**Time is of the essence - investigating kinetic interactions between drug, endogenous neuropeptides and receptor**
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# Chapter 1

## General introduction

The search for effective drugs treating diseases has been an age-old quest. While drug design and development have witnessed major progress over the last decades, one of the main challenges still resides in the lack of efficacy. Consequently, traditional lead selection procedures like Lipinski's rule of five and affinity-based selection need to be reconsidered. Over the past 10-years, binding kinetics, i.e. the association and dissociation rate of a drug to and from its target, have been proposed as better predictive parameters in assessing the potential of novel drugs [1-6]. Although the importance of binding kinetics is increasingly recognized, there is still a need for robust assays suitable to study association and dissociation rates of potential drug candidates. Additionally, many successful drugs achieve their effect by competing with endogenous ligands for the same binding site. Therefore, understanding the pharmacological and physiological behavior, such as binding kinetics, of endogenous ligands in the human body is crucial. This is of particular importance for endogenous ligands since they are often released temporally at locally high concentrations. Finally, to bridge the gap between *in vitro* and *in vivo* studies, functional assays that can reliably translate binding kinetics to *in vitro* functional effects are crucial.

To illustrate the importance and relevance of the research performed in this thesis, this chapter provides a general introduction. Firstly, the superfamily of G protein-coupled receptors (GPCRs) will be introduced, followed by an introduction of the sub-family of neuropeptide receptors which are predominantly GPCRs. Consequently, the background of two well-known neuropeptide receptors, namely the gonadotropin-releasing hormone (GnRH) receptor and neurokinin 1 (NK1) receptor, will be outlined as the experimental chapters of this thesis are centered around these receptors. Furthermore, the concept of binding kinetics will be defined, including the challenges of measuring these kinetic binding parameters. Lastly, the aim and outline of this thesis will be explained.

## G protein-coupled receptors

The GPCR family is one of the largest and most diverse receptor families and nearly 800 genes encoding GPCRs have to date been identified [7]. GPCRs are composed of seven transmembrane helices with extracellular and intracellular loops and an extracellular (N-terminal) and intracellular (C-terminal) tail. GPCRs are coupled to intracellular G proteins and can be activated by a wide range of ligands, such as peptides, neurotransmitters, hormones, growth factors, odorant molecules and even photons [8] (Figure 1). GPCR activation results in a conformational change of the receptor, causing GDP to be exchanged for GTP. Consequently, this leads to dissociation of the Gαβγ-heterotrimer into the βγ-dimer and the α-subunit. The four main Gα-subunits are; $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12/13}$. The activation and inhibition of diverse G protein-dependent pathways makes GPCRs essential in cell

signaling [9]. Targeting the GPCR super-family has led to approximately 30% of the marketed drugs and to date GPCRs are vital targets in drug research due to their role in (patho-) physiology throughout the body [10].

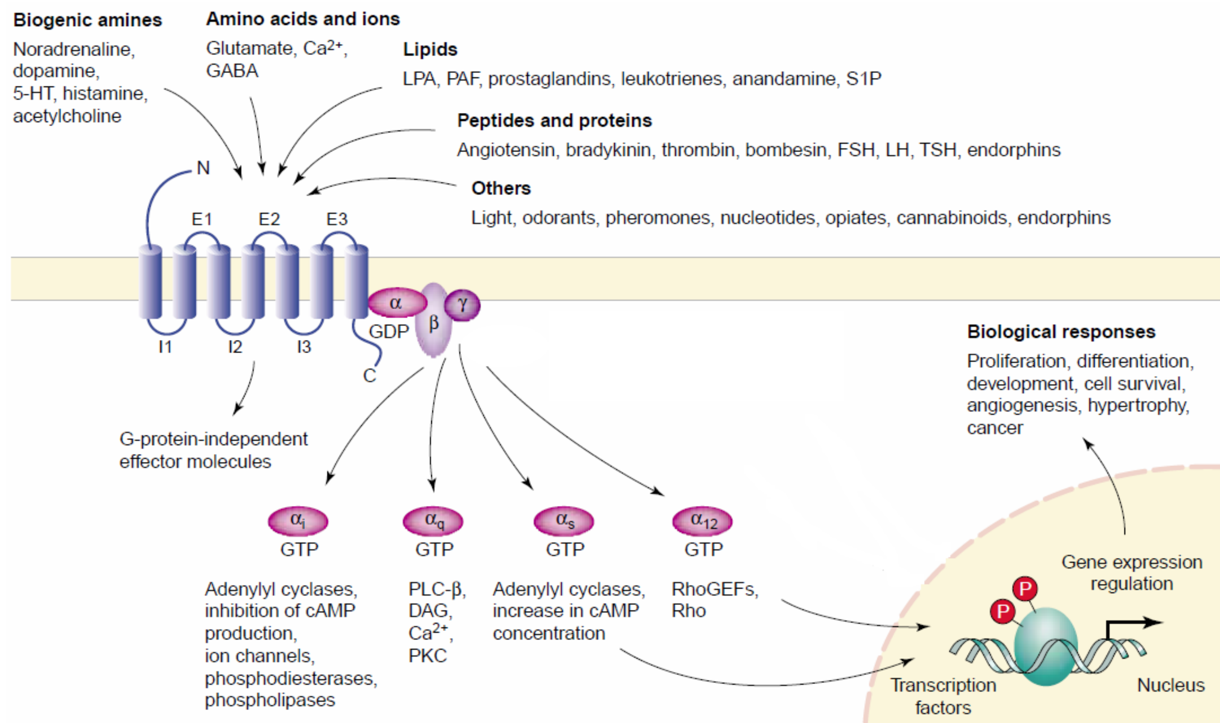


**Figure 1:** Schematic overview of ligands binding to G protein-coupled receptors and their four main signaling pathways. A wide range of ligands can bind and activate GPCRs through G protein-dependent (i.e. $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12}$ proteins) and G protein-independent (e.g. β-arrestin) pathways. These signaling pathways can regulate pivotal cellular functions such as proliferation [8].

## Neuropeptide receptors

Neuropeptides are (poly)peptides and can be short as kisspeptin-10 (e.g. 3 amino acids) or as long as neurexophilin-1 (e.g. 250 amino acids). Neuropeptides mediate neuronal communication by binding to neuropeptide receptors expressed on either neuronal substrates such as glial cells or on non-neuronal target cells [11]. The neuropeptide receptor family consists of over 44 receptor families which are predominantly GPCRs. Neuropeptide receptors and their endogenous ligands are involved in numerous behavioral and physiological functions such as blood pressure, body temperature, feeding behavior, pain regulation, reproduction, learning, memory and sleep [12]. Consequently, neuropeptide transmission is an attractive focal area for drug design in numerous therapeutic areas, such as inflammatory conditions, epilepsy and psychiatric diseases [13-15].

*GnRH receptor*

One of the most well-known neuropeptide receptors is the gonadotropin-releasing hormone (GnRH) receptor. This receptor binds endogenous GnRH and upon activation stimulates the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH). The GnRH receptor belongs to the superfamily of GPCRs and is (predominantly) coupled to $G\alpha_{q/11}$ proteins This receptor is involved in maintaining hormone levels in both males and females which makes it an attractive drug target in the treatment of hormone-dependent diseases such as fertility disorders, precocious puberty, and cancers of the endometrium, ovary, prostate and mammary [16, 17]. Sustained receptor exposure to GnRH or GnRH analogs leads to desensitization of GnRH receptor-mediated gonadotropin secretion. This desensitization or blockade of the GnRH receptor is called chemical castration and underlies the therapeutic use of GnRH analogs. The first GnRH analog to reach the market was nafarelin acetate in 1998 and soon after many more GnRH analogs were FDA approved, such as leuprolide acetate, goserelin acetate, degarelix and triptorelin [18-20]. To date, many peptide GnRH receptor agonists and antagonists are on the market to treat hormone-dependent disorders [17, 21-24] and available patient information suggest that the pharmacokinetic and pharmacodynamic profiles are very comparable. Accordingly, insights into the *in vitro* binding parameters, such as drug-target binding kinetics, could improve the understanding of the mechanism of action of these well-known drugs.

*NK1 receptor*

Another well-known neuropeptide receptor is the neurokinin 1 (NK1) receptor or tachykinin 1 receptor. This receptor belongs to the tachykinin receptor family that consists of NK1, NK2 and NK3 receptors. The NK1 receptor belongs to class A GPCRs and is functionally coupled to $G\alpha_{q/11}$ proteins and $G\alpha_s$ proteins. Multiple endogenous tachykinins bind to the NK1 receptor, including Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). Each tachykinin has a specific rank order to activate tachykinin receptors with regard to potency and affinity, namely SP > NKA > NKB for the NK1 receptor, NKA > NKB > SP for the NK2 receptor and NKB > NKA > SP for the NK3 receptor. The NK1 receptor plays an imperative role in the brain with respect to the regulation of affective behavior and emesis, as well as nociception in the spinal cord [25].

Presently, only two drugs are on the market targeting the NK1 receptor for the treatment of chemotherapy-induced nausea and vomiting (CINV). Aprepitant, a high affinity, selective NK1 receptor antagonist was FDA approved in 2003 [26]. This small molecule

antagonist was the first NK1 antagonists to reach the market as previous clinical trials were predominantly aimed towards clinical pain states [27]. Interestingly, a distinguishing feature of aprepitant is its so-called PK/PD discrepancy *in vivo*, i.e. aprepitant levels in the brain were below the limit of quantification while a strong inhibitory effect was still present, which researchers attest to its slow receptor dissociation rate [28]. In 2014, a combination drug of a NK1 small molecule antagonist (netupitant) and a 5-$HT_3$ antagonist, was approved for the treatment of CINV [29]. *In vitro* studies demonstrated that netupitant was wash-out resistant for up to 5 hours and the action of netupitant was therefore deemed insurmountable [30]. These two drug examples allude to the importance of being aware of and consequently optimizing kinetic binding parameters.

## Binding kinetics, a retrospective analysis

Traditional drug discovery programs are predominantly focused on equilibrium-based parameters such as $K_i$ and $IC_{50}$ values. However, candidate drugs with high affinity and potency often fail in clinical trials due to target toxicity and/or lack of *in vivo* efficacy [31, 32]. Therefore, other, more predictive parameters than affinity and potency values are warranted. Binding kinetics are a collective term for the association ($k_{on}$) and dissociation ($k_{off}$) rate constant of a drug to and from its target. Additionally, the so-called drug-target residence time is reflective of the life-time of the drug-target complex and is defined as the reciprocal of $k_{off}$ [3]. Over the past 10 years binding kinetics are increasingly acknowledged to be vital for the mechanism of action of a potential drug [33]. Moreover, many blockbuster drugs have been retrospectively been examined for their binding kinetics and were found to have distinct kinetic profiles [34]. For example, quetiapine, a dopamine $D_2$ receptor antagonist approved for the treatment of schizophrenia and bipolar disorder, has significantly less adverse effects and on-target toxicity in comparison to other dopamine $D_2$ receptor antagonists due to its fast dissociation rate [35]. However, more often slow dissociation rates are favorable. Tiotropium, a muscarinic $M_3$ receptor antagonist, is a well-known long-acting muscarinic antagonist. Since the muscarinic $M_3$ receptor is mainly targeted to treat chronic diseases, a long duration of action is desirable to achieve prolonged efficacy and thus improve patient compliance [36]. Another advantage, aside from the long duration of action of tiotropium, is that it has kinetic selectivity (i.e. faster dissociation rates from other muscarinic receptor subtypes) over other muscarinic receptors thereby minimizing off-target toxicity [37]. Finally, negative allosteric CCR5 modulator maraviroc, was the first drug targeting CCR5 to get FDA approval and proved to be highly efficacious in inhibiting HIV cell infection[38]. Watson and colleagues reported very slow dissociation rates for this compound and the reversal of antagonism rate was found to be longer than 136 hours at room temperature [39]. All these case studies

demonstrate the importance of binding kinetics in achieving high *in vivo* efficacy and/or minimizing (target) toxicity.

## Challenges of incorporating binding kinetics in the drug discovery paradigm

While the previous examples greatly emphasize the impact of binding kinetics, kinetic binding parameters are often only taken into account in retrospect, if at all. Concerns are regularly expressed about suitable high-throughput assays to study binding kinetics in a time-efficient manner, such that they might be introduced in an earlier stage of the drug discovery process.

### *Labeled binding assays*

The most recognized assays to study binding kinetics are radioligand binding experiments, where the ligand of interest is radiolabeled and association and dissociation experiments are performed to directly measure $k_{on}$ and $k_{off}$ values. However, since radiolabeling every potential drug candidate is very costly and time consuming, novel protocols and techniques have been proposed over the past years [34, 40].

In 1984, Motulsky and Mahan introduced a pharmacological approach in which the binding kinetics of unlabeled ligands can be quantitatively measured by only using one labeled tracer[41]. This so-called competition association method has to date been used to determine the binding kinetics of numerous potential drug candidates [42-44]. Recently, a more medium-throughput dual-point competition association assay was developed [45]. This assay makes use of only two time points and the specific binding of the labeled tracer at these time points generates a qualitative measure of the dissociation kinetics of the (competitive) unlabeled ligand. This screening assay has already been successfully applied to multiple targets [46-48].

Considering the disadvantages of working with radioactivity, alternative labeling techniques have been explored. Schiele *et al* developed a universal homogeneous kinetic probe competition assay (kPCA) that allowed accurate and cost-effective measurements of binding kinetics in a high-throughput format [49]. They compared binding kinetics of three target groups (GPCRs, protein-protein interactions and enzymes) measured with radioligand binding studies, kPCA and surface plasmon resonance (SPR) spectroscopy. Results were highly correlated and the authors proposed that the time-resolved fluorescence energy transfer (TR-FRET) method used for kPCA combines the time resolution of SPR and related biosensors while maintaining the versatility of radioligand binding studies. Notably, one of the

disadvantages of kPCA is the need for not only a fluorescently labeled tracer but also an engineered fluorescently labeled receptor.

### *Label-free binding assays*

Alternative methods to measure binding kinetics are label-free techniques such as SPR and surface acoustic wave (SAW) biosensors [50, 51]. These assays enable real-time quantitative measurements of association and dissociation rates of unlabeled ligands targeting membrane proteins. Advantages of both assays are the capability of using relatively small quantities of materials in addition to the high time resolution [52-54]. The need for having an immobilized receptor protein represents a serious disadvantage when studying GPCR binding as these proteins rapidly disintegrate when taken out of their natural environment.

More recently, a label-free mass spectrometry (MS) ligand binding assay was developed for the adenosine $A_1$ and $A_{2A}$ receptors [55]. The authors were able to perform saturation, association, dissociation and displacement studies without an internal standard making it a true label-free assay suitable to study binding kinetics. Results from the MS experiments were highly correlated to radioligand binding studies. An inconvenience of this assay is the need for an elaborate sample quantification procedure that needs technical expertise.

### *Functional assays*

Another method to qualitatively study binding kinetics of agonists and antagonists is by measuring their functional effects.

To examine the binding kinetics of agonists, a functional wash out can be conducted. Cells are pre-incubated with the agonist of interest to allow the binding of agonist to the receptor. Consequently, cells are washed and the effects of agonist binding can be measured. In theory, agonists with fast dissociation kinetics should be readily washed out while slowly dissociating agonists should still be bound to the receptor thereby maintaining most of the functional effect [56].

The binding kinetics of antagonists can be measured by examining their functional insurmountability. For these experiments cells are pre-incubated with a competitive antagonist prior to addition of an (endogenous) agonist. The maximal response of the agonist with and without antagonist pre-incubation can then be compared. If the maximal response of the agonist is significantly decreased upon antagonist pre-incubation, the antagonist is deemed insurmountable which is often correlated to its slow dissociation rate [57, 58]. A

drawback of functional assays predicting binding kinetics is that these only provide an indication for the dissociation rate of a ligand while the association rate might also be of importance.

## Objectives and outline of this thesis

### *Objective*

The objective of this thesis was to provide kinetic binding parameters of well-known neuropeptides and competitive drugs targeting the GnRH receptor and NK1 receptor to advance the understanding of these ligand-receptor interactions. Additionally, we aimed to design, validate and compare various kinetic assays to supply a more diverse toolbox suitable for studying binding kinetics. The kinetic assays that were used and discussed in this thesis are radioligand binding, TR-FRET, label-free xCELLigence and real-time cAMP assays (Figure 2). Lastly, correlations between binding kinetics and functional effects *in vitro* were explored. A schematic overview of the contents of this thesis is presented in Figure 3.

### *Outline*

In **Chapter 2** the kinetic profile of neuropeptide – receptor interactions is reviewed to provide a clear overview of the importance of binding kinetics and other kinetic interactions. This chapter also includes the potential of neuropeptide receptors in drug discovery. Furthermore the relevance of not only characterizing the drug candidate but also the endogenous ligand and target, with particular focus on their kinetic aspects, is explained.

The binding kinetics of well-known GnRH receptor agonists are analyzed in **Chapter 3**. For this purpose two kinetic binding assays were designed, validated and compared (Figure 2A and 2B).

Endogenous GnRH and a slowly dissociating analog (buserelin) were further studied in **Chapter 4**. The receptor activation profiles induced by both agonists were examined with a label-free impedance-based assay measuring changes in cell morphology (Figure 2C). This assay allowed for real-time measurements of cellular effects. A wash-out assay was also designed to examine the long-lasting effects of both agonists.

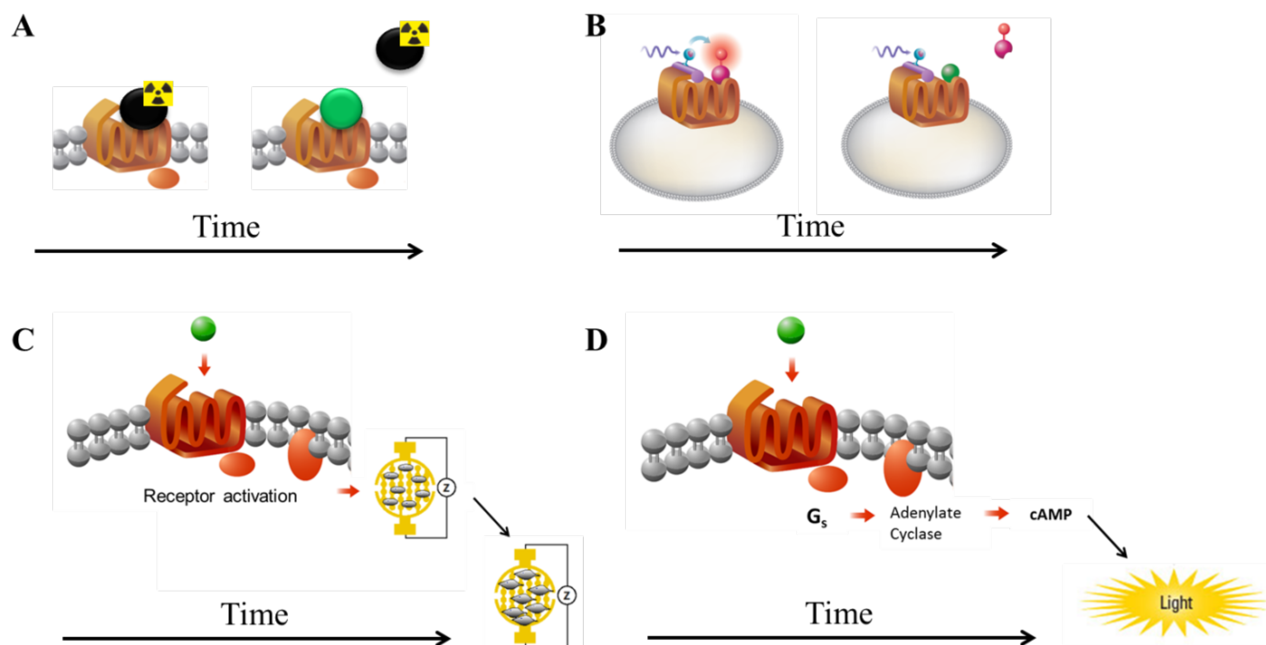


**Figure 2:** Schematic depiction of the kinetic binding assays (A and B) and kinetic functional assays (C and D) used in this thesis. (A) Radioligand binding assay. Assay requirements are cell membrane preparations and high affinity radiolabeled tracer. Over time the unlabeled ligand of interest will displace the radiolabeled tracer and from this the $k_{on}$, $k_{off}$ and residence time (RT) values of the unlabeled ligand can be calculated. (B) TR-FRET™ assay. Assay requirements are whole cells with a SNAP-tagged receptor and a high affinity fluorescent tracer. When the fluorescent tracer and tagged receptor are in close proximity a FRET signal can be detected, over time the unlabeled ligand of interest will displace the fluorescent tracer and from this the $k_{on}$, $k_{off}$ and RT values of the unlabeled ligand can be calculated. (C) Real-time functional label-free xCELLigence assay. Assay requirements are whole cells, no tracer or labeling necessary. Receptor activation can be followed over time by monitoring the cell morphology through impedance. (D) Real-time functional GloSensor™ cAMP assay. Assay requirements are whole cells transfected with GloSensor plasmid, this cAMP-biosensor undergoes a conformational change upon cAMP binding, followed by the turnover of Luciferin resulting in an increase in luminescence. cAMP production can be followed over time by monitoring luminescence.

In **Chapter 5** the binding kinetics of well-known endogenous tachykinins targeting the NK1 receptor are examined using radioligand binding studies (Figure 2A). Moreover, functional parameters such as potency and maximal response values were determined in label-free impedance-based experiments (Figure 2C).

In **Chapter 6** the relationship between *in vitro* drug-target binding kinetics and cellular responses is investigated to improve the understanding of drug efficacy *in vivo*. The functional effects of slowly (aprepitant) and fastly (DFA) dissociating NK1 receptor antagonists were examined in the presence of endogenous agonists SP or NKA. Two different kinetic functional assays were compared, namely a real-time morphology-based assay and a real-time cAMP assay (Figure 2C and 2D). Moreover, we examined the onset of receptor activation, providing a novel method to examine binding kinetics in a functional assay.

**Chapter 7** provides an overall conclusion of the novel findings presented in this thesis and new perspectives and opportunities for the research toward GPCRs, including neuropeptide receptors, and kinetic interactions are discussed. Hopefully this thesis will inspire researchers in academia and industry to implement kinetic binding studies to their research programs.

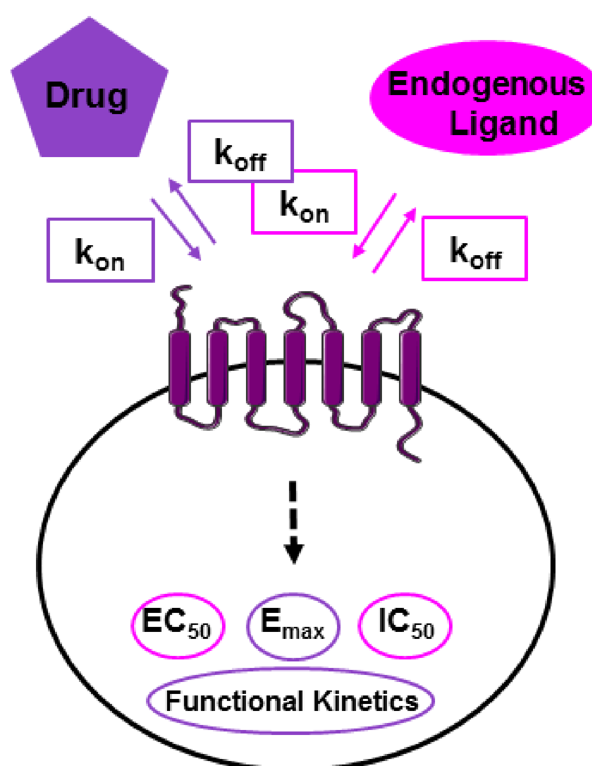


**Figure 3:** Schematic overview of the contents of this thesis. The main focus of this thesis is on the binding kinetics ($k_{on}$, $k_{off}$ and RT) of endogenous ligands and competitive drugs targeting the GnRH receptor or the NK1 receptor. Furthermore, the translation of these varying binding kinetics to *in vitro* functional effects, such as $E_{max}$, are explored.

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