAMP levels show, both receptors have similar functional properties in terms of the efficacy and potency for the orthosteric and allosteric ligands. EC_{50} values for wildtype receptor and the receptor sensor were 1.6 ± 0.3 and 2.8 ± 0.5 for carbachol and 31.4 ± 0.4 and 36.4 ± 0.4 for carbachol in presence of 100µM gallamine, respectively (Figure 4.1.7).

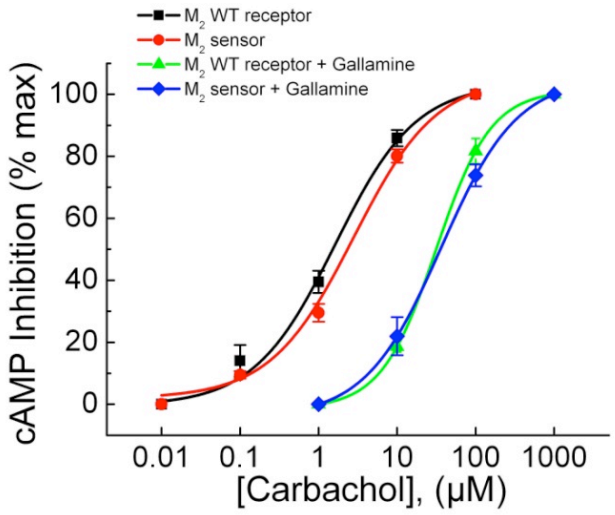


Figure 4.1.7

Inhibition of forskolin-induced cAMP signals by the M_2 mAchR wt receptor and the M_2 receptor. Concentration-response curves for carbachol in absence and presence of 100 µM gallamine are presented (data are shown as means ± SEM, n=4-5).

In conclusion, the experiments to test the functionality of both receptor sensor constructs strongly suggest that despite structural modifications which were introduced in the sensor construct, both receptor sensors remain functional in terms of affinity for various ligands, and downstream-signaling.

4.1.4 FRET-measurements

As described in "Introduction" and "Methods", the FRET-technique provides a unique tool to visualize conformational changes in GPCRs. This study aimed at the analysis of conformational changes in the M_2-receptor using the newly developed FRET-based receptor sensor. Using this technique, the conformational changes in the receptor upon activation with different orthosteric and allosteric ligands and the real-time kinetics of these changes were measured.

4.1.4.1 Agonists

The typical mAChR agonists acetylcholine and carbachol were chosen for the FRET measurements. Stimulation of the sensor expressing cells with the full receptor agonist acetylcholine resulted in a fast reversible decrease in FlAsH and concomitant increase in CFP fluorescence, indicative of a decrease in FRET (Figure 4.1.8). The changes in CFP and FlAsH fluorescence were clearly agonist-dependent and had maximal amplitudes of ~ 5-6 %. This type of experiments has also been performed for carbachol giving similar results.

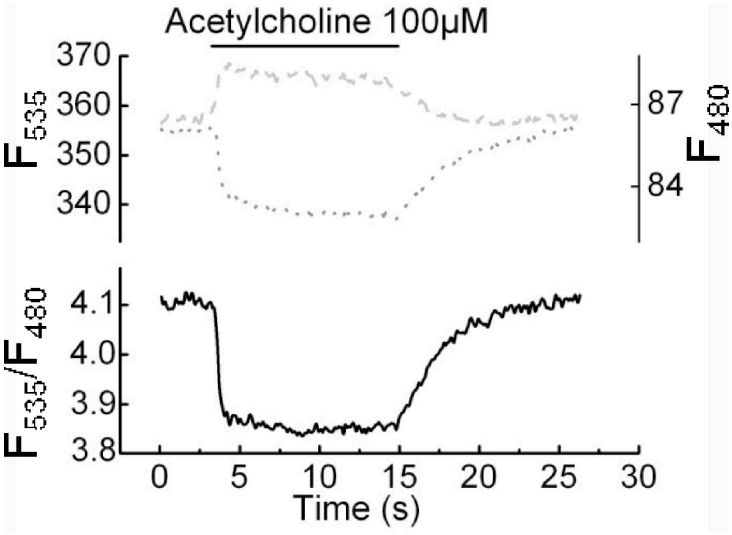


Figure 4.1.8 *Representative FRET-trace recorded from TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor upon stimulation with 100 µM acetylcholine. An increase in the F_{480} and a decrease in the F_{535} channel indicates a decrease in FRET between CFP and FlAsh as shown as a decrease of the F_{535}/F_{480} ratio (cycle time 100 ms, illumination time 80 ms, n=4).*

Next, various concentrations of the two agonists, acetylcholine and carbachol, were used to establish the concentration-response dependencies (Figure 4.1.9).

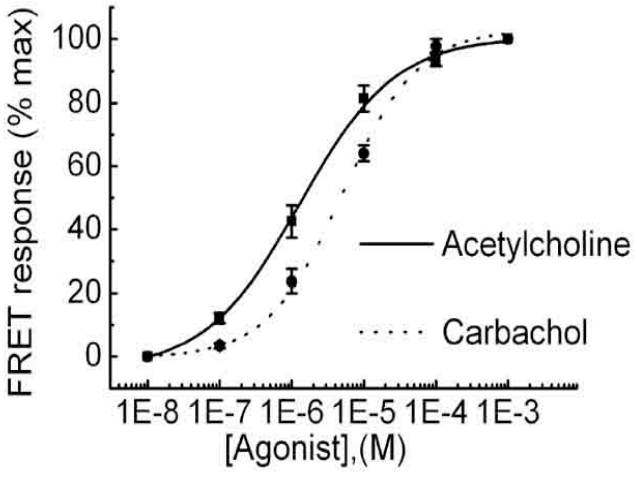


Figure 4.1.9 *Concentration-response curves for TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor with acetylcholine and carbachol.*
The carbachol curve is shifted to the right, indicating a higher EC_{50} value (data are shown as means ± SEM, n=6-8)

EC_{50} values were 1.86 ± 0.3 µM for acetylcholine and 5.22 ± 0.4 µM for carbachol. Comparison of the data from these concentration-response FRET-measurements with results described by Maggio et al (Maggio et al. 1994) revealed that the obtained values were in good agreement with the published data for the wild type M_2 receptor. Maggio et al used COS-7 cells transfected with the wild type M_2 receptor and described IC_{50}-values of 0.87 ± 0.17 µM for acetylcholine and 2.14 ± 0.6 µM for carbachol.

In addition, it was attempted to measure the concentration-response dependencies for the partial agonists pilocarpine and oxotremorine-M. Unfortunately the FRET signals induced by these ligands were too small (about 2 % change) to obtain reliable data.

For further experiments an agonist concentration of 100 µM was chosen, since this concentration was saturating for both agonists.

4.1.4.2 Antagonists

To investigate whether the sensor reports the effects of receptor antagonists, cells were first stimulated with the agonists acetylcholine (Figure 4.1.10A) or carbachol (Figure 4.1.10B), and then a saturating concentration of atropine in the continuous presence of the respective agonist was applied. This antagonist is known to have a rather high affinity for the muscarinic M_2-receptor (Lazareno and Roberts 1989). While atropine alone did not cause any change in FRET, addition of atropine in the presence of agonists led to a rapid and complete reversal of the signal caused by acetylcholine or carbachol, which was significantly faster than the simple wash-out of the agonists (Figure 4.1.10 C, D).

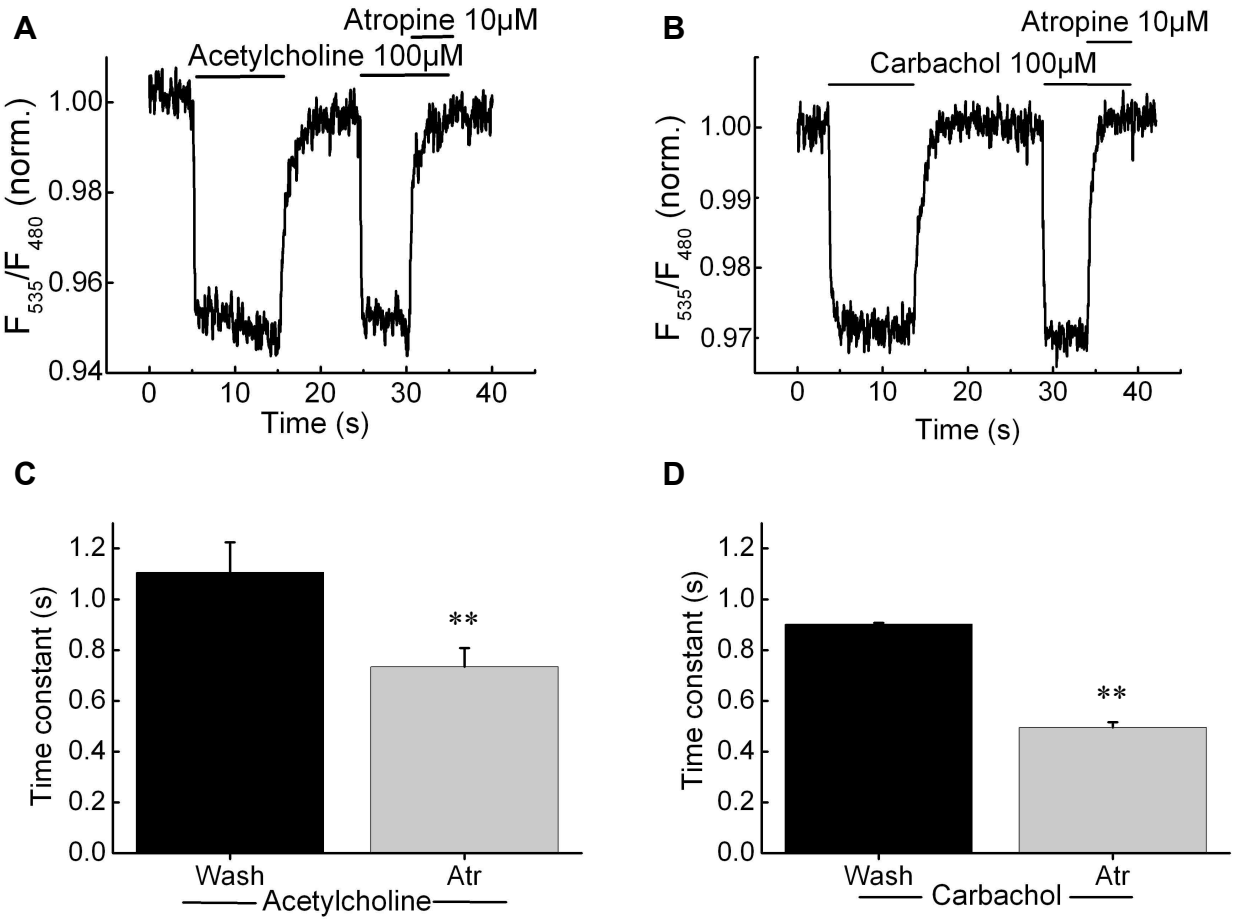


Figure 4.1.10 *Inhibition of agonist-induced receptor activation by atropine. TsA201 cells were transfected with the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor. Addition of atropine results in a fast reversal of the FRET signal evoked by the agonists acetylcholine (**A**) and carbachol (**B**). The kinetics of the agonist wash-out and atropine effects were compared by calculating time constants of the FRET ratio decay from the ratio traces for acetylcholine (**C**) and carbachol (**D**). Statistical significance was assessed using a T-test, ** $p<0,01$ (A,B show corrected, normalized values for FRET emission, data in **C**,**D** are shown as means ± SEM of kinetic FRET measurements, cycle time 100 ms, illumination time 80 ms, n=6-7)*

4.1.4.3 Allosteric ligands

The FRET experiments with allosteric ligands turned out to be complicated since many of the recently developed rather selective allosteric ligands for the M_2 mACR, such as the new bisquarternary dimers of strychnine or brucine (Zlotos et al. 2003) or DUO3 and WDUO3 (Muth et al. 2003) show fluorescence in the relevant spectral range. Thus, the choice was limited to a small number of allosteric ligands. The selected ligands were gallamine, tacrine and dimethyl-W84 (Figure 4.1.11), all of them are well described in the literature (Holzgrabe et al. 2000; Huang et al. 2005; Szilagyi and Lau 1993; Trankle et al. 2005).

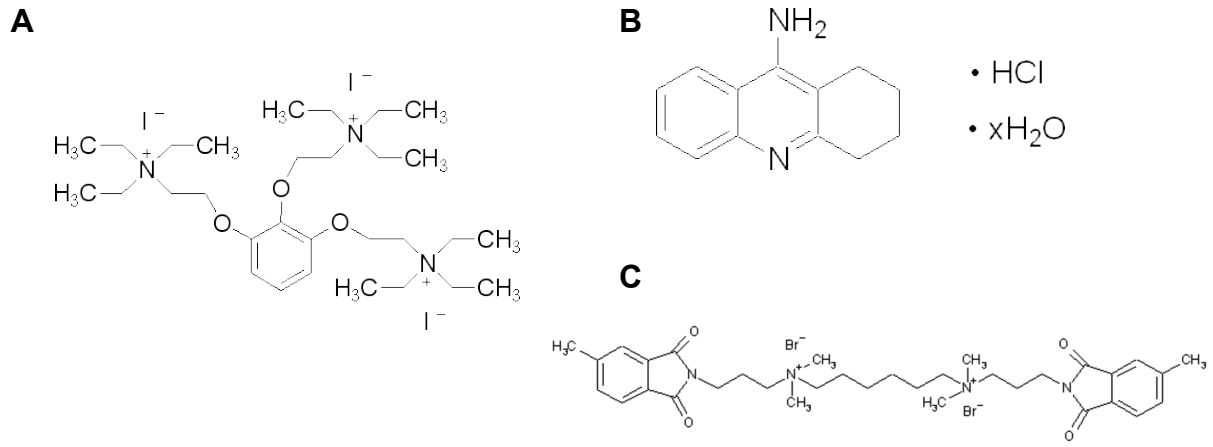


Figure 4.1.11 *Chemical structures of **A** gallamine, **B** tacrine, **C** dimethyl-W84 (Images taken from www.sigmaaldrich.com and www.enzolifesciences.com)*

While gallamine and dimethyl-W84 have been described to have some selectivity for the M_2-receptor subtype, tacrine is rather unspecific.

First, it was investigated whether addition of the allosteric ligands to non-stimulated receptors evokes a change in the FRET-signal. When given on their own, without simultaneous addition of orthosteric ligand, no change in the FRET signal for gallamine and tacrine was observed (Figure 4.1.12).

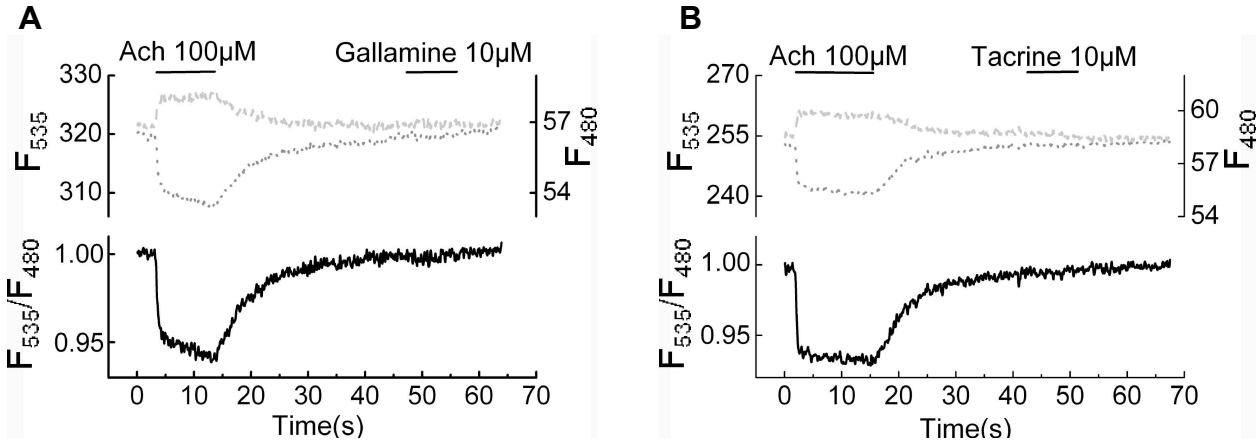


Figure 4.1.12 *Representative, corrected and normalized traces of FRET measurements with TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor for acetylcholine (100 µM)* ***A*** *+ gallamine (10 µM) (n=7) and* ***B*** *+ tacrine (10µM) (n=5)(cycle time 100 ms, illumination time 80 ms).*

Dimethyl-W84 alone showed a slight change of the FRET signal, which might be due to the fluorescent quenching, but this change was very small compared to the FRET-signals from acetylcholine and carbachol (Figure 4.1.13).

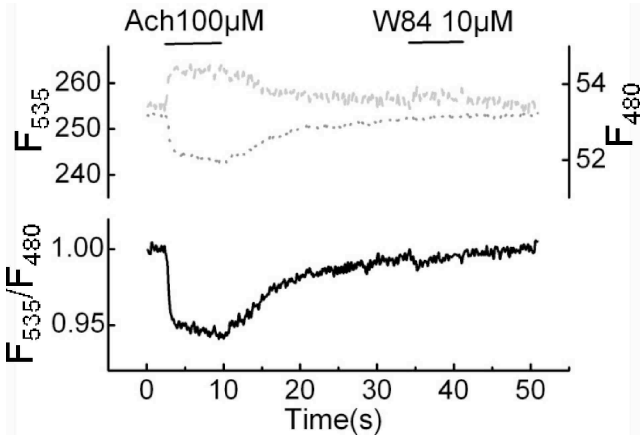


Figure 4.1.13 *Representative, corrected and normalized traces of FRET measurements with TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor for acetylcholine and dimethyl- W84 (cycle time 100 ms, illumination time 80 ms, n=6).*

In the next series of experiments the allosteric ligands were applied to the activated receptors, i.e. during their stimulation with acetylcholine or carbachol. In this case we could observe a reversible increase of FRET for all three allosteric ligands (Figures 4.1.14-4.1.16), suggesting that the allosteric ligands might induce a conformational change in the agonist-activated

receptor. However, the intensity of the observed FRET-changes were different for the various allosteric ligands, which might be the case if the changes in receptor conformation were different for every ligand. To test this hypothesis experimentally we, first, applied all allosteric ligands at sub-saturating concentrations of 10 µM to compare their effects on the acetylcholine (A) and carbachol (B) stimulated receptors. Under these conditions the weakest FRET signal was observed for tacrine, which showed only a change of about 10 % and 30 % when added on top of acetylcholine and carbachol, respectively (Figure 4.1.14).

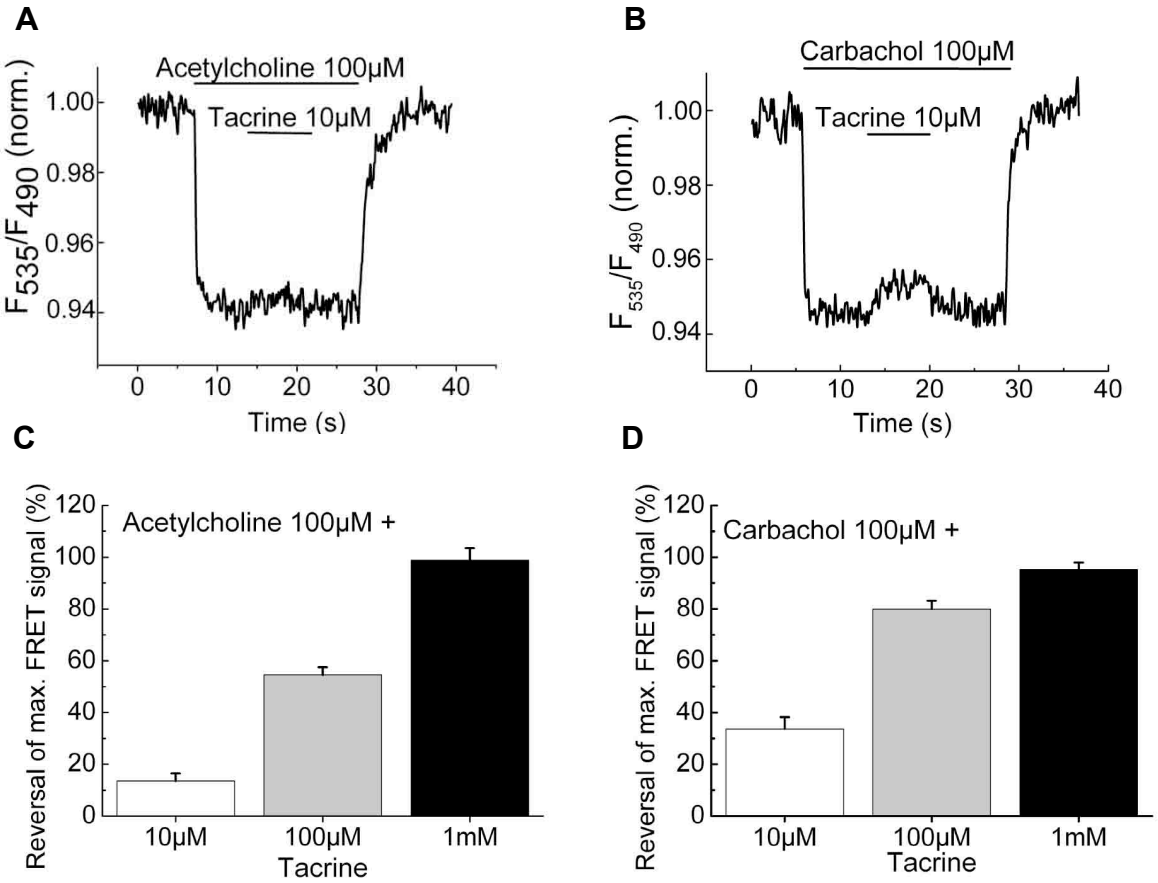


Figure 4.1.14 *Representative, corrected and normalized FRET traces with TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor for* ***A*** *acetylcholine with tacrine and* ***B*** *carbachol with tacrine. Columns for* ***C*** *acetylcholine and* ***D*** *carbachol show the amplitudes of the FRET-signals induced with different concentrations of tacrine (cycle time 100 ms, illumination time 80 ms, for C, D data are shown as means ± SEM, n=5-7).*

Gallamine at 10 µM inhibited the agonist-induced FRET signals by about 20 % in case of acetylcholine and by 40 % for carbachol (Figure 4.1.15).

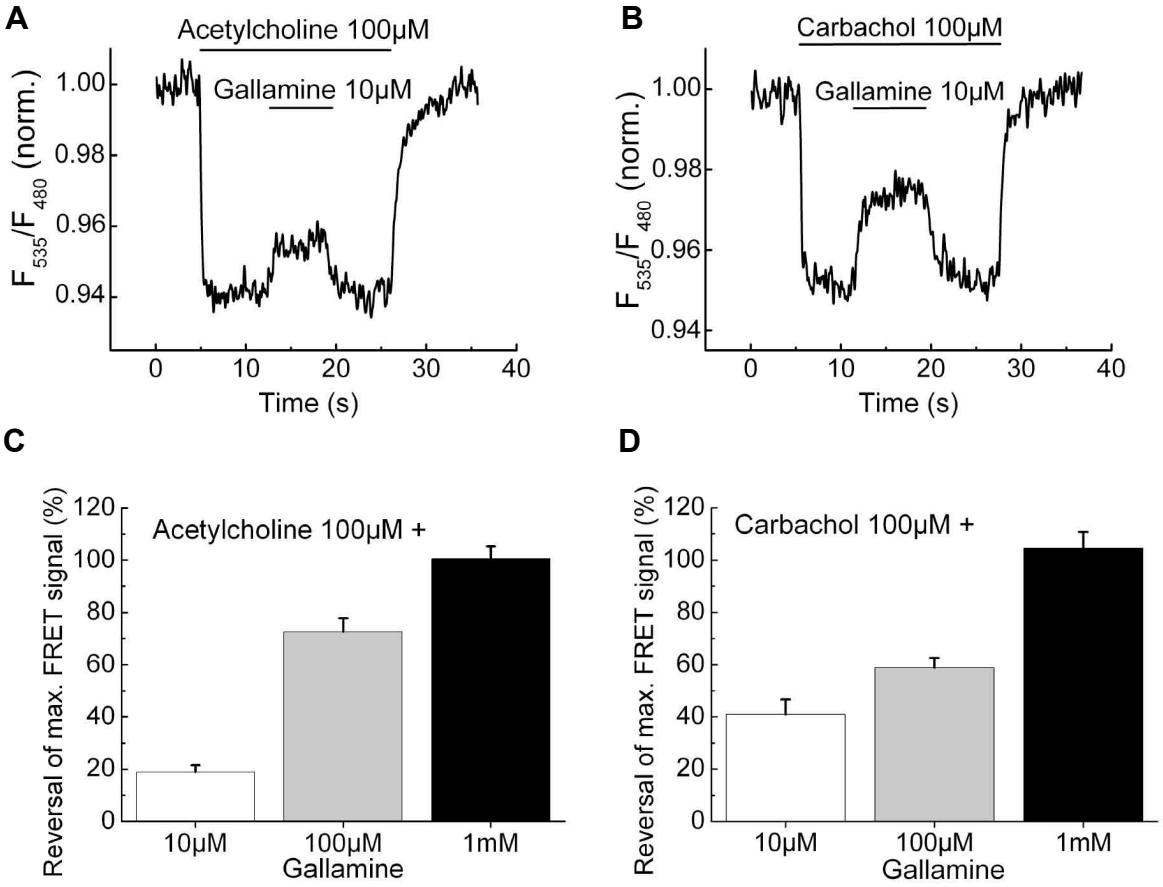


Figure 4.1.15 *Representative, corrected and normalized FRET traces with TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor for* ***A*** *acetylcholine with gallamine and* ***B*** *carbachol with gallamine. Columns for* ***C*** *acetylcholine and* ***D*** *carbachol show the amplitudes of the FRET-signals induced with different concentrations of gallamine (cycle time 100 ms, illumination time 80 ms, for C, D data are shown as means ± SEM n=5-9).*

The biggest change of FRET among all allosteric antagonists was observed for dimethyl-W84. Already at a concentration of 1 µM it inhibited the agonist-evoked FRET-signal by 25 % when given on top of acetylcholine and by about 40% with carbachol (Figure 4.1.16).

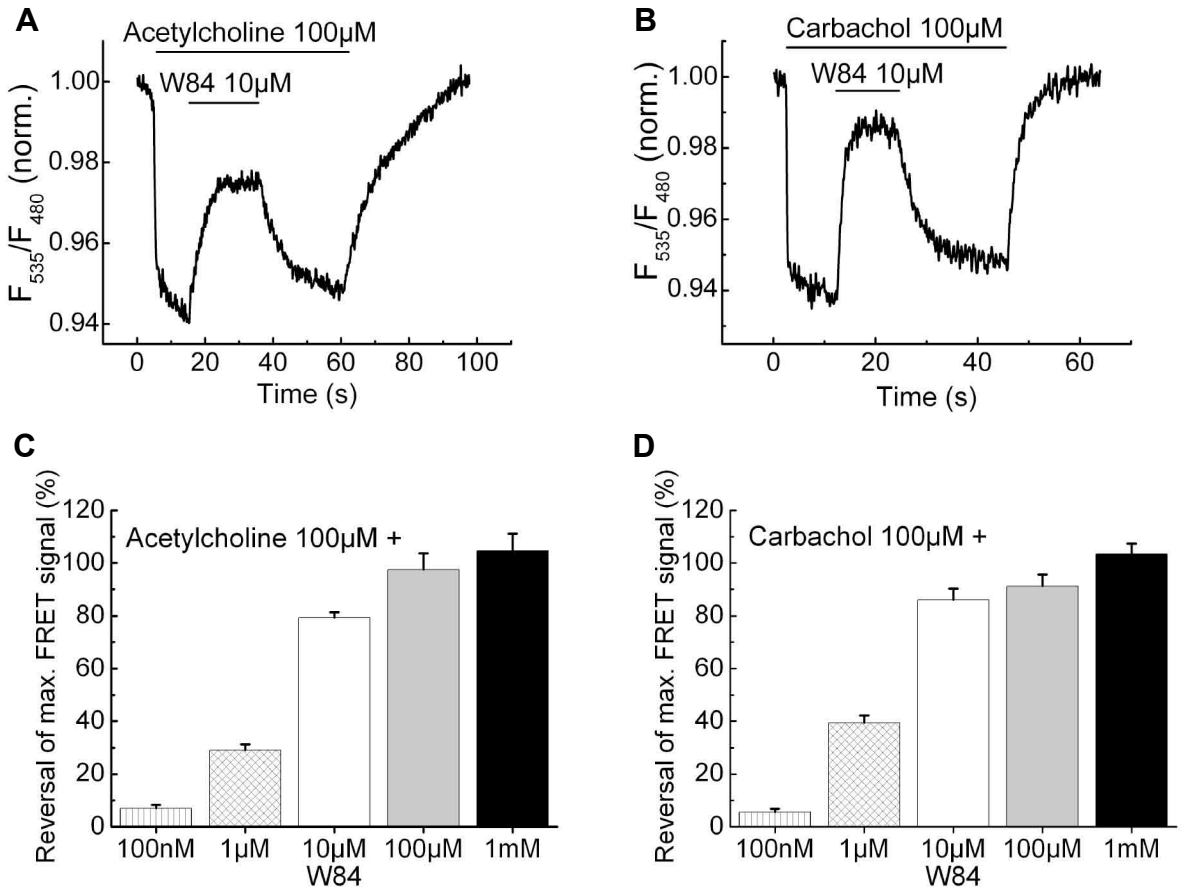


Figure 4.1.16 *Representative, corrected and normalized FRET traces with TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor for **A** acetylcholine with dimethyl-W84 and **B** carbachol with dimethyl-W84. Columns for **C** acetylcholine and **D** carbachol show the amplitudes of the FRET-signals induced with different concentrations of dimethyl-W84 (cycle time 100 ms, illumination time 80 ms, for C, D data are shown as means ± SEM, n=5-7).*

The amplitudes of the FRET signals evoked by tacrine and other allosteric ligands were concentration-dependent, showing a complete inhibition if the agonist effects at 100 µM to 1 mM of the allosteric antagonists (Figure 4.1.12-4.1.15, C and D).

In summary, these measurements indicate that allosteric antagonists show no effect when applied to non-stimulated receptors but rapidly decrease the FRET signal in presence of orthosteric agonists. The inhibitory effects of allosteric ligands are concentration-dependent and vary in their potencies depending on the nature of the allosteric ligand.

4.1.4.4 Kinetic measurements

Using the FRET-based sensor designed in the present study, for the first time, it became possible to measure the real-time kinetics of allosteric receptor modulation. These measurements should reveal whether there are any differences between orthosteric and allosteric ligands and among the allosteric ligands regarding the molecular mechanisms on the receptor. Both allosteric ligands and orthosteric antagonists alter the FRET-signal in a similar way: the FRET-change upon agonist addition is reverted corresponding to the switching of the receptor into some type of "inactive conformation". Conversely, receptor stimulation with full agonists results in a decrease of the FRET-signal, which shows the receptor switching into an "active conformation". To compare how different ligands act at the receptor the kinetics of the FRET signals induced by these various ligands were studied.

First, the correct concentration of antagonists had to be found which saturated the kinetics of receptor modulation (Figure 4.1.17). The measurements revealed that the maximal kinetics was achieved for atropine at the concentration of 10 μM, for methoctramine at 100 μM.

The allosteric ligands were taken at the highest concentration of 1 mM, because higher concentrations led to non-specific disturbances in the measurements. For gallamine, different concentrations were tested to show that the used concentration achieved the maximal speed (Figure 4.1.15). Whether gallamine behaves even faster at more than 1 mM could not be tested because problems with the FRET-measurements occurred at concentrations higher than 1 mM.

We measured the off-kinetics for FRET-signals induced by these ligands by calculating the time constants of the FRET-signal responses after addition of the ligand (as displayed in Figure 4.1.19). The kinetics of the ligands were studied upon application at saturating concentrations on top of either acetylcholine or carbachol (Figure 4.1.18). The obtained time constants are shown in Table 3.

τ (ms)	Acetylcholine	Carbachol
Atropine	677 ± 68	494 ± 20
Methoctramine	439 ± 70	373 ± 33
Tacrine	444 ± 74	340 ± 48
Dimetyl-W84	212 ± 46	175 ± 30
Gallamine	87,2 ± 6,6	105 ± 17

Table 3 *Receptor time-constants calculated from kinetic FRET-measurements for different antagonists and allosteric ligands from at least 5 different experiments per ligand (data are shown as means ± SEM)*

Gallamine, tacrine and dimethyl-W84 revealed different kinetics of receptor deactivation. Gallamine and dimethyl-W84 showed surprisingly rapid kinetics, which were significantly faster than the effects of the receptor antagonists atropine and methoctramine and still faster than the washout kinetics. Tacrine, however, did not have a significantly faster kinetics than the orthosteric antagonists (Table 3).

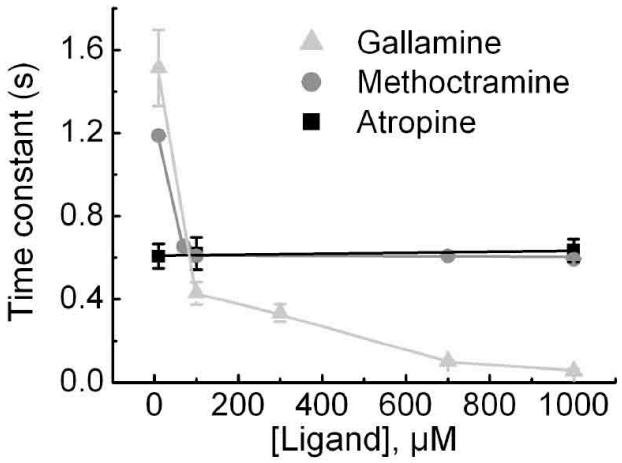


Figure 4.1.17 *Time constant curve calculated from the time constants of kinetic FRET measurements with TsA201 cells transiently transfected with the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor. Different concentrations for atropine, gallamine and methoctramine were used, for each concentration, at least 5 experiments were performed to obtain representative values. Kinetic saturation is reached with c_{Atr} =10 µM, c_{Gall}=1 mM and c_{Meth}=100 µM. These concentrations were used to perform kinetic measurements shown in Figure 4.1.18 (Data are shown as means ± SEM).*

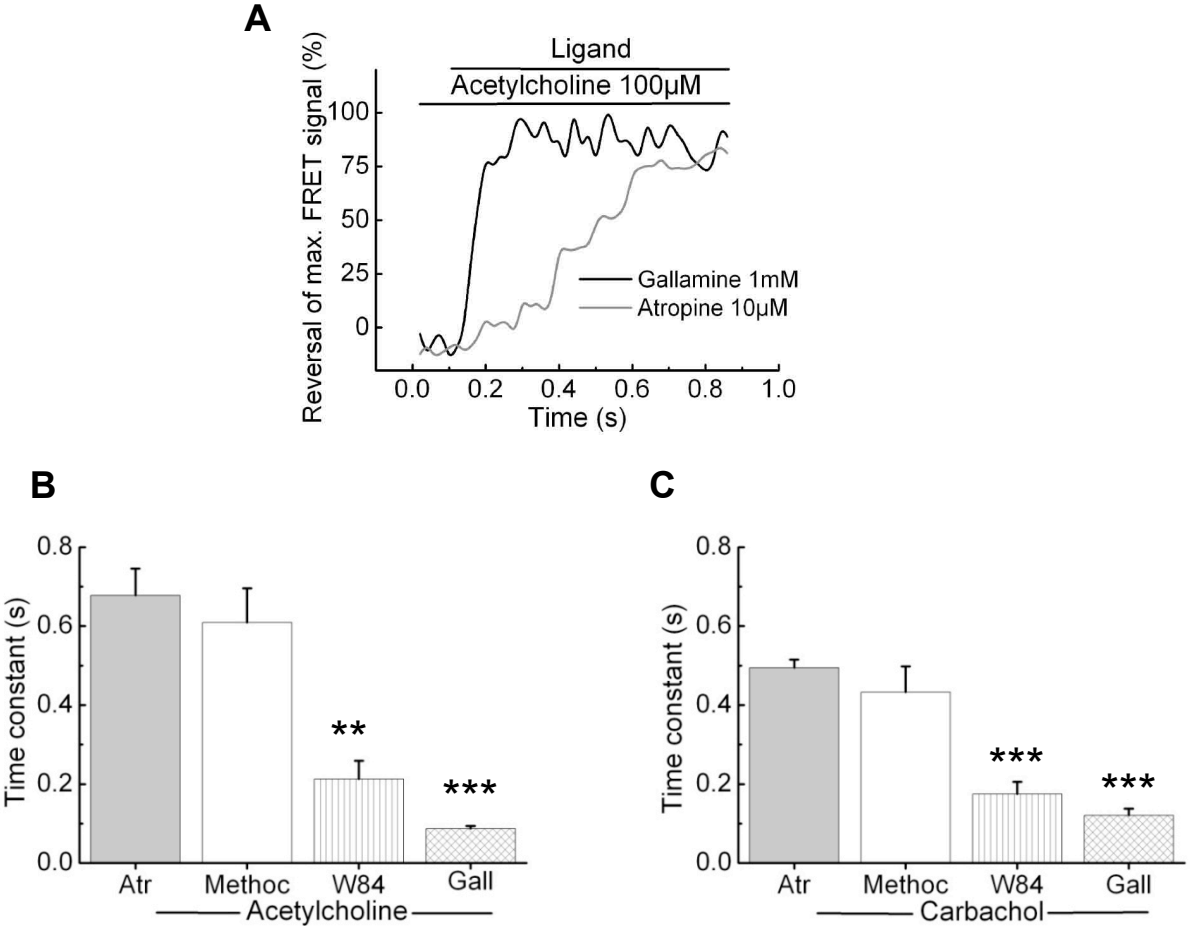


Figure 4.1.18 *Kinetics of FRET-changes induced by different muscarinic M_2-receptor ligands added in saturating concentrations. TsA201 cells were transiently transfected with the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor, for the measurements cycle times of 50 ms (gallamine) or 100 ms (other ligands) and illumination times of 40 ms(gallamine) or 80 ms (other ligands) were applied.* ***A*** *Kinetics of inhibition of the acetylcholine stimulated FRET-signal by gallamine and atropine in direct comparison. For column diagrams acetylcholine* ***B*** *and carbachol* ***C*** *were applied at 100 µM and the kinetics of the FRET-response upon addition of the antagonists atropine (10 µM, grey bars) and methoctramine (100µM, white bars) were measured. Allosteric ligands were used at 1mM on top of the orthosteric agonists.*

Time constants in ***B*** *and* ***C*** *are shown as means ± SEM and were determined as τ =63% of the FRET signals (n=6-9). Statistical significance of the differences was assessed using a T-test; *** p<0,001*

The kinetic traces for all ligands were accurately fitted using a monoexponential function (Figure 4.1.19).

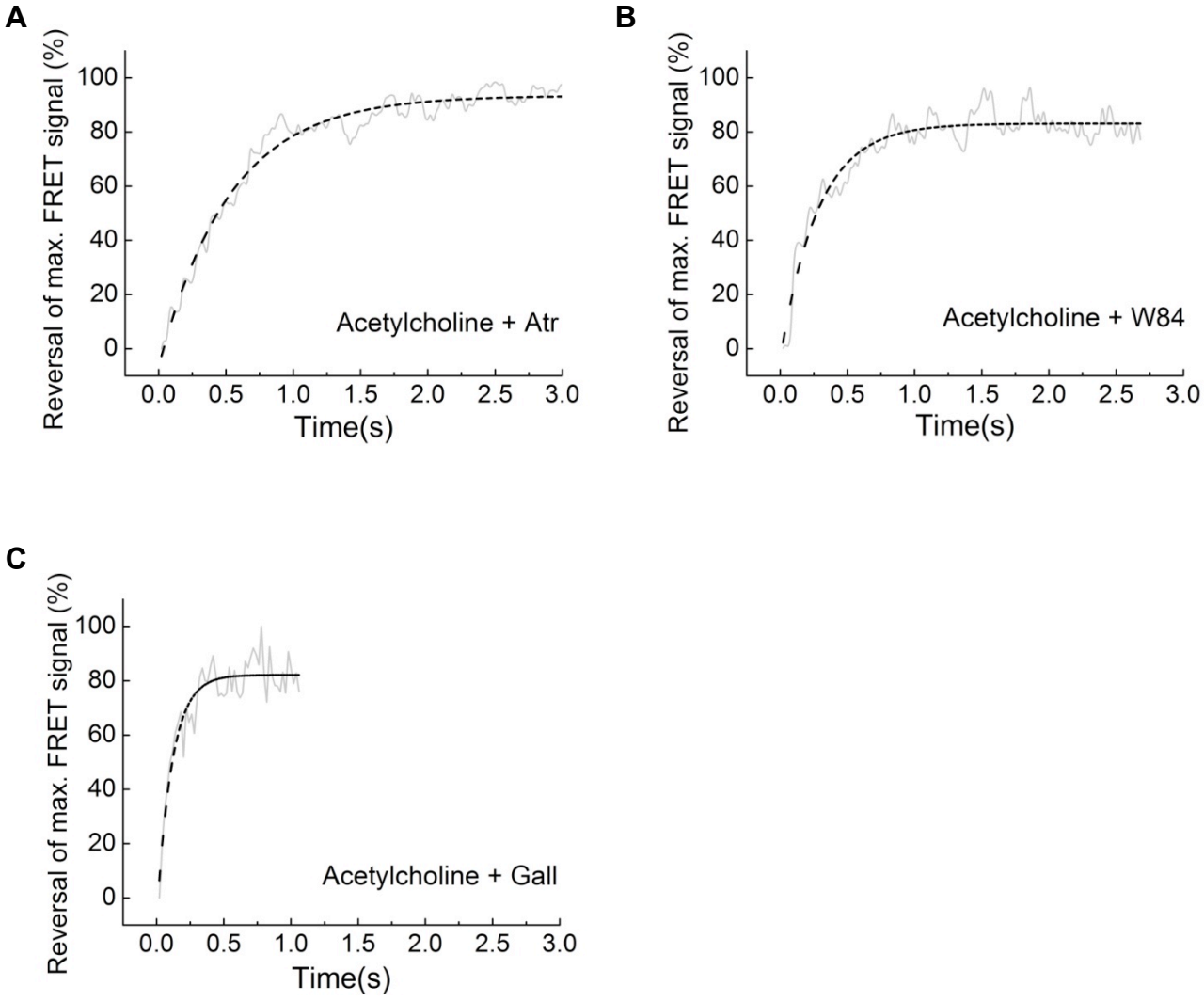


Figure 4.1.19 *Representative individual FRET traces obtained from kinetic FRET-measurements for* ***A*** *atropine (10µM),* ***B*** *dimethyl-W84 (1mM) and* ***C*** *gallamine (1mM) applied with 100 µM acetylcholine and fitted with a monoexponential function to estimate the τ values shown in Figure 4.1.18. Experiments were performed in TsA201 cells transiently expressing the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor. Cycle time and illumination time were 50 ms and 40 ms for gallamine and 100 ms and 80ms for atropine and dimethyl-W84, respectively (n=6-9).*

Some alkane-bis ammonium compounds similar in structure to dimethyl-W84 have been reported to interact with acetylcholinesterases (Trankle et al. 1998). To exclude that such effects interfere with the kinetic measurements, a batch of transfected cells were pre-incubated with physostigmine to block esterase activity. Those cells were then measured with 100 µM acetylcholine and 1mM dimethyl-W84. Obtained kinetics were similar to previously measured kinetics for non-physostigmin treated cells with τ values of 282 ± 63 ms without preincubation and 288 ± 40 ms with previous physostigmine incubation (Figure 4.1.20).

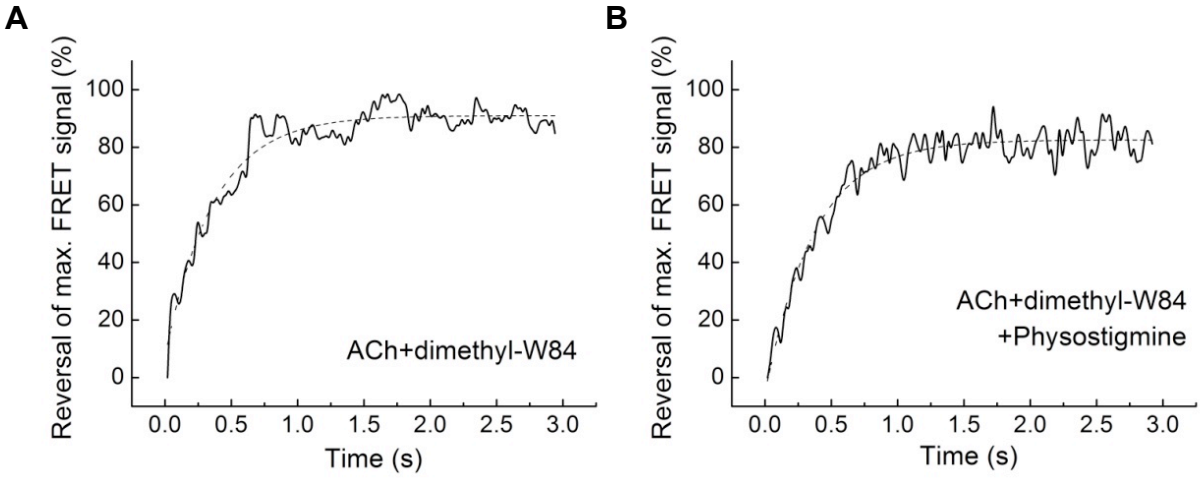


Figure 4.1.20 *Representative kinetic FRET-traces for with dimethyl-84 with (**B**) and without preincubation with physostigmine (**A**). Preincubation with 10µM of the esterase inhibitor physostigmine (2 h, 37°C) has no effect on the kinetics of the FRET response of dimethyl-W84. Measurements were performed as described in Figures 4.1.18 (n=3).*

Next, the observed rate constant values. k_{obs}, for atropine, methoctramine and gallamine were calculated and compared (Figure 4.1.21). They showed a dramatic difference between the kinetics for the antagonists and gallamine.

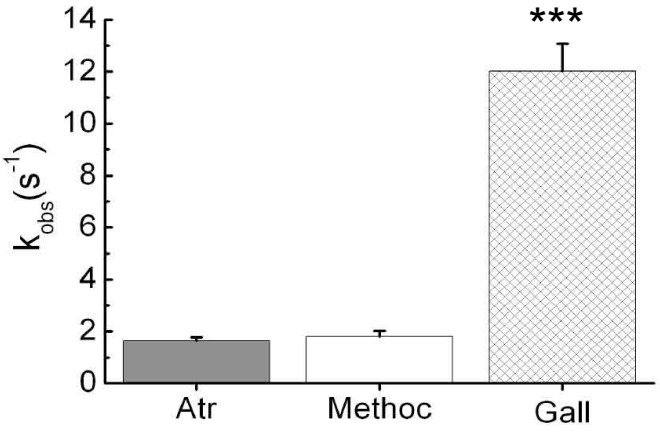


Figure 4.1.21 *k_{obs} diagram for methoctramine, atropine and gallamine calculated from obtained time-constants (Table 3) (all at 1mM) (data are shown as means ± SEM, n=6-9)*

The fact that the allosteric ligand inactivate the receptor much faster than orthosteric antagonists suggests that the allosteric ligands might actively induce receptor conformation. By this mechanism the allosteric ligand inhibit the activity of the receptor when applied in combination with receptor agonists.

4.2 M_4 mAChR

The M_4 mAChR (Figure 4.2.1), as described in the introduction has been suggested as a promising target for the treatment of schizophrenia. A lot of efforts are made at the moment to develop a functional allosteric enhancer for this receptor. In the present study it was attempted to generate an analogous muscarinic M_4-receptor sensor to analyze the action of such substances.

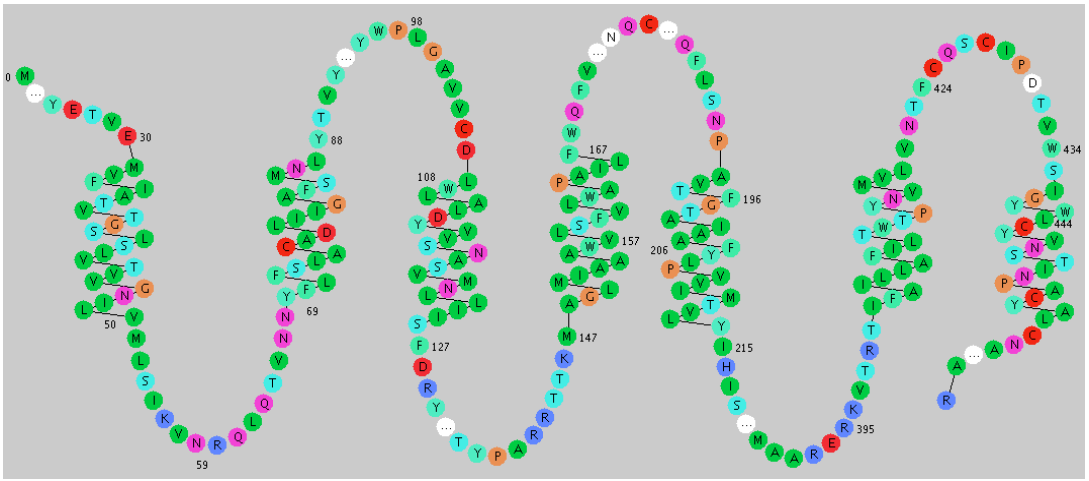


Figure 4.2.1 *Two-dimensional representation of the wild type muscarinic M_4-receptor*

1–31 N-Terminus, extracellular, 32–54 TMD1, 55–68 ICL 1, cytosolic; 69–89 TMD2; 90–106 ECL1, extracellular; 107–128 TMD3; 129–148 ICL2, cytosolic; 149–171 TMD4; 172–193 ECL2, extracellular; 194–216 TMD5; 217–401 ICL3, cytosolic; 402–422 TMD6; 423–436 ECL3, extracellular, 437–456 TMD7; 457–479 C-Terminus, cytosol

TMD: Transmembrane domain, ECL: extracellular loop, ICL: intracellular loop

(www.gpcr.org, www.uniprot.org/uniprot/P08173)

4.2.1 Cloning of a functional M_4-construct

Similar to the M_2 mAChR, it was attempted to generate a functional receptor sensor construct for the FRET-measurements.

4.2.1.1 First attempts

As previously described for the M_2 mAChR, cell surface expression is a crucial parameter for the success of FRET experiments. Since the M_4-receptor exhibits many structural similarities with the M_2-receptor (Figure 4.2.2), the first construct was designed in a similar way to the already functional FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor.

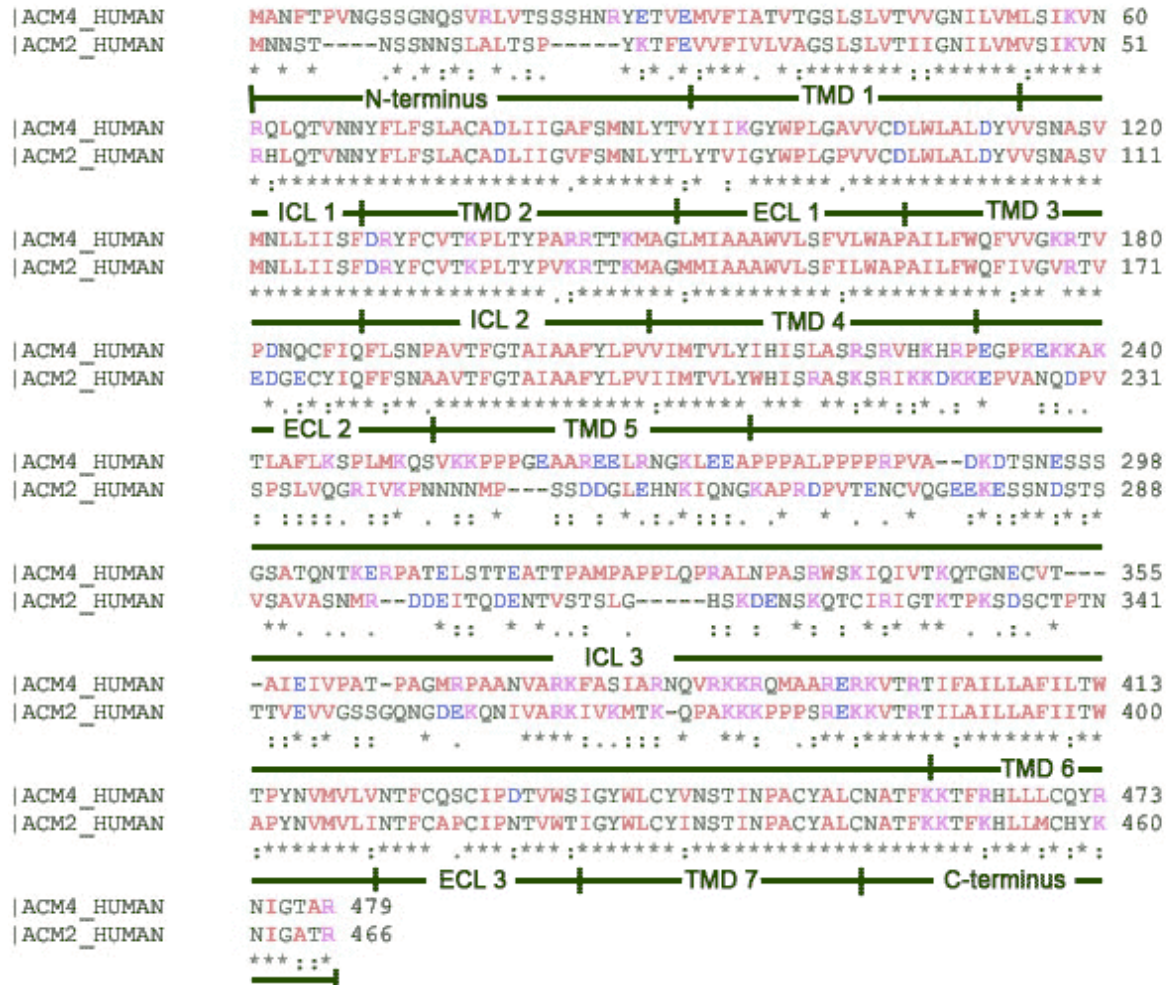


Figure 4.2.2 *Alignment of the human M_4- and the M_2-muscarinic receptor displays sequence similarities between the two proteins. The fully conserved residues, strong and weak conservation are marked as star, semicolon and colon, respectively (alignment created with www.ebi.ac.uk/clustalw/).*

TMD: Transmembrane domain, ECL: extracellular loop, ICL: intracellular loop

The receptor - G-protein activation sites in ICL3 (Okamoto and Nishimoto 1992) were preserved. The FLAG-motif was attached at the amino acid D2, the ICL 3 was shortened between amino acids K238 and I379, The FlAsH motif CCPGCC was inserted into the shortened loop. The five amino acid linker GSGEG was fused to the C-terminus after the amino acid R478. Unfortunately microscopic analysis revealed that this construct lacked the necessary cell surface staining.

Next, it was tried to leave the ICL 3 in its original length. The FlAsH motif was therefore positioned between amino acids K238 and A239. All other modifications were maintained leading to the construct FLAG-M_4-ll3-FlAsH-GSGEG-CFP.

This construct as well did also not provide sufficient cell surface expression. Using confocal microscopy the receptor was localized exclusively in intracellular vesicles.

Suspecting that the FlAsH motif might be the reason for the membrane trafficking problem, two YFP/CFP constructs were constructed. In both above constructs, FLAG-M_4-sl3-FlAsH-GSGEG-CFP and FLAG-M_4-ll3-FlAsH-GSGEG-CFP, the FlAsH motif was replaced by the YFP. Unfortunately, these constructs showed almost no receptor expression all.

4.2.1.2 Membrane expression

The difficulties with the membrane expression of the M_4-receptor seemed to exceed the problems with the M_2-muscarinic receptor. Personal communication with the group of Ralf Schülein, Leibniz-Institut für Molekulare Pharmakologie, Berlin, Germany, suggested that, similar to the M_2-receptor, expression of the M_4-receptor seems to undergo a very strict control mechanism in the endoplasmatic reticulum, so that only a small amount of the synthesized protein reaches the membrane. A further modification to bypass this mechanism might be provided with addition of the signal peptide of the endothelin receptor which is responsible for the trafficking the N-terminal tail of the endothelin-receptor B (ET_B) across the membrane of the endoplasmatic reticulum (Kochl et al. 2002).

Kochl et al described 26 amino acids at the N-terminus of the ET_B-receptor, which are crucial for trafficking of the receptor across the membrane of the ER.

We fused these 26 amino acids (MQPPPSLCGRALVALVLACGLSRIWG) to amino acid D2 in the M_4mAChR using a sequential PCR. This was performed with the described constructs FLAG-M_4-sl3-FlAsH-GSGEG-CFP and FLAG-M_4-ll3-FlAsH-GSGEG-CFP, resulting in EndBSigPep-M_4-sl3-FlAsH-GSGEG-CFP and EndBSigPep-M_4-ll3-FlAsH-GSGEG-CFP. Of

those two constructs, only the construct with the shortened ICL 3, EndBSigPep-M_4-sl3-FlAsH-GSGEG-CFP, exhibited improved membrane staining.

With this constructs, first FRET measurements could be performed. Unfortunately, the FRET-signal never exceeded a 3 % change, which was considered too low to monitor the changes brought about by allosteric ligands.

5 Discussion

5.1 Generation and optimization of a M_2 mAChR FRET-sensor construct

In recent years many FRET-based sensors for various GPCRs have been developed. However, no functional constructs for the muscarinic M_2- and M_4- receptors have been described. Despite being important targets for drug development, with M_2 for cardiac diseases and M_4 for Alzheimer's disease and schizophrenia, no real-time data on the mechanisms and kinetics of receptor activation and inhibition for these two receptors have been reported.

In the present study the first functional M_2 mAChR sensor was designed and optimized to meet several criteria, which would allow using it for measuring conformational changes in this receptor by FRET in living cells. Based on the secondary structure of the receptor, several modifications were been introduced to ensure reproducible measurements of receptor activation. Considering the amino acid composition and the two-dimensional representation of the M_2 mAChR, two issues get apparent:

1. With about 180 amino acids the ICL3 of receptor is very long (Figure 4.1.1) and

2. the C-terminal tail, consisting of only about 22 amino acids, is rather short, especially when compared to the other types of muscarinic receptors M_1, M_3 and M_5.

To achieve high FRET efficiency it is essential that the two fluorophores, which are attached to the receptor in the sensor construct, are in a proper spatial orientation and on optimal distance of about 5 nm. With a loop of about 180 amino acids length the distance between the more distant parts of ICL3 and the receptor C-terminus might be too large to allow for an effective energy transfer. Therefore, shortening of the loop without disturbing receptor functionality seemed to be essential to obtain an optimal sensor construct. Previous studies from our laboratory indicated the shortening of the ICL3 in e.g. α_{2A}-adrenergic receptor (Vilardaga et al. 2003) allowed to create a fully functional receptor sensor showing high FRET-efficiency and robust changes in FRET upon receptor activation.

These studies suggested also that the length of the C-terminal receptor tail might be an important factor for such a construct. Both subtypes of muscarinic receptors used in this study have a C-terminus of about 22 amino acids, which might be too short and due to that, after attachment of CFP, cell surface expression might be impaired. Moreover, the C-terminus of

these receptors harbors a palmitoylation site. Hayashi et al demonstrated that the putative palmitoylation site for the M_2-muscarinic receptor, C457, is also necessary for G-protein coupling to the receptor (Hayashi and Haga 1997), which might be disturbed when a fluorescent protein such as CFP is fused in its close proximity. For the neural cell adhesion molecule N-CAM, Little et al showed that the palmitoylation is needed to anchor the protein to the cell membrane (Little et al. 1998), and thus blocking this site with CFP could potentially interfere with the trafficking of the receptor to the plasma membrane. Considering these structural features of the receptors, it seemed reasonable to lengthen the C-terminal tail of both receptors with a flexible five amino acid linker. Both shortening of the ICL3 and elongation of the C-Terminus seemed to be justified modifications to obtain a functional sensor, which would deliver reproducible FRET-data.

With all mentioned optimizations of the construct, it was possible to create a functional construct that showed robust changes in FRET (Figure 4.1.8). However, the transfection levels per well transfected HEK TsA201 cells did not exceed 30%. Data from radioactive binding assays (Figure 4.1.4) revealed that the overall expression of the sensor was good, but the confocal microscopy showed that still too many receptor molecules remained in intracellular vesicles. The reasons for this remain elusive, though it has been known for several GPCR, including muscarinic M_2 and M_4 mACh receptors, that their expression is strictly controlled at the level of ER in a way that high percentages of newly synthesized receptors do not leave the ER due to these processes (Alken et al. 2009; Dong et al. 2007; personal communication with Ralf Schülein, Berlin). Taking into account that several modifications had to be done to the receptor construct, one could expect folding problems and thus deteriorated membrane trafficking of the receptor sensor.

Nevertheless addition of the HA signal peptide to the already used FLAG-epitope finally resulted in increased membrane trafficking of the receptor sensor. The robust FRET signal changes upon receptor stimulation remained unchanged by this alteration.

5.2 Functionality of the M_2 mAChR sensor

Since many alterations have been made to the primary structure of muscarinic M_2-receptor, the question arose whether this sensor still remained functional in terms of its ability to couple to its downstream signaling pathways. As mentioned before, the muscarinic M_2 and M_4 receptors predominantly activate the G_i proteins. To ensure that both M2-sensor constructs constructs are able to activate G-proteins several methods were used.

For the Flag-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor ERK 1/2 activation assays and patch clamp GIRK measurements were used.

The AlphaScreen ERK 1/2 assay, performed with transiently transfected HEK 293 cells and the Flag-M_2-sl3-FlAsH-GSGEG-CFP receptor sensor, failed to give any result. As the control batch with CHO cells stably expressing the M_2 wt mAChR gave normal results, possible reasons could be that the expression of the construct on the membrane of the HEK293 cells was insufficient for this assay. Furthermore, this ERK assay is quite susceptible to stress, for example due to harsh pipetting or sudden movements of the plate. Especially HEK cells seem to react quite sensitive to such treatment when used in ERK assays. Unfortunately, even the assays made with transiently transfected CHO cells did not improve the results.

While the ERK alpha assay performed in a batch of cells is typically dependent on sufficient membrane expression of the receptor, the second approach of measuring GIRK-channel currents by patch clamp recordings is based on single cell experiments. Thus, especially with the observed expression problems, this method seems to be more reliable in terms of functionality assessment, as receptor expression of the muscarinic receptors appears to be crucial for functional responses (Schwarz et al. 1993). The GIRK-channel measurements using the Patch Clamp technique showed efficient activation of the channels by the transiently transfected M_2-receptor sensor, so that reliable and reproducible experiments could be made. These experiments revealed an $EC_{50} \approx 100 \pm 7$ nM for acetylcholine (Figure 4.1.5). This value is comparable with data described by others for the wild type receptor in a similar experimental system (Ben-Chaim et al. 2003), which showed an $EC_{50} \approx 40$ nM for acetylcholine.

These measurements confirmed that the modifications made to obtain the muscarinic M_2-receptor construct Flag-M_2-sl3-FlAsH-GSGEG-CFP did not hinder the activation of G_i-proteins.

In line with these findings, the HA-Flag-M_2-sl3-FlAsH-GSGEG-CFP sensor also remained functional. Radioligand binding assays performed to determine ligand affinity confirmed that the affinities for the ligands carbachol and gallamine were comparable to those of the wild type M_2 mAChR (Figure 4.1.6).

After stimulation with carbachol, the receptor construct inhibited forskolin-induced cAMP production. This inhibitory response was affected in presence of gallamine and the result was also comparable with the wild type M_2 mAChR (Figure 4.1.7).

Summarizing the results of the functionality assays it can be noted that the structural alterations made on both receptor sensor constructs did not change receptor functionality in terms of receptor expression, ligand affinity and downstream-signaling. Thus it can be assumed that results obtained in the following FRET-experiments reflect the behavior of the M_2 wt mAChR.

5.3 FRET-measurements with orthosteric ligands

The full agonists acetylcholine and carbachol were chosen for the FRET-measurements to monitor the conformational changes in the Flag-M_2-sl3-FlAsH-GSGEG-CFP sensor during receptor activation. Both agonists have been well described under various experimental conditions. Upon addition of acetylcholine and carbachol to TsA201 cells transiently transfected with this receptor sensor, a rapid, reversible decrease of the FlAsH/CFP FRET-ratio was observed. The speed of the receptor activation measured in such experiments was $\tau = 113 \pm 21$ ms for acetylcholine and $\tau = 126 \pm 33$ ms for carbachol. These agonist-induced FRET-signals were concentration-dependent. The concentration-response curves showed the following EC_{50}-values: $EC_{50} \approx 1.86 \pm 0.3$ µM for acetylcholine and $EC_{50} \approx 5.22 \pm 0.4$ µM for carbachol (Figure 4.1.9). These values are in good agreement with already published IC_{50} data for the wild type M_2-receptor using [^{3}H]NMS in competitive binding assays (Maggio et al. 1993; May et al. 2007a). One possible explanation for the slight differences might be different expression levels, which, due to various amounts of spare receptors, are crucial for such concentration-response dependencies. These experiments confirm that the FLAG-M_2-sl3-FlAsH-GSGEG-CFP sensor reacts to full agonists in a manner comparable with the wild type receptor.

Next, FRET measurements were performed for the antagonists atropine and methoctramine. Both antagonists are well described in the literature. While atropine has been characterized as a non-selective muscarinic receptor antagonist, methoctramine exhibits certain selectivity for the M_2 receptor subtype (Eglen and Harris 1993; Melchiorre et al. 1987; Wess et al. 1988). In FRET experiments the antagonist atropine behaved as expected and showed a complete inhibition of the agonist-induced FRET signals (Figure 4.1.10 A,B). Atropine completely reversed the agonist induced FRET-signal when given at saturating concentrations. The kinetics of this effect were significantly faster than the washout kinetics, (Figure 4.1.10 C,D). Interestingly, the high affinity of both antagonists also resulted in a persistent inactivation of

the receptor. No further stimulation with agonists was possible for up to 10 min after treatment with 10 μM atropine or 100 μM methoctramine.

5.4 FRET-measurements with allosteric ligands

5.4.1 Allosteric ligands induce conformational changes in the receptor

Allosteric modulators of GPCRs have been described as "ligands that bind to an allosteric site on a GPCR to modulate the binding and/or signaling properties of the orthosteric site" (May et al. 2007b). In this study, for the first time allosteric ligands were characterized using FRET and a live-cell imaging method. This method enables to monitor changes in receptor conformation and also to determine kinetic time-constants for this receptor switch.

First, it had to be determined whether the M_2 mACR sensor can be utilized to study allosteric receptor modulation. In this study gallamine, tacrine and dimethyl-W84, which are very well characterized negative allosteric modulators of muscarinic receptors, were used. Dimethyl-W84 and gallamine have been described as relatively selective ligands for the M_2-receptor, whereas tacrine is regarded to be a rather unselective allosteric ligand for all muscarinic receptor subtypes (Holzgrabe et al. 2000; Huang et al. 2005; Szilagyi and Lau 1993; Trankle et al. 2005).

As expected for allosteric ligands in general, all three ligands did not change the FRET-signal in the absence of an agonist. (Figures 4.1.12-4.1.13). After addition of an agonist, all used allosteric ligands were able to reverse the FRET-signal induced by an agonist in a concentration-dependent manner, indicating that the active receptor conformation changes was due to binding of an allosteric ligand to the activated receptor. This inhibition was dependent on the nature of the allosteric ligand and the used concentration, but it always reached the maximum FRET-signal inhibition for every ligand at high concentrations (Figures 4.1.14-4.1.16 C,D).

The inhibition of the active receptors by allosteric ligands seen in these experiments could be explained with an active switch due to binding of the allosteric ligand. This induced conformational change would then lead to a receptor conformation with lower affinity for the agonist or with a lower overall affinity. This mechanism has been suggested in previous publications (Christopoulos and Kenakin 2002; May et al. 2007b) and it also agrees with the allosteric two-state model. Until now it has remained unclear whether this switch in the

conformation is directly induced by binding of allosteric ligands or whether the receptor first has to modulate binding of the orthosteric agonist/antagonist, for example by removing it from the binding pocket. To distinguish between these two possibilities and to delineate the mechanisms how allosteric ligands act at the receptor, kinetic measurements of their FRET signals were performed.

5.4.2 Kinetics of the FRET responses for allosteric ligands

Allosteric ligands are known to bind to an allosteric site, which is distinct from the orthosteric site. To determine, whether the allosteric ligands affect the agonist kinetics of antagonist and allosteric binding were compared.

If allosteric ligands can bind to the receptor and actively induce distinct conformational states, the kinetics of the FRET signals might be faster than the kinetics of the antagonists, which first needs displacement of the agonist from the orthosteric site and then drive the receptor into its inactive conformation.

As indicated in "Results" (Figure 4.1.18), the allosteric ligands gallamine and dimethyl-W84 exhibited significantly faster kinetics than methoctramine and atropine reversing agonist-signals. Interestingly, slight differences in the kinetics for the allosteric ligand dimethyl-W84 were observed when the receptor was activated by two different agonists, carbachol and acetylcholine (Figure 4.1.18 B,C). A possible explanation for this might be the higher affinity of acetylcholine compared to carbachol, which might prevent a faster change of the signal due to more stable binding effects, which also could affect the speed of the conformational change. The rate constants of dimethyl-W84 were $\tau = 212 \pm 46$ ms with acetylcholine and $\tau = 175 \pm 30$ms with carbachol. This is also in agreement with the assumption that acetylcholine binds with a higher affinity to the orthosteric binding site of the recepor which could hinder conformational changes brought about by dimethyl-W84. Furthermore acetylcholinesterases, as described for alkane-bis-ammonium compounds by Ohnesorge et al. (Ohnesorge 1969), do not influence the outcome of dimethyl-W84 kinetic measurements. Whereas in radioligand binding experiments the incubation time is in the minute range, FRET-experiments take place in the second to millisecond range. In addition, kinetic measurements were performed at saturating ligand concentrations. According to that, the effect of acetylcholine esterases in FRET-measurements seems to be negligible, see Figure 4.1.20.

Gallamine seems to have a very interesting behavior regarding its kinetics (Figure 4.1.21). Despite having a lower affinity, its kinetics for both orthosteric agonists were even faster than

those of dimethyl-W84 (τ = 87.2 ± 6.6 ms with acetylcholine and τ = 105 ± 17 ms with carbachol, see Table 3). This difference in the kinetics might be due to sterical hindrances, since dimethyl-W84 is a much bulkier molecule. The obtained time-constant values for gallamine and dimethyl-W84 might suggest that allosteric modulation results in actively induced conformational changes in the receptor. In contrast to gallamine and dimethyl-W84, tacrine did not exhibit faster kinetics than the compared antagonists. Before allosteric receptor modulation had been extensively studied, tacrine had been considered as a muscarinic receptor antagonist (Pearce and Potter 1988). It is also known that it inhibits the acetylcholine esterase and it was therefore used in Alzheimer treatment (Greenlee et al. 2001). In literature, tacrine is described as "atypical allosteric ligand" (Gregory et al. 2007; Trankle et al. 2005) due to its binding to a "non-classical" allosteric site. This atypical binding profile might explain the findings in this study. Personal communication with Arthur Christopoulos, Monash University Melbourne, indicated that the role of tacrine as an allosteric ligand is not yet clear and that some of its behaviors, such as a steep concentration-effect curve (slope factor >1), indicate additional binding features.

5.5 Generation of the M_4 mAChR sensor construct

Unfortunately it was not possible to generate a functional M_4-receptor sensor during this study. Despite the structural similarities between the M_2 and the M_4 mAChR, the construct of a similar design, which was developed for the M_2-receptor, did not work for the M_4-receptor.

In addition, only very few mutational and functionality data exist for the M_4-receptor, so that the available data and extrapolation onto the M_2-receptor were considered when designing the constructs. Van Koppen et al showed that a M_4-receptor construct with a shortened ICL3 with deleted amino acid residues between R263 and E395 could be expressed in CHO cells in comparable amounts to the wild type M_4 mAChR and the binding studies with [^{3}H]-QNB revealed sufficient membrane expression (Van Koppen et al. 1994). According to that it can be speculated that the problems with the generation of a functional M_4-receptor-construct and its low poor expression are related to the presence of the CFP on the C-terminus or an insertion of the FlAsH motive into the ICL3.

6 Summary

Allosteric modulators have been proposed as promising new compounds to modify protein function. Allosteric binding sites have been discovered for several G-protein-coupled receptors, including M_{1-5} muscarinic receptors. Since these receptors play a pivotal role in the regulation of a plethora of organ functions, it is particularly important to investigate the mechanisms of allosteric modulation.

To study molecular mechanisms of allosteric modulation in the M_2 muscarinic receptor, a new FRET-based sensor was designed. CFP fused to the C-terminus of the receptor and a small fluorescent compound FlAsH, which labels a specific binding sequence in the third intracellular loop, were used as donor and acceptor fluorophores, respectively.

The first part of the study was to design a functional FRET receptor sensor. After several optimization steps the constructs FLAG-M_2-sl3-FlAsH-GSGEG-CFP and HA-FLAG-M_2-sl3-FlAsH-GSGEG-CFP were generated which showed good cell-surface expression, robust changes in FRET and the ability to deliver reproducible data. The second part of this thesis sought to elucidate the mechanisms of the allosteric ligand binding and their effects on the receptor conformation.

The described modifications, which were introduced in the wild type M_2 mAChR to create the FRET sensor can alter receptor functionality and influence receptor expression. Radioligand binding studies revealed that the used transfection method provided sufficient receptor expression but, unfortunately, about 60 % of the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor remains in the cytosol. However, this was sufficient to perform FRET experiments. Patch clamp GIRK-measurements with acetylcholine evinced that the new M_2-sensor was able to activate G_i-proteins. Also, radioligand-binding assays with the second construct HA-FLAG-M_2-sl3-FlAsH-GSGEG-CFP showed ligand affinity comparable to the wildtype receptor. Furthermore inhibition of forskolin-stimulated cAMP production was indistinguishable from the behaviour of the wildtype receptor. According to that, the full functionality of both receptor constructs could be confirmed.

FRET measurements with the full muscarinic receptor agonists carbachol and acetylcholine confirmed that the FLAG-M_2-sl3-FlAsH-GSGEG-CFP receptor construct showed rapid changes in FRET upon addition of both ligands, which were concentration-dependent. Concentration response curves and the resulting EC_{50} values of both agonists were similar to those already published in literature. In addition, the orthosteric antagonists atropine and

methoctramine inhibited the FRET changes induced by the agonists. This inhibition was significantly faster than the washout kinetics, pointing to an active displacement of the agonists by the antagonists.

Allosteric ligands gallamine, tacrine and dimethyl-W84 did not alter receptor conformation when added without an orthosteric ligand. However, when applied in addition to muscarinic agonists, all three substances inhibited the FRET-signal. The extent of this inhibition was dependent on the used concentration of the allosteric ligands. These results reveal that conformational changes brought about by allosteric ligands can be measured with the FRET technique.

Furthermore real-time FRET-based kinetic measurements could be performed in living cells and showed that the allosteric ligands gallamine and dimethyl-W84 alter receptor conformation significantly faster than the antagonists atropine and methoctramine. This data indicate that allosteric ligands actively induce the conformational changes in the receptor.

7 Zusammenfassung

Allosterische Modulatoren, welche es ermöglichen die Funktionen einiger Proteine zu verändern, scheinen eine vielversprechende neue Substanzgruppe zu sein. Bereits bei mehreren GPCR konnten allosterische Bindungsstellen identifiziert werden, so auch in der Gruppe der muscarinischen Acetylcholin-Rezeptoren. Da gerade diese Rezeptorgruppe eine große Rolle im Organismus spielt und dort viele verschiedene Organfunktionen beeinflusst, stellt das Aufdecken der Mechanismen allosterischer Liganden gerade hier ein wichtiges Ziel dar.

Um die Mechanismen allosterischer Bindung am M_2-muscarinergen Rezeptor auf molekularer Ebene zu untersuchen, wurde ein FRET-Rezeptor Sensor hergestellt. Die GFP-Mutante CFP wurde dazu mit dem C-terminalen Ende des Rezeptors verbunden, und die Bindungssequenz für das FlAsH-Molekül, ein sogenannter Hairpin-binder, wurde in die Aminosäuresequenz der dritten intrazellulären Schleife eingebracht. Diese beiden Fluorophore fungieren als Donor und Akzeptorpaar im FRET-Sensor-Konstrukt.

Da es bis jetzt keine Daten zu einem solchen muscarinischen FRET-Rezeptor-Sensor gibt, musste zunächst ein funktionelles Konstrukt erstellt werden. Nach Optimierung von Expression und FRET-Signalstärke gelang es mit den Konstrukten FLAG-M_2-sl3-FlAsH-GSGEG-CFP und HA-FLAG-M_2-sl3-FlAsH-GSGEG-CFP zwei Sensoren herzustellen, welche den nötigen Anforderungen entsprechen.

Im zweiten Teil der Doktorarbeit sollten dann die Mechanismen der allosterischen Rezeptormodifikatoren genauer beleuchtet werden.

Durch die extensive Modifikation am M_2 mAChR Konstrukt musste zunächst, die Funktionalität des Rezeptors sichergestellt werden. Durch Radioliganden-Bindungs-Experimente stellte sich heraus, dass nach Transfektion der Rezeptor sehr gut in der Zelle exprimiert wird, doch zu 60% im Zellinneren verbleibt. Dies reichte jedoch aus, um FRET-Experimente durchzuführen. Messungen mit der Patch-Clamp Methode zeigten, dass der M_2-Rezeptor Sensor in der Lage ist, G_i-Proteine zu aktivieren. Außerdem zeigten Radioliganden Bindungs Assays, dass die Liganden Affinität des zweiten Konstruktes HA-FLAG-M_2-sl3-FlAsH-GSGEG-CFP vergleichbar mit der des Wildtyp-Rezeptors ist. Desweiteren ist das Konstrukt weiterhin in der Lage das FRET-Signal von Forskolin-stimulierten cAMP zu inhibieren.

Somit scheint die Funktionalität beider Rezeptorkonstrukte, trotz Modifikationen, erhalten geblieben zu sein.

FRET-Experimente unter Zugabe der Agonisten Acetylcholin und Carbachol zeigten auf, dass das FLAG-M_2-sl3-FlAsH-GSGEG-CFP Rezeptor-Konstrukt konzentrationsabhängige und schnelle Änderungen des FRET-Signals aufweist. Auch die dabei ermittelten Werte der Konzentrations-Wirkungs-Kurven stimmten mit bereits veröffentlichten Werten überein.

Die Antagonisten Atropin und Methoctramin führten zu einer Blockade des durch den Agonisten induzierten Signals, welches gleichzeitig schneller als das Auswaschen des Agonisten war.

Alleinige Zugabe der gewählten allosterischen Liganden, Gallamin, Tacrin und Dimethyl-W84 änderte das FRET-Signal nicht. Erst durch vorherige Stimulation mit einem orthosterischen Agonisten waren sie in der Lage, das FRET-Signal des Agonisten zu blockieren. Die Stärke dieser Inhibition hing von der Konzentration des jeweiligen allosterischen Liganden ab.

Diese Ergebnisse offenbaren, dass die Konformationsänderungen des Rezeptors nach Zugabe eines allosteren Liganden mit der FRET-Methode gemessen werden kann.

Darüber hinaus konnte durch kinetische Messungen aufgedeckt werden, dass die allosteren Liganden Gallamin und W84 die Konformation des Rezeptors schneller ändern, als die Antagonisten Atropin und Methoctramin.

Dieses Ergebnis bekräftigt die Annahme, dass allosterische Liganden aktiv eine bestimmte Konformationsänderung des Rezeptors herbeiführen.

8 Appendix

8.1 Abbreviations

Å	Ångström, internationally recognized unit of length (0.1 nm)
Aβ	Amyloid-β peptide
AC	Adenylate cyclase
AChEI	Achetylcholine esterase inhibitor
AD	Alzheimer's disease
APP	Amyloid precursor protein
ATCM	Allosteric ternary complex model
ATSM	Allosteric two-state model
ATP	Adenosine-tri-phosphate
bp	Base pairs
BRET	Bioluminescence resonance energy transfer
cAMP	Cyclic adenosine-mono-phosphate
cDNA	Complementary desoxyribonucleic acid
CFP	Cyan fluorescent protein
C-terminus	End of a protein terminated by a free carboxyl group
CTM	Cubic ternary receptor model
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP's	Mixture of the deoxyribonucleotides dATP, dGTP, dCTP, dUTP
EC_{50}	Half maximal effective concentration
ECFP	Enhanced cyan fluorescent protein

ECL 1/2/3	Extracellular loop 1-3
EDT	Ethandithiol
EMEA	European medicines agency
Epac	Exchange protein directly activated by cAMP
ETM	Extended two state model
EYFP	Enhanced yellow fluorescent protein
FCS	Fetal calf serum
FDA	Food and drug administration
FlAsH	Fluorescein-based arsenical hairpin binder
FLIM	Fluorescence lifetime imaging microscopy
FP	Fluorescent protein
FRET	Fluorescence resonance energy transfer
G_s, G_i, G_o,G_q	Different subtypes of the G-protein α-subunit
GFP	Green fluorescent protein
GIRK	G-protein coupled inwardly rectified potassium channel
GIT	Gastrointestinal tract
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
[^{3}H]NMS	Radioactively tagged N-metylscopolamine
[^{3}H]QNB	Radioactively tagged 3-Quinuclidinyl benzilate
HBSS	Hank's buffered salt solution
ICL 1/2/3	Intracellular Loop 2/3
IP_3	Inositol-1,4,5-triphosphate
IUPHAR	International Union of Pharmacology
KO-mouse	Knock Out mouse, mouse lacking a certain genetic information
LB medium	Lysogeny broth medium

L-DOPA	Levodopa
mAChR	Muscarinic acetylcholine receptor
Nac	Nucleus accumbens
NC-IUPHAR	Nomenclature committee of international union of pharmacology
N-terminus	Start of protein terminated with a free amino-group
p.a.	Pro analysi
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PDE	Phosphodiesterase
PIP_2	Phosphatidyinositol-4,5-bisphosphate
PLC Cβ	Phospholipase Cβ
PTX	Pertussis toxin
RAM	Rabbit anococcygeus muscle
ReAsH	Resorufin-based arsenic hairpin binder
TMD	Transmembrane domain
VTA	Ventral tegmental area
YFP	Yellow fluorescent protein

8.2 List of Oligonucleotides

CFP Not rev	*AAA GC GG CC GC GAT ATC TTA CTT GTA CAG CTC GTC CAT*
CFP XbaIforw	*AAA TCT AGA GTG AGC AAG GGC GAG GA*
CFP XhoI rev	*AAA CTC GAG TTA CTT GTA CAG CTC GTC C*
CFPXbaGSGEGoBafw	*AAA TCT AGA GGC TCC GGA GAA GGC GTG AGC AAG GCC*
CFPXbaIGSGEGfor	*AAA TCT AGA GGA TCC GGA GAA GGC GTG AGC AAG GCC*
EndSigPepIIfw	*GCG GAC GCG CCC TGG TTG CGC TGG TTC TTG CCT GCG GCC TGT CGC GGA*
EndSigPepM2fw	*CCT GCG GCC TGT CGC GGA TCT GGG GAA ATA ACT CAA CAA ACT CCT CTA AC*
EndSigPepM4fw	*CCT GCG GCC TGT CGC GGA TCT GGG GAG CCA ACT TCA CAC CTG TCA AT*
HindIII EndSigPepIIIfw	*AAA AAG CTT ATG CAG CCG CCT CCA AGT CTG TGC GGA CGC GCC CTG GTT G*
HindIIIM4_fw	*AAA AAA ATG GCC AAC TTC ACA CCT GTC*
HindIIISignalpeptid	*AAA AAG CTT ATG AAG ACG ATC ATC GCC CTG AGC TAC ATC TTC TGC CTG GTA T*
M2 Xba1rev	*AAA TCT AGA AGC GCC TAT GTT CTT ATA ATG*
M2BamHIEcoRIfw	*GGA TCC AAA GAA TTC AAG CAG CCT GCA AAA AAG AAG C*
M2EcoBaml3rev	*GAA TTC AAA GGA TCC AGT CAT CTT CAC AAT CTT GCG G*
M2EcoBams3rev	*GAA TTC AAA GGA TCC GTC TTG GTT GGC AAC AGG CT*

M2EcoXhoI3rev	*GAA TTC TTT CTC GAG AGT CAT CTT CAC AAT CTT GCG G*
M2EcoXhoIsl3rev	*GAA TTC TTT CTC GAG GTC TTG GTT GGC AAC AGG CT*
M2FlasHEcoRIrev	*AAA GAA TTC ACA GCA TCC AGG ACA GCA GTC TTG GTT GGC*
M2HindIIIFLAGfor	*AAA AAG CTT ATG GAC TAC AAG GAC GAT GAT GAC AAG AAT AAC TCA ACA A*
M2HindIIIfor	*AAA AAG CTT ATG AAT AAC TCA ACA AAC TCC TCT*
M2KQPEcoRIfor	*AAA GAA TTC AAG CAG CCT GCA AAA AAG AAG CC*
M2P229EcoRI for	*AAA GAA TTC CCC GTT TCT CCA AGT CTG GTA C*
M2T367EcoRIflr	*AAA GAA TTC ACT AAG CAG CCT GCA AAA AAG AAG*
M2XbaGrev	*AAA TCT AGA ACC CTT TGT AGC GCC TAT GTT CTT ATA*
M2XbaGSGEGrev	*AAA TCT AGA TCC CTC TCC TGA ACC CTT TGT AGC GCC*
M2XhoIEcoRI_fw	*CTC GAG AAA GAA TTC AAG CAG CCT GCA AAA AAG AAG C*
M4BamHIEcoRIfw	*GGA TCC AAA GAA TTC ATC GCT CGC AAC CAG GTG CGC*
M4BamHIFlasHrev	*AAA GGA TCC ACA GCA TCC AGG ACA GCA TTT CTT CTC CTT CGG GCC CTC GG*
M4BamHIfw	*AAA GGA TCC ATC GCT CGC AAC CAG GTG CGC*
M4BamHIrev	*AAA GGA TCC TTT CTT CTC CTT CGG GCC CTC GGG CC*
M4EcoRIfw	*AAA GAA TTC ATC GCT CGC AAC CAG GTG CGC*
M4FlasHforw	*TGC TGT CCT GGA TGC TGT GGA TCC CGC CCA GCC ACA GAG CTG TCC A*

M4FlasHnew fw	*TGC TGT CCT GGA TGC TGT GTG GCC CGC AAG TTC GCC*
M4FlasHrev	*GGA TCC ACA GCA TCC AGG ACA GCA TTC CTT GGT GTT CTG GGT GGC A*
M4FSEIFlAsHrev	*ACA GCA TCC AGG ACA GCA TCC CGG CCG GCC TCC CG*
M4KpnIFlagfw	*AAA GGT ACC ATG GAC TAC AAG GAC GAT GAT GAC AAG GCC AAC TTC ACA CC*
M4l3EcoRIBamHIr	*GAA TTC AAA GGA TCC GCT GGC GAA CTT GCG GGC*
M4long3FlTMD6fw	*TGC TGT CCT GGA TGC TGT AGC ATC GCT CGC AAC CAG GTG*
M4long3FlTMD6rev	*ACA GCA TCC AGG ACA GCA GGC GAA CTT GCG GGC CAC GTT*
M4s3EcoRIBamHIr	*GAA TTC AAA GGA TCC TTT CTT CTC CTT CGG GCC CTC GGG CC*
M4XbaIrev	*AAA TCT AGA CTA CCT GGC AGT GCC GAT GTT CC*
Signalpep Flag	*GCT ACA TCT TCT GCC TGG TAT TCG CCG ACT ACA AGG ACG ATG ATG*
YFP BamHI-forw	*AAA AAA GGA TCC GTG AGC AAG GGC GAG GAG C*
YFP Eco RI_rev	*AAA AAA GAA TTC CTT GTA CAG CTC GTC CAT GC*
YFPXhoI-fw	*CTC GAG GTG AGC AAG GGC GAG GAG C*

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10 Acknowledgment

This PhD thesis was carried out from January 2006 to January 2010 supervised by Dr. rer. nat. Viacheslav Nikolaev and Prof. Martin Lohse at the Pharmacological Department of the University of Würzburg.

I would like to thank the people, who supported me during these 4 years.

First of all, I would like to thank Prof. Lohse, who did support me during this time with his knowledge and his kindness. Thank you for this interesting thesis, for our discussions and your confidence in me.

Also I am indebted to Dr. Nikolaev, he was always there when help was needed and added great ideas as well as encouragement, if something went wrong. He lifted my spirits up and provided new perspectives.

Thank you to my fellow colleagues at the Department of Pharmacology, you gave me skilled advice and emotional backup.

I especially would like to thank Nicole Ziegler, who became a very good friend. I hope we will stay in touch for a long time.

I am also indebted to Leif Hommers, who patiently performed all GIRK-measurements and presented great results.

Thanks to Christian Dees, everyday you had a friendly word for me, provided excellent support at the lab and made me smile a lot.

Nadine Fröhlich, thanks a lot for not giving up on my “baby”, your improvements added the “final edge”.

Thank you Ulrike Zabel for your latest PCR-tricks and a lot of laughter in our office, I will miss that.

My friends at the DDBL, Monash University, Melbourne, thank you for those wonderful days, I enjoyed working with you so much.

Thanks to Prof. Arthur Christopoulos, who gave me new insights on allosteric mechanisms and all the support I could hope for.

Thank you Dr. Katie Leach and Dr. Celine Valant for all your efforts and the time you spend with me to make the best of my short visit.

Last but not least I would like to thank the people, who have supported me for so many years.

My dear friends Beli, Krissy, Martina, Bea and Anja, you made studying and learning so easy. Our discussions made me realize, how much I enjoy pharmacological sciences.

My mother and my father, thank you for enabling me to follow my every dream.

Julia, my sister, I really loved your infinite interest in my topic and your questions.

Lilli, my sweet daughter you make everything worthwhile.

My beloved husband Thomas, you always supported me and made me confide in myself when I was doubtful. Thanks for your understanding and your love.

11 Curriculum Vitae

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Auslandsaufenthalt

Okt.-Nov. 2008	*Laboraustausch mit der Arbeitsgruppe von Prof. A. Christopoulos, Monash University, Melbourne, Australien*

Veröffentlichungen

2010

A FRET-based M2 muscarinic receptor sensor reveals rapid kinetics of allosteric modulation.

Maier-Peuschel M, Frolich N, Dees C, Hommers LG, Hoffmann C, Nikolaev VO, Lohse MJ.
J Biol Chem. 2010 Jan 18. ***Epub ahead of print***

2008

A FRET-based M_2-muscarinic receptor sensor to study mechanisms of allosteric modulation
Maier-Peuschel M., Nikolaev V.O., Hoffman C., Lohse M.J.
Naunyn Schmiedebergs Arch Pharmacol. 2008 April, ***Abstract***

Transgenic mice ubiquitously expressing camp sensor EPAC1-camps for in situ and in vivo camp imaging
Nikolaev V.O., Jacobs S., Maier-Peuschel M., Schmitteckert E.M., Engelhardt S., Calebiro D., Schulz S., Lohse M.J.
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A novel modification of ERK1/2 results in hypertrophy specific signaling
Lorenz K., Schmitt J.P., Schmitteckert E.M., Maier-Peuschel M., Quitterer U., Lohse M.J.
Naunyn Schmiedebergs Arch Pharmacol. 2008 April, ***Abstract***

Preise und Stipendien

2008

Stipendium „Jubiliäumsstiftung“ der Julius-Maximilians Universität Würzburg
Travel Grant Awardee der Victor Chang Stiftung, Sydney

Poster Prize, Symposium „Molecular Pharmacology of G-protein coupled receptors“, 13.-15. November, Sydney