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

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Citation

Nederpelt, I. (2017, April 6). *Time is of the essence - investigating kinetic interactions between drug, endogenous neuropeptides and receptor*. Retrieved from <https://hdl.handle.net/1887/47526>

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Issue Date: 2017-04-06

Chapter 7

Conclusions and future perspectives

In this thesis the binding kinetics of endogenous neuropeptides and drugs targeting two well-known neuropeptide receptors (i.e. the GnRH receptor and NK1 receptor) have been investigated. Kinetic binding and functional assays have been designed and validated to examine the differences in binding kinetics of the above-mentioned ligands. This final chapter focusses on providing a conclusion to the previous chapters while highlighting ongoing challenges with regard to binding kinetics. Moreover, opportunities for further research toward binding kinetics and neuropeptide receptors are discussed.

Conclusions

Kinetic assays contribute to a more complete pharmacological profile of ligands

Assays applied in current drug discovery are mainly used for equilibrium assessments of drug candidates. However, since kinetic binding parameters are increasingly recognized as important considerations in drug discovery a need for kinetic assays is imminent. Throughout all the chapters of this thesis, several binding and functional assays have been designed to serve kinetic binding assessments of various ligands. In Chapter 3 kinetic radioligand binding studies were compared to kinetic time-resolved fluorescence resonance energy transfer (TR-FRET) studies. For both assays a competition association assay was developed and validated and results obtained from the two methods were highly correlated. In Chapter 4 a functional wash-out experiment using the label-free real-time xCELLigence was performed with the two most kinetically diverse agonists from Chapter 3. The results from these experiments were in agreement with the kinetic data obtained with the kinetic radioligand and TR-FRET binding assays. Moreover, in Chapter 6 a medium-throughput kinetic screening assay was used to qualitatively examine the dissociation rates of a library of antagonists. Two exemplary antagonists with contrasting dissociation rates were further examined with two kinetic functional assays. Kinetic functional data from a real-time impedance-based morphology assay and a novel real-time cAMP assay corresponded well with the kinetic screening assay. Furthermore, data from both kinetic functional assays were highly correlated. In conclusion, kinetic binding and kinetic functional assays are very suitable and transferable for the investigation of kinetic ligand-receptor interactions.

Binding kinetics of endogenous neuropeptides are very diverse

Neuropeptides are 3-100 amino acid long polypeptides and are synthesized by neurons. They can bind neuropeptide receptors and together they are involved in many physiological and behavioral functions, making neuropeptides and their cognate receptors an attractive target to treat a wide range of diseases. While drug discovery programs predominantly focus on characterizing the drug candidate, knowledge of the pharmacological profile of the endogenous ligand and its target is essential when orthosteric drugs are desired. Chapter 2 reviews the kinetic profile of three exemplary neuropeptide receptors and their endogenous ligands (i.e. GnRH receptor, CRF1 receptor and NPY receptor). The neuropeptide binding kinetics, release rate and receptor internalization rates were very different for all three receptor-neuropeptide pairs emphasizing the importance and variability of kinetic profiles. Moreover, in Chapter 5 the binding kinetics of multiple endogenous ligands

targeting the NK1 receptor were determined. These ligands proved to have very different binding kinetics, particularly association rates were very variable.

Differential binding kinetics can have differential functional effects in vitro

While binding kinetics are progressively acknowledged as pivotal pharmacological parameters of a drug candidate, understanding of the translation of binding kinetics to *in vitro* and *in vivo* functional effects is still largely absent. The *in vitro* functional effects of neuropeptide agonists with variable dissociation rates were examined in Chapter 4. Long lasting receptor activation was evident for a slowly dissociating agonist while a fastly dissociating analog failed to show persistent receptor activation. Additionally, Chapter 5 demonstrated that differences in association rates lead to altered potency and efficacy values *in vitro*. Moreover, differential binding kinetics of endogenous ligands and their competing antagonists play an important role in the interaction of the ligands with the receptor. In Chapter 6, it was illustrated that slowly dissociating antagonists can have superior efficacy to its fastly dissociating counterpart. Additionally, slowly dissociating antagonists can cause a reduced rate of signal transduction. Moreover, the binding kinetics of the competing endogenous ligands also proved to be of importance. Antagonistic effects were significantly bigger in the presence of a slowly associating in comparison to a fastly associating endogenous ligand.

In summary, this thesis provides a large variety of kinetic assays that can be used to qualitatively and quantitatively determine receptor binding kinetics of ligands of interest. Additionally, the binding kinetics of endogenous neuropeptides can be very different and should therefore be considered when designing orthosteric drugs. Moreover, the combination of endogenous ligands and competitive drugs with differential binding kinetics can have significantly different functional effects *in vitro*. Finally, a wide range of kinetic assays, improved knowledge of endogenous ligand binding kinetics and a good understanding of the translational effects of binding kinetics could improve drug discovery today and decrease drug attrition rates in the future.

Lessons learned

Assay considerations

The search for optimal assay conditions is particularly challenging when designing an assay to study binding kinetics for neuropeptide ligands. Firstly, kinetic binding assays that utilize a tracer ligand are heavily reliant on the binding kinetics of the tracer. If the aim of the project is to find slowly dissociating ligands and the binding kinetics of tracer are very fast,

the mathematical model to evaluate the results is unable to provide quantitative data. To acquire quantitative parameters, the binding kinetics of the tracer ligands and unlabeled ligands should ideally be in the same time range (i.e. seconds, minutes, hours). Secondly, kinetic binding assays are often carried out at room temperature or sometimes at even lower temperatures. In these cases, the obtained kinetic binding parameters are not measured at physiological temperature (37 °C). It should be taken into account that binding kinetics will be significantly faster at higher temperatures [1-3]. Lastly, the type of kinetic assay can also be of influence. For instance, functional assays are often implemented as endpoint measurements, e.g. accumulation of a protein or 2nd messenger measured after a certain incubation time. Consequently an over- or underestimation of pharmacological parameters can occur due to inadequate incubation times. Conversion of endpoint assays into real-time measurements could circumvent this limitation.

Pharmacological profile of the drug, receptor and its endogenous ligands in the human body

The need for fast or slow binding kinetics of a drug candidate is always relative to its target system and therefore understanding of the pharmacological profile of the entire system is crucial. Firstly, the pharmacokinetic (PK) half-life of the drug candidate needs to be considered. For example, if the PK half-life is slower than the residence time (RT) of the drug the latter becomes less relevant. Conversely, if the RT of the drug is slower than the PK half-life, binding kinetics play a pivotal role in dictating the duration of action of the drug, assuming target engagement *in vivo*. Secondly, the internalization and desensitization rate of the targeted receptor should be taken into account. For instance, if the internalization rate of the receptor is faster than the dissociation rate of the drug the latter becomes trivial for agonist (and arguably antagonist) drugs. Correspondingly, if the receptor internalizes slowly, binding kinetics of agonist (and arguably antagonist) drugs can be very pertinent as the drug effect is hardly limited by receptor internalization. Finally, the kinetic profiles of both the drug and the endogenous ligands should be deliberated. Since the majority of drugs target the orthosteric binding site of the receptor, they are in constant competition with endogenous ligands. Concentrations of endogenous ligands in the human body often fluctuate significantly and binding kinetics can also be quite variable. Consequently, the binding kinetics and release rate of endogenous ligands should be determined in anticipation of achieving optimal binding kinetics of the drug candidate.

Future perspectives

This thesis predominantly focused on binding kinetics, ranging from designing and comparing kinetic assays to determining kinetic parameters of well-known drugs and endogenous ligands and translating binding kinetics to *in vitro* functional effects. The following paragraphs will discuss some future perspectives for neuropeptide receptors and GPCRs in general.

Increasing the output of kinetic assays

Within this thesis we have discussed multiple assays suitable for qualitative and quantitative measurements of ligand-receptor binding kinetics. Additional, more high-throughput screening methods could aid in the applicability of kinetic assays in drug discovery programs. Guo *et al.* have developed a medium-throughput screening assay to qualitatively estimate dissociation rates of ligands [4]. To date this screening assay has been successfully applied to multiple GPCRs [5-7], including the NK1 receptor in this thesis. However, a screening assay suitable for the estimation of association rates is still lacking. Additionally, while some binding assays can be transformed in high-throughput formats, radioligand binding assays are often performed in very low throughput formats. Glickman *et al.* have reviewed the potential of using scintillation proximity assays (SPA) in high-throughput screening and kinetic measurements [8]. To date only a few research groups have used this SPA assay to study binding kinetics of GPCR ligands. In 2007, the kinetics of small molecule GnRH antagonists were qualitatively assessed in a high-throughput format [9] and more recently, a quantitative determination of the binding kinetics of human adenosine A₁ receptor ligands was reported [10].

When researchers are interested in examining binding kinetics of antagonists in functional assays, insurmountability assays are typically the assay of choice. However, this assay is heavily reliant on assay conditions. For instance, a recent study of Vauquelin *et al.* demonstrated the importance of pre-incubation times, where too short a pre-incubation of antagonist might not be sufficient for decreasing the maximal response [11]. Importantly, “too short” pre-incubation times are dependent on the association rate of the antagonist and assay temperatures and might therefore be different per ligand and assay. Moreover, the time at which measurements are terminated could result in skewed results depending on the equilibrium between receptor, agonist and antagonist. Additionally, the kinetic data from functional assays such as the real-time cAMP assay and the real-time impedance-based morphology assay are often disregarded, as results from only one time-point are considered. Chapters 4 and 6 demonstrate the value of acknowledging functional kinetics and further studies of this concept on other targets would be beneficial.

Beyond binding kinetics, what happens after a ligand binds to its target?

Throughout the chapters in this thesis the *in vitro* translational effects of binding kinetics have been explored, including the kinetics of cellular responses. To gain more insights into these receptor activation kinetics more information should be gathered about the individual kinetics of all proteins and enzymes involved in the final response. To illustrate the kinetic information available to date, the kinetics of the $G\alpha_q$ pathway are reviewed here (Figure 1). The $G\alpha_q$ pathway is initiated by binding of an agonist to the $G\alpha_q$ coupled receptor leading to a conformational change which results in the exchange of GDP for GTP and activation of the G protein. Consequently, phospholipase C (PLC) is activated and induces hydrolysis of phosphoinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3). IP_3 subsequently binds to IP_3 receptors resulting in the release of Ca^{2+} into the cytosol, which induces the activation of myosin light chain kinases (MLCKs). Finally, upon activation of MLCKs actin filaments are contracted. All these processes together occur within seconds but information on the kinetic parameters of components of the $G\alpha_q$ pathway is limited and the representation is not consistent throughout the literature (e.g. *rate* constants versus *time* constants). It should be noted that these (rare) reports of kinetics of the activation and production of these downstream proteins could be receptor and pathway specific. For instance, G protein coupling and activation could be different for all G protein subtypes, i.e. $G\alpha_s$ protein coupling being faster or slower than $G\alpha_q$ coupling. Another important factor to consider is the kinetic differences in G protein and β -arrestin coupling. A study of Nuber *et al.*, reported a tau value (time constant) of 2.2 s for β -arrestin coupling to the β_2 -adrenergic receptor [12], versus 0.05-25 s for G protein coupling. Arguably, the GPCR under investigation could also influence the kinetics of G protein coupling. Moreover, it should be noted that ligand-receptor binding kinetics could influence the rate of G protein or β -arrestin coupling and activation.

Furthermore, the chapters in this thesis have demonstrated that varying binding kinetics can have divergent functional effects. For example, in Chapter 4 persistent signaling responses were observed for a slowly dissociating GnRH receptor agonist. Conversely, in Chapter 5 a positive correlation between potency values and association rate was found for a set of NK1 receptor agonists. Additionally, while slow dissociation rates are often thought to increase E_{max} values the opposite was observed with the NK1 receptor agonists. Arguably, slowly dissociating agonists could be more efficacious in inducing receptor internalization while fastly dissociating agonists are less efficacious in inducing this process, thereby maintaining full receptor activation and a higher maximal effect. Lastly, in Chapter 6 it was demonstrated that a slowly dissociating NK1 antagonist can significantly decrease the initial kinetics of receptor activation. Moreover, these effects were stronger for a slowly associating

agonist than for a fastly associating agonist. These results indicate that the signaling kinetics can be significantly altered with divergent binding kinetics.

Altogether, the short review and examples in this thesis could provide a foundation for further research towards not only target binding kinetics but also the kinetics involved after a ligand is bound to its receptor. Additionally, knowledge of the kinetics of signaling pathways could provide more detailed input for mathematical models used to predict the translational effects of binding kinetics.

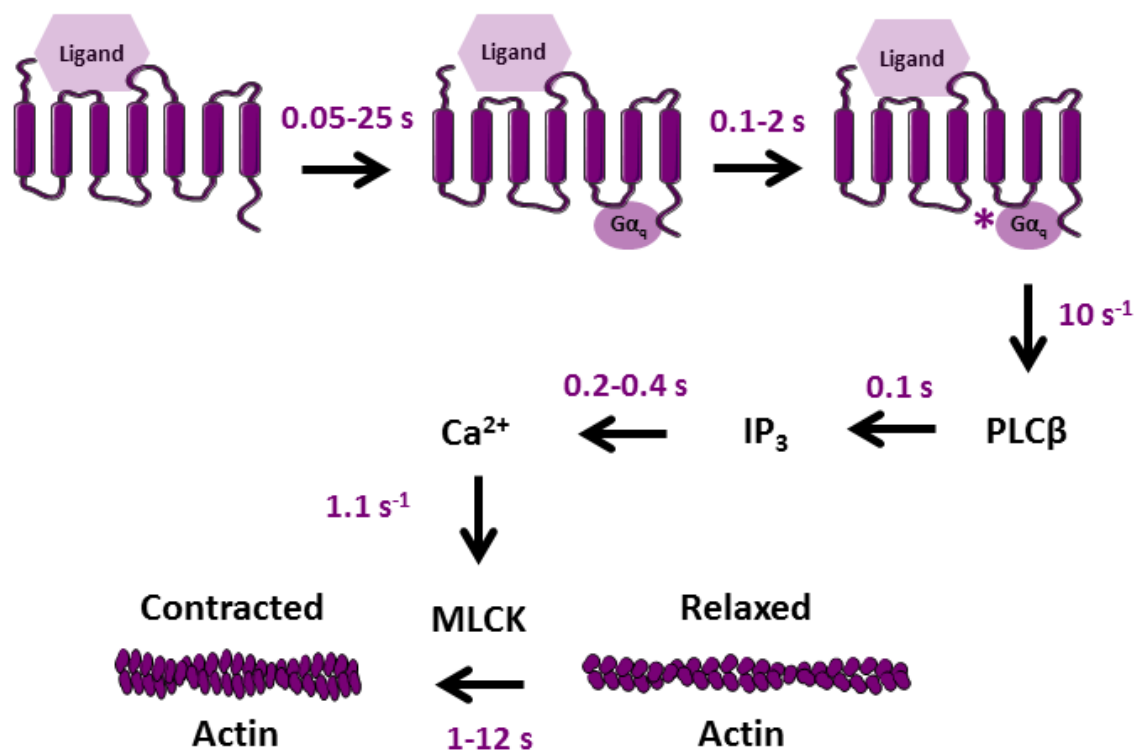


Figure 1: Upon agonist binding to a Gα_q coupled receptor, the receptor undergoes a conformational change allowing the exchange of GDP to GTP and the time constant (tau) for this process ranges from 0.05 to 25 seconds [13-15], followed by activation of the G protein (*) within 0.1 to 2 seconds [13, 14, 16]. Following G protein activation, phospholipase C is activated with an activation rate of 10 s⁻¹ [17]. Consequently, PLC induces hydrolysis of phosphoinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-triphosphate (IP₃) with a maximum production after approximately 0.1 seconds [17, 18]. IP₃ can then bind to IP₃ receptors which results in the opening of Ca²⁺ channels, releasing Ca²⁺ into the cytosol within 0.2-0.4 seconds [19-21]. In turn, Ca²⁺ induces the activation of myosin light chain kinases (MLCKs) with a rate of 1.1 s⁻¹ [22]. Activation of MLCKs will lead to actin contraction after 1-12 seconds [23, 24].

The potential role of binding kinetics in biased signaling

To date, the concept of biased signaling has gained increasing consideration in the GPCR field. Biased signaling or biased agonism can be described by the ability of an agonist to selectively activate a specific signaling pathway (Figure 2). For instance, ligand A may predominantly activate G proteins (Figure 2A) while ligand B may prefer β -arrestin activation (Figure 2B). Concurrently, ligands can also differentiate between different G proteins where ligand C could favor $G\alpha_i$ signaling while ligand D could favor $G\alpha_q$ signaling (Figure 2C). Biased agonism and its role in GPCR activation has already been extensively studied [25-27]. Interestingly, a recent publication proposed a new role of ligand-receptor binding kinetics in apparent biased agonism [28]. The authors examined the binding kinetics and functional effects of several dopamine D_2 receptor (D_2R) agonists. They were able to correlate differential binding kinetics to various biased signaling profiles of D_2R agonists. Moreover, it was shown that agonist bias could be reversed over time in different cell signaling processes. This research article provides a new perspective on binding kinetics and its role in functional processes and presents great potential for further research.

Illustratively, biased signaling at neuropeptide receptors such as the opioid receptor family is frequently observed [29-33]. The opioid receptor family consists of three members, i.e. δ opioid, κ opioid and μ opioid receptors, the latter being the most studied. For instance, Thompson *et al.* studied the potential for biased agonism of endogenous opioids [34]. They reported that several endogenous ligands showed distinct biased signaling profiles in comparison to the other endogenous ligands. The binding kinetics of these endogenous ligands have not been examined thus far and considering our findings in Chapter 5 it would be worthwhile to study the kinetic binding parameters of these ligands. Consequently, it should be investigated if the binding kinetics can be correlated with the various signaling profiles reported in the study of Thompson *et al.* [34].

Another neuropeptide receptor that is known for biased signaling is the parathyroid hormone receptor type 1 (PTHr1) [35]. Recent studies have proposed that various PTH analogs can induce differential signaling pathways. More interestingly, it has been suggested that persistent signaling is induced by ligands that prefer G protein-independent pathways while ligands that prefer G protein-dependent pathways induce a more transient response [36]. Although the binding kinetics of the examined ligands were not reported, the authors did briefly discuss the possibility of long residence time being involved in inducing these persistent signaling profiles. Similarly, Hothersall *et al.* recently reviewed the role of residence time in sustained signaling profiles [37] which agreed with our findings in Chapter

4. To test if long residence time is involved in persistent signaling profiles, the binding kinetics of PTH analogs should be examined.

In conclusion, specific knowledge of how ligand binding kinetics can influence biased profiles of agonists for neuropeptide receptors, and GPCRs in general, could improve the discovery of novel drugs targeting this receptor family.

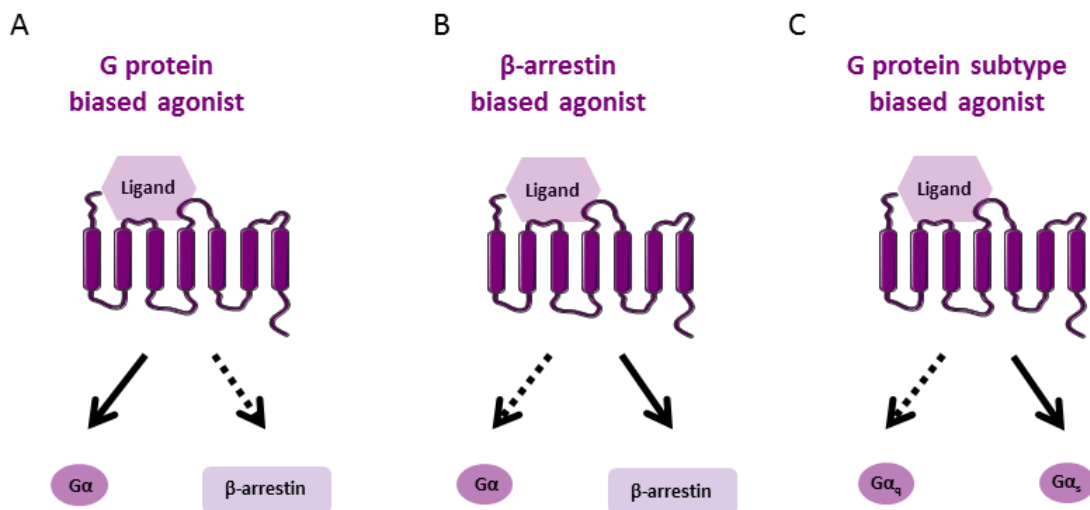


Figure 2: Schematic overview of various types of biased agonists. Upon agonist binding, biased agonism can be observed towards G protein activation (A), β-arrestin activation (B) or a specific G protein subtype, such as Gα_s (C).

The future of binding kinetics at neuropeptide receptors

Neuropeptide receptors present an attractive drug target in the treatment of a wide range of therapeutic areas such as cancer, inflammation and reproduction [38]. In view of the latter, this receptor family is well-known for its role in the hypothalamic-pituitary-gonadal axis, playing a crucial role in reproductive functions. Over the past decade, kisspeptin and its receptor KISS1R (also known as GPR54) have gained interest in the treatment of sex hormone-dependent disorders such as infertility and precocious puberty [39]. More interestingly, increasing evidence indicates the benefits of long-acting kisspeptin analogues targeting KISS1R [39-43]. While current research is mainly focused on increasing the metabolic and plasma half-life of KISS1R ligands, another approach could be to design drugs with slow dissociation kinetics. It would be interesting to examine the binding kinetics of kisspeptin and its analogues and establish a structure-kinetics relationship (SKR) study with the aim of finding slowly dissociating ligands for KISS1R.

Kinetic ambition

This thesis evolves around the kinetic binding interactions between the endogenous ligand, the drug and the receptor followed by the cellular response. Three main conclusions are drawn;

- 1) Both kinetic binding assays such as radioligand binding and TR-FRET studies, and kinetic functional assays such as real-time cAMP and real-time morphology studies, are very suitable to qualitatively and quantitatively study the binding kinetics of numerous ligands.
- 2) The binding kinetics of endogenous neuropeptides are very divergent.
- 3) Differential binding kinetics will translate into differential functional effects *in vitro*.

In conclusion, the toolbox of kinetic assays is expanding which allows more accessible and high-throughput measurements of binding kinetics. Secondly, these kinetic assays enable the assessments of kinetic binding parameters of endogenous ligands and drug candidates. Lastly, including kinetic binding studies in the drug discovery paradigm will improve the understanding of drug-target interactions, translation to functional effects and predictions of *in vivo* responses.

Finally, I am hopeful that this thesis will contribute to an increased understanding of ligand-receptor interactions and that it provides a larger toolbox suitable for studying these kinetic interactions. My ambition is to transform binding kinetics into traditional, indispensable, drug discovery parameters and thereby improve the success rate of drug discovery in the future.

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