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# CHAPTER 1

## General Introduction

# 1



## ABOUT THIS THESIS

During the course of our lives we all come across the use of medicines at many points in time. Given the enormous number of medicines taken today it is hard to imagine a world with only a small number of medicines available. Yet back around a century in time this was actually the case. The number of medicines available was much smaller and those available were mostly derived from plants. Many diagnoses that were grim or even life threatening at that time, are nowadays handled with relative ease using available medicines.

Drug research as we know it today, evolved from the maturation of chemistry and pharmacology to a point where molecules could be designed and the effect on the human body could be described and understood.<sup>1</sup> In 1905 John Newport Langley introduced the concept of a drug receptor<sup>2</sup> and around the same time Paul Ehrlich aimed to understand the selectivity of molecules and came up with the famous expression “*corpora non agunt nisi fixata*” (drugs do not act unless they are bound).<sup>3</sup> Over the 20<sup>th</sup> and 21<sup>st</sup> century the art of drug discovery progressed enormously to a point where large compound libraries are screened towards a specific drug target in order to find a hit molecule. Subsequently, this hit compound can be altered and improved through rational design to obtain a lead candidate which can be further evaluated and may eventually become a new medicine.

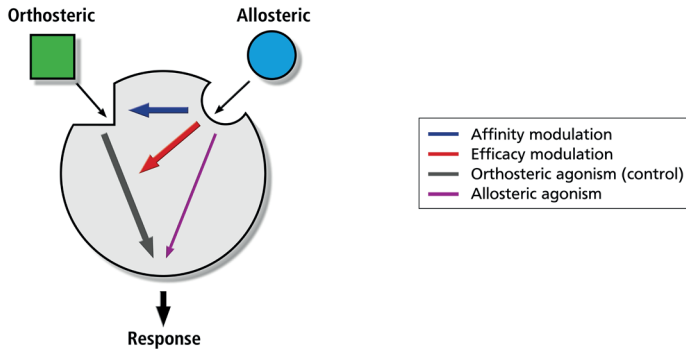
Cell-cell communication in the central nervous system (CNS) occurs through secretion of signaling molecules, so-called neurotransmitters. These neurotransmitters exert their action via binding to different receptors, including the metabotropic glutamate receptor 2 (mGlu<sub>2</sub>). For multiple CNS diseases involving a distorted glutamatergic signaling this receptor may be a potential drug target. Therefore, pharmaceutical industry has embarked on the design and synthesis of potential medicines acting via this receptor. Unfortunately this has not resulted in a marketed drug so far. For a better understanding of the receptor, this thesis zooms in at a molecular level to study the mechanism of action of the mGlu<sub>2</sub> receptor from different perspectives with a focus on binding kinetics. This chapter provides a background for the research presented followed by the aim and outline of this thesis.

### GPCRs

G protein-coupled receptors (GPCRs) are critical signal transduction gatekeepers and represent the largest protein family in the human genome with more than 800 members.<sup>4</sup> Structurally, these receptors share a common architecture of seven transmembrane helices connected by three intracellular and three extracellular loops, and are classified into five subgroups: class A (rhodopsin), class B (secretin), class C (glutamate), adhesion and frizzled/taste2.<sup>5,6</sup> Located in the cell membrane, GPCRs recognize a variety of extracellular stimuli including photons, ions, small molecules, peptides and proteins. Upon receptor activation by its endogenous agonist, a conformational change in receptor structure occurs inducing downstream signalling events via different intracellular proteins including heterotrimeric G proteins (hence the name GPCR), arrestins and kinases.<sup>7</sup> These signal-transduction pathways are very diverse between individual GPCRs as different downstream signalling pathways can be activated and multiple of these pathways can be activated at the same time. The pathways that are being activated can even differ between receptor ligands, so-called biased signalling.<sup>8</sup> Signalling events via GPCRs are vital for a good functioning of the human body, but may be distorted in diseases. Therefore, many drugs have been successfully developed that target these receptors and currently more than 30% of drugs on the market act via these receptors.<sup>9</sup>

### Allosteric modulation

For decades, the development of drug substances for GPCRs was focused on the orthosteric binding site, which is the site where the endogenous receptor ligand binds. However, like many proteins most GPCRs provide one or more other, so-called allosteric, binding sites that are topographically distinct from the orthosteric binding site. Ligands that bind such a secondary binding site are called “allosteric modulators” and in the case of GPCRs they may alter the protein conformation of the receptor such that the affinity and/or signalling efficacy of the endogenous orthosteric agonist is modulated as depicted in figure 1. The concept of allosteric modulation was first described and formalized in the 1960s by Monod, Wyman and Changeux when they observed enzyme inhibition by a compound that bound a secondary binding site remote from the substrate binding site.<sup>10</sup> Ligands that potentiate the affinity and/or efficacy of the endogenous agonist are referred to as positive allosteric modulators (PAMs), ligands that inhibit these parameters are referred to as negative allosteric modulators (NAMs) and ligands that bind the allosteric binding site without altering the action of any orthosteric ligands are known as neutral or silent allosteric ligands (NALs/SALs).<sup>11</sup> Across the five major classes of GPCRs allosteric modulators have been described and at present there are two marketed drugs that allosterically modulate GPCR function: Cinacalcet, a calcium sensing receptor (CaSR) PAM and Maraviroc, a C-C chemokine receptor 5 (CCR5) NAM.<sup>12</sup>

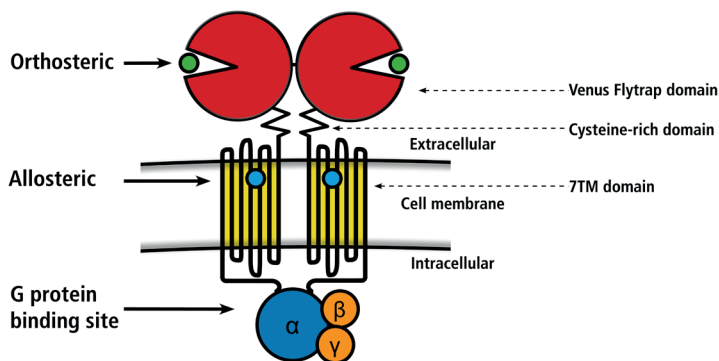


**Figure 1. Schematic representation of modes of action of allosteric modulators.**

Allosteric ligands (blue) bind to a binding site topographically distinct from the orthosteric binding site to modulate affinity (dark blue) and/or efficacy (red). Some allosteric modulators can directly activate the receptor (purple). Figure was adapted from Conn *et al.* (2009).<sup>13</sup>

### Metabotropic glutamate receptors

In contrast to the electrical signalling within neurons, communication between neurons is performed by neurotransmitters, which are exchanged via synapses. Glutamate serves as neurotransmitter at the majority of excitatory synapses in the central nervous system (CNS): around 60% of neurons in the human brain use glutamate as their primary neurotransmitter.<sup>14</sup> Glutamate is the major mediator of sensory information, motor coordination, emotions and cognition.<sup>15</sup> Upon neuronal repolarization, glutamate stored in specific vesicles is released into the synapse where it exerts its fast action via ionotropic glutamate (iGlu) receptors and modulates synaptic activity via metabotropic glutamate (mGlu) receptors.<sup>16</sup>



**Figure 2. Schematic representation of an mGlu receptor dimer embedded in the cell membrane.**

The large extracellular VFT domain is connected to the 7TM domain via a cysteine-rich domain. The 7TM domain contains seven alpha-helices that cross the cell membrane and are connected by three intracellular and three extracellular loops. Orthosteric ligands (green) bind in the glutamate binding site which is located in the VFT domain. Allosteric modulators (blue) bind in an allosteric binding pocket found in the 7TM domain. Upon receptor activation a single G protein is activated and released from the receptor initiating subsequent downstream signalling events.

mGlu receptors are class C GPCRs and were first described in the 1980s as phospholipase C-coupled receptors.<sup>17-19</sup> Structurally, class C GPCRs are characterized by a large extracellular orthosteric ligand binding domain, the so-called Venus Flytrap domain (VFT), which is connected to the typical seven transmembrane (7TM) domain via a cysteine-rich domain (CRD),<sup>20,21</sup> as shown schematically in figure 2. They are obligatory dimers that are predominantly expressed as homodimers linked by a disulfide bond in the VFT.<sup>22,23</sup> Eight mGlu receptors have been found that are divided into three subgroups, group I (mGlu<sub>1,5</sub>), group II (mGlu<sub>2,3</sub>) and group III (mGlu<sub>4,6-8</sub>), based on sequence homology, second messenger coupling and pharmacology.<sup>24,25</sup> In contrast to group I mGlu receptors that are mainly expressed on post-synaptic neurons, groups II & III mGlu receptors are mainly presynaptic receptors that inhibit neurotransmitter release at various types of synapses.<sup>15</sup>

### The mGlu<sub>2</sub> receptor

Activation of the mGlu<sub>2</sub> receptor, which is expressed throughout the central nervous system, reduces glutamate release into the synapse. Hence, it has emerged as a therapeutic target for psychiatric diseases characterized by glutamatergic dysfunction.<sup>21,26</sup> mGlu<sub>2</sub> receptor activation can reduce glutamate hyperfunction in diseases like schizophrenia and anxiety,<sup>27,28</sup> whereas mGlu<sub>2</sub> receptor blockade can decrease glutamate hypofunction in depression and cognition.<sup>29,30</sup> Although the structure of the mGlu<sub>2</sub> VFT domain is known,<sup>31</sup> the current structural understanding of the 7TM domain is based on NAM-bound crystal structures of the mGlu<sub>1</sub> and mGlu<sub>5</sub> receptors.<sup>32-35</sup> A variety of glutamate-like agonists has been developed, of which LY2140023, a prodrug of LY404039 showed improvement in positive and negative symptoms in schizophrenic patients, which unfortunately could not be confirmed in later clinical studies.<sup>28,36</sup> Inhibition of the mGlu<sub>2</sub> receptor can be achieved by (orthosteric) antagonists of which LY341495 is the most studied.<sup>37</sup>

Development of orthosteric ligands presents challenges for selectivity and brain penetration and therefore discovery efforts have largely shifted to the development of allosteric modulators that bind in a less conserved pocket in the 7TM domain.<sup>13</sup> Many mGlu<sub>2</sub> PAMs showed activity in *in vivo* experimentation<sup>12</sup> and two PAMs have advanced into clinical trials: AZD8529<sup>38</sup> and JNJ-40411813/ADX71149.<sup>39,40</sup> Reported negative allosteric modulators (NAMs) include RO4491533<sup>41</sup> and decoglurant of which the latter has advanced into clinical trials as well.<sup>30</sup>

### Binding Kinetics

The idea that binding kinetics of the drug-receptor complex are important for drug efficacy in addition to the occupied receptor binding sites was first described in 1961 by William Paton in his 'rate theory'.<sup>42</sup> However, since then the majority of new models describing drug



efficacy, such as the ‘two-state receptor model’, considered efficacy in terms of the ability to stabilize an active receptor conformation under equilibrium conditions.<sup>43</sup> Typically, affinity and efficacy are measured under equilibrium conditions, whereas in open systems like the human body both drug and target have fluctuating concentrations. Over the last decade it has become increasingly apparent that not only a ligand’s affinity is crucial for a good efficacy, but that also the ligand’s binding kinetics – described by the second order association rate constant  $k_{on}$  and the first order dissociation rate constant  $k_{off}$  – are important parameters to be optimized to improve efficacy.<sup>44,45</sup> In addition to classical equilibrium assays, affinity ( $K_D$ ) can be determined by  $k_{off} / k_{on}$ . Based on the  $k_{off}$  the so-called drug-target residence time (RT) can be calculated. This parameter is indicative for the lifetime of the drug-target complex and is defined as the reciprocal of the dissociation rate constant,  $1/k_{off}$  (Fig. 3).<sup>46</sup>

Binding kinetics		Residence time	Affinity
$R + L \xrightleftharpoons[k_{off}]{k_{on}} RL$		$RT = \frac{1}{k_{off}}$	$K_D = \frac{k_{off}}{k_{on}}$

**Figure 3.** Binding of a ligand (L) to a receptor (R) is described by the association rate constant ( $k_{on}$ ) and dissociation of the ligand-receptor complex (LR) is described by the dissociation rate constant ( $k_{off}$ ). From these rate constants, residence time (RT) and affinity ( $K_D$ ) can be derived.

The relevance of RT was retrospectively shown for multiple marketed GPCR drugs.<sup>47</sup> For example the successful anti-asthma drug tiotropium – the drug that was spent the most money on in The Netherlands in 2016<sup>48</sup> – is a long RT muscarinic acetylcholine  $M_3$  receptor antagonist, which results in longer duration of action and subsequently a lower side-effect burden.<sup>49</sup> Although RT and  $k_{off}$  have been the focus of most kinetic studies,  $k_{on}$  has also been described to be important for fast drug action, a high receptor occupancy and even a longer duration of action.<sup>50–52</sup> Recently, the importance of  $k_{on}$  was further emphasized when it was shown that extrapyramidal side effects induced by  $D_2$  receptor antagonists are linked to  $k_{on}$  rather than  $k_{off}$  as was the general consensus so far.<sup>53,54</sup>

### Covalent probes

A special kinetic profile can be obtained by changing a ligand such that it binds its target covalently, resulting in ‘ultimate’ RT and continuous target occupancy. Such covalent ligands have proven to be successful medicines for various indications, exemplified by well-known drugs such as aspirin, penicillin and omeprazole. However, covalent molecules are generally avoided in drug discovery due to safety concerns. The development of covalent ligands was inspired by the rhodopsin receptor that forms a covalent bond with its native ligand 11-*cis*-retinal.<sup>55</sup> As such, this covalent interaction is involved in our vision via rod cells in the eye.

Introduction of covalent ‘warheads’ into ligands that were optimised for non-covalent affinity may result in highly targeted, selective lead molecules that thereby overcome some of the difficulties of off-target binding.<sup>56</sup> Generally, covalent GPCR ligands consist of a high affinity pharmacophore and a reactive electrophilic warhead that is able to form a covalent bond with a specific nucleophilic amino acid residue in the receptor binding pocket. Commonly used warheads include reactive thiols, Michael acceptors, isothiocyanates and the fluorosulfonyl moiety.<sup>57</sup> Over the last decade the structural understanding of GPCRs has vastly increased by the great number of receptor crystal structures obtained. To facilitate this receptor crystallization, covalent ligands can be used to stabilize the receptor in a specific conformation. This approach was successfully used to obtain the crystal structures of the Adenosine A1 and multiple  $\beta$ 2 adrenergic receptors amongst others and could be of special interest for crystallization of active state receptors as these are notoriously difficult to crystallize.<sup>58,59</sup> Further functionalisation of covalent ligands for affinity-based protein profiling (AfBPP) can be achieved by photoaffinity labelling. As direct substitution of a photoreactive tag likely decreases affinity, this is commonly accomplished via a ‘clickable’ handle on the covalent ligand which can be used to attach a photoreactive tag following receptor binding and covalent bond formation,<sup>60</sup> as was recently shown for an mGlu<sub>5</sub> NAM.<sup>61</sup> In summary, over the last years covalent GPCR ligands have emerged as valuable tool compounds for characterization of receptor structure, mechanism of action, expression patterns in native tissues and binding kinetics.<sup>57</sup> Additionally, covalent allosteric modulators are more likely to be used as therapeutics due to their preferential safety profile as they only exert their effect whilst the endogenous agonist is bound.<sup>62</sup>

### Label-free assays

Within the field of *in vitro* pharmacology a wide variety of different assays for GPCRs is used to characterize novel molecules. These assays can be divided into two groups: binding assays that evaluate how well (affinity) and fast (binding kinetics) ligands bind the receptor and functional assays that evaluate how well ligands exert their functional effect(s) (potency and efficacy). The majority of these methods are dependent on labels that are attached to ligands or the receptor itself. Radioligand binding is a robust and well-characterized method within the field and is used in this thesis to evaluate ligand binding and functional responses mediated by the G protein.<sup>63</sup> These assays however require expensive and specialized laboratories and careful handling regarding safety and waste disposal. Therefore, other methods have been developed which often use fluorescent labels, such as BRET and FRET assays.<sup>64</sup> Labelling of compounds or receptors with bulky fluorescent groups may alter the properties of these molecules thereby potentially leading to false positive or false negative results. Furthermore classical functional assays are mostly pathway-biased as they often focus on a single signalling pathway.

New assays are emerging that lack the need for any kind of label. These so-called label-free assays can be used to assess ligand binding and functional responses in real-time. To measure binding kinetics and affinity, surface plasmon resonance (SPR) and surface acoustic wave (SAW) biosensors have been developed, which were followed more recently by ligand binding assays using mass spectrometry (MS).<sup>65</sup> Whereas these assays use isolated protein or membrane preparations, other biosensor-based label-free assays assess functional responses of GPCR ligands on whole cells by via ligand-induced morphological changes.<sup>66</sup> These biosensors measure changes in cell morphology either optically, such as EPIC,<sup>67</sup> or by changes in impedance, such as xCELLigence.<sup>68</sup> Cell types used include recombinant cell lines, cell lines endogenously expressing the receptor of interest and even cells derived from patients.<sup>69</sup> By eliminating the need for labels, these whole cell biosensor-based assays have the capability of assessing endogenous receptor function in real-time under more physiologically relevant conditions.<sup>70</sup> Hence, label-free assays may provide more translational insights into the *in vitro* – *in vivo* translation.

## OBJECTIVES AND OVERVIEW OF THIS THESIS

### Aim

During the drug development process many potential medicines are withdrawn as they are unsafe or lack efficacy. The translational step between *in vitro* and *in vivo* experiments is not as predictive as one would desire. Therefore, many promising compounds fail during *in vivo* experiments or even in later stages of the drug development process, thereby spilling lab animals, time and money. This is not different for the mGlu<sub>2</sub> receptor for which no marketed drug is available so far, despite huge discovery efforts. Taken together, there is a need to improve the understanding of key *in vitro* parameters that drive *in vivo* efficacy. Hence, the aim of this thesis was to provide detailed insights into binding kinetics of PAMs at the mGlu<sub>2</sub> receptor and to evaluate the potential of kinetic profiling of an established mGlu<sub>2</sub> PAM library for prediction of *in vivo* efficacy. This required in depth understanding of the mechanism of allosteric modulation from a functional and a kinetics perspective. An additional aim was to develop a label-free assay which allows evaluation of functional kinetics, constitutive receptor activity and inverse agonism. The last aim was to design, synthesize and characterize a novel covalent mGlu<sub>2</sub> PAM.

### Outline of this thesis

**Chapter 2** focuses on the characterization of the PAM JNJ-46281222 that is used throughout this thesis as pharmacological tool compound. Additionally, the molecular mechanism of allosteric modulation of the mGlu<sub>2</sub> receptor is evaluated. In **Chapter 3** this mechanism

is further evaluated from a binding kinetics perspective. Kinetic parameters for orthosteric mGlu<sub>2</sub> ligands including endogenous glutamate are determined in the absence and presence of allosteric modulators and functional assays are performed to study the interplay between the orthosteric and allosteric binding sites. **Chapter 4** describes the synthesis and biological evaluation of a novel series of novel mGlu<sub>2</sub> PAMs. Affinity, potency and binding kinetics are determined followed by extensive structure-kinetics relationships (SKR) in addition to the more classical structure-activity relationships (SAR). The kinetic profiles of the PAMs are evaluated by comparison of kinetic parameters with *in vitro* equilibrium parameters and ultimately with *in vivo* efficacy data. **Chapter 5** describes the design, synthesis and pharmacological evaluation of the first covalent PAM probe within the family of class C GPCRs, which presents a novel pharmacological tool with ‘ultimate’ binding kinetics and a wide range of applications in the mGlu<sub>2</sub> receptor field. Additionally, the binding mode of this pharmacological tool compound is studied using computational and receptor mutagenesis approaches. In **Chapter 6** an impedance-based label-free biosensor assay is developed in order to study mGlu<sub>2</sub> pharmacology and in particular constitutive receptor activity and inverse agonism. The results and forthcoming future opportunities are summarized in **chapter 7**. Hopefully, the results obtained in this thesis will contribute to an increased understanding of the key *in vitro* parameters necessary for *in vivo* and *in patient* efficacy, ultimately resulting in novel safe and efficacious drugs.

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