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# Multi-biomarker pharmacokinetic-pharmacodynamic relationships of central nervous systems active dopaminergic drugs

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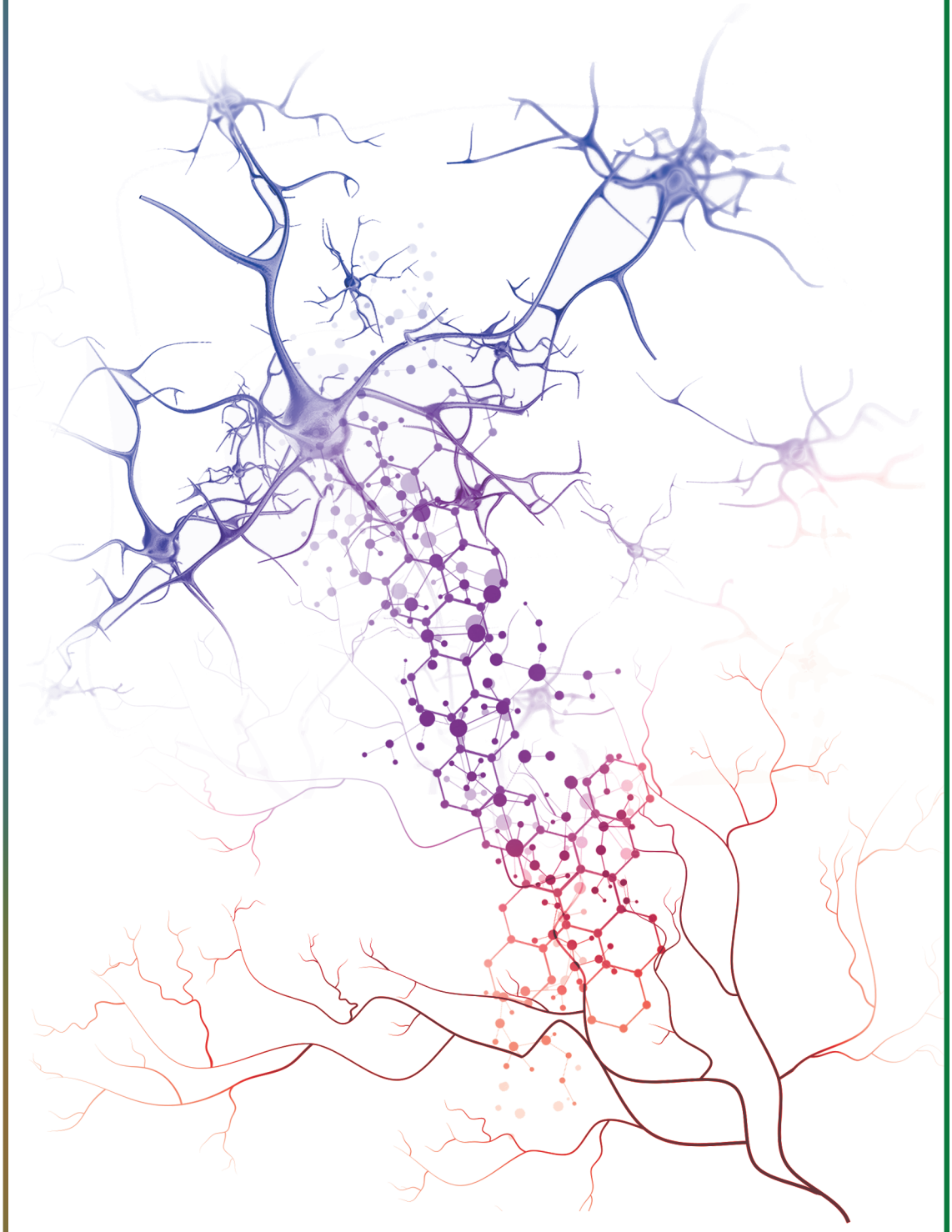



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# Multi-Biomarker PK/PD Relationships

## of Central Nervous Systems Active Dopaminergic Drugs



Willem van den Brink

# Multi-Biomarker Pharmacokinetic-Pharmacodynamic Relationships of Central Nervous Systems Active Dopaminergic Drugs

**Willem van den Brink**

**Ph.D. Thesis, Leiden University, November 2018**

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# CHAPTER 1

## DYNAMICAL ANALYSIS OF BLOOD-BASED SYSTEMS BIOMARKERS OF CENTRAL NERVOUS SYSTEM DRUGS

## Scope and intent of investigations

The body is a complex of interacting networks, and therefore the disciplines of pathology and pharmacology are shifting from a purely reductionist approach to a method that also includes an holistic approach [1–3]. Moreover, the biological processes related to these networks behave dynamically in response to drugs. The understanding of the dynamics of the biological processes improves the success rates in drug development [4,5]. In this thesis, we will focus on central nervous system (CNS) drug development as an example of a complex system that may suffer from a large series of serious pathologies. Neurological disorders and mental illnesses, hereafter named CNS diseases, are among the main contributors to the global burden of disease affecting millions of people worldwide [6–8]. Yet, CNS drug development is hampered by low success rates (<10%) and long duration of development (~12.6 years) [9,10]. Among other reasons, this can be attributed to lack of understanding of underlying pathological and pharmacological processes and the lack of validated biomarkers that represent these processes. Indeed, biochemical pathway analysis in cerebrospinal fluid (CSF) show a multitude of pathways involved in CNS diseases, with large overlap among CNS diseases [11]. *In silico* evaluation predicted that schizophrenia is characterized by a disbalance among the different neurochemical pathways throughout the brain that could partly be restored by antipsychotic drug treatment [12]. One of the questions is how we can translate these insights into methodologies that can be applied in CNS drug development, also considering the limitations of sampling from the human brain. In this thesis, we will therefore focus on two questions:

1. How can the relation between drug dose and the dynamic systems response be quantified *in vivo*?
2. How can blood-based markers that represent central drug effect be obtained?

### *Section I – General introduction*

In **Chapter 2** we first give a general introduction into the fields of pharmacokinetic/pharmacodynamic (PK/PD) modeling and pharmacometabolomics and how these approaches have been applied in CNS drug development. As an example, PK/PD modeling enabled interspecies translation of the prolactin response, which is a biomarker of dopamine $D_2$ receptor antagonist effect [13]. Another example showed how pharmacometabolomics reveals new lipid biomarkers to evaluate and understand the relation between $D_2$ antagonist treatment and weight gain [14]. The chapter subsequently discusses the challenges of integrating PK/PD modeling and pharmacometabolomics to enable the dynamic evaluation of the systems response upon drug treatment. A specific attention is paid to interspecies scaling in translational drug development.

**Chapter 3** describes an overview of biochemical and endocrine markers in the brain and in plasma that have been associated with dopaminergic agents. Dopamine drugs are, for

example, used to treat schizophrenia ($D_2$ antagonists) or Parkinson's disease ($D_2$ agonists). In addition to their interference with the dopamine pathway they also influence other neurochemical pathways [15]. Furthermore, since the $D_2$ system is involved in the control of hormone release of the pituitary, peripheral hormone concentrations are expected to change upon administration of dopaminergic agents [16–18]. Finally, the $D_2$ system is not only functional in the brain, in fact, it is widely distributed throughout the body being expressed in, for example, the gut, the adrenal glands and the kidney [19]. The aim of this chapter is to obtain an overview of the different biochemical and endocrine pathways in the brain and in plasma that are perturbed by these agents, to subsequently point directions to further improve biomarker-driven CNS drug development. Special attention will be paid to potential blood-based biomarkers that reflect drug effects in the brain.

### *Section II – The dynamical neuroendocrine systems response to study dopamine $D_2$ drug effects*

The neuroendocrine system provides a tight connection between the brain and the periphery. Its biological function is to control peripheral processes from the brain, such as stress and reproductive function. One of these neuronal pathways is the dopaminergic tuberoinfundibular pathway. It is well-known that activation of this pathway leads to enhanced release of dopamine into the pituitary, where it inhibits the prolactin release from the lactotrophs [17]. With regard to CNS drug development, this connection has received much attention for the discovery of blood-based biomarkers that reflect central drug effect [16].

In **Chapter 4** we use the selective dopamine $D_2$ antagonist remoxipride as a paradigm compound to evaluate the effect of $D_2$ antagonism on the release of pituitary hormones and neuropeptides. While prolactin has been widely used to dynamically evaluate $D_2$ antagonistic drug effects [13,16,20], the other pituitary hormones and neuropeptides are not so often used for such evaluation. Given the biological relation between dopamine and multiple hormones, the aim of this chapter is to dynamically evaluate the neuroendocrine systems response upon remoxipride treatment in rats.

To place the results of remoxipride into perspective, in **Chapter 5**, the neuroendocrine systems response is evaluated with the selective $D_2$ agonist quinpirole as paradigm compound. $D_2$ agonists may be expected to interact with the neuroendocrine system inversely to $D_2$ antagonists, but this is not necessarily the case (**Chapter 3**). Comparing agonistic with antagonistic interactions is envisioned to provide more insight into the dopamine specific effects. While single administration dynamics provide insight into the short-term mechanistic interaction between the drug and the biological system, it does not take into account longer-term processes, such as tolerance and sensitization. As these processes

involve behavioral changes upon quinpirole treatment in rats [21], the neuroendocrine systems dynamics are evaluated with single and multiple quinpirole administration.

### *Section III – The dynamical biochemical systems response to study dopamine $D_2$ drug effects*

The neuroendocrine system only represents a small part of the system-wide dopaminergic effects. Moreover, it utilizes one of the mechanisms through which blood-based biomarkers of neurological effects can be obtained. Indeed, neurochemical markers may also distribute from the brain into plasma to be discovered as blood-based biomarker. Pharmacometabolomics has proven useful for discovery of systems biomarkers of CNS drug effects and diseases [22,23]. For example, schizophrenia involves disturbances multiple metabolic pathways, including energy metabolism, neurotransmitter metabolism, fatty acid biosynthesis, and phospholipid metabolism, that are partially restored following risperidone treatment. Biomarkers such as myo-inositol, uric acid, and tryptophan were found important to distinguish disease and treatment groups [24]. Thus, pharmacometabolomics provides a powerful approach for CNS drug biomarker discovery. At the same time, there is no methodology available that quantifies the dynamical pharmacometabolomics response upon drug treatment. A logical step is the integration of PK/PD modeling and pharmacometabolomics, as is discussed in **Chapter 2**.

In **Chapter 6** a methodology is developed to describe the pharmacometabolomics data by a PK/PD model, in order to reveal the systems-wide pharmacodynamics of remoxipride in plasma. The aim of this chapter is to quantify the multiple dose-response relationships underlying the systems-wide effects of remoxipride in terms of pharmacologically meaningful parameters such as potency ($EC_{50}$), maximal effect ($E_{MAX}$) and endogenous metabolite turnover rate ($k_{OUT}$). Here, it is important to describe the pharmacokinetics of remoxipride simultaneously with the pharmacodynamics, in order to account for potential temporal delays between drug concentration and biomarker profile. Additionally, biomarkers that represent the diverse response patterns are presented for future validation.

The PK/PD-metabolomics method is taken a step further in **Chapter 7** in which we describe multiple biomarker responses in plasma as well as in brain extracellular fluid upon administration with quinpirole. Again, it is important to describe the pharmacokinetics and the pharmacodynamics simultaneously, but now with an additional layer of complexity: biomarker responses in brain as well as in plasma. This provides us the opportunity to identify response patterns that are specific for the brain or the periphery. Moreover, for biomarkers that show a response in brain as well as in plasma, we can indicate the target site of action by comparing the temporal response patterns in both biofluids. We show that multiple biomarkers respond in the brain and in plasma with different pharmacodynamic

characteristics (e.g. $EC_{50}$, $E_{MAX}$, $k_{OUT}$). We also present potential (blood-based) biomarkers of quinpirole effects for future validation.

*Section IV – General discussion and conclusion*

In **Chapter 8** we give an overall reflection on the results in the different chapters, discuss the implications of our findings, and provide directions for future research on the integration of PK/PD modeling and pharmacometabolomics in CNS drug development.

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# CHAPTER 2

## BUNDLING ARROWS: IMPROVING TRANSLATIONAL CNS DRUG DEVELOPMENT BY INTEGRATED PK/PD-METABOLOMICS

W.J. van den Brink, T. Hankemeier, P.H. van der Graaf, E.C.M. de Lange

Expert Opinion on Drug Discovery 2018 13(6):539-550

## Abstract

Introduction: Central nervous system (CNS) diseases affect millions of people worldwide, and the number of people is quickly growing. Unfortunately, the success of new CNS drugs in clinical development is less than 10%, and this has been contributed to the complexity of the CNS, unexpected side effects, difficulties for drugs to penetrate the brain and the lack of biomarkers.

Areas covered: First, we discuss how pharmacokinetic/pharmacodynamic (PK/PD) models are designed to predict the dose-dependent time course of effect, and how they are used to translate drug effects from animal to men. Then, we discuss how pharmacometabolomics provides insight into system-wide pharmacological effects and why it is a promising method to study interspecies differences. Third, we advocate the application of PK/PD-metabolomics modeling to advance translational CNS drug development by discussing its opportunities and challenges.

Expert opinion: It is envisioned that PK/PD-metabolomics will increase understanding of CNS drug effects and improves translational CNS drug development to increase success rates. Successful further development of this concept will need multi-level and longitudinal biomarker evaluation over a large dose range, multi-tissue biomarker evaluation, and the generation of a proof of principle by application to multiple CNS drugs in multiple species.


**Key words:** Biomarkers; CNS drug development; Interspecies scaling; Pharmacometabolomics; PK/PD modeling; Systems pharmacology

### Highlights

1. Translational CNS drug development is shifting from an empirical to a mechanistic approach
2. PK/PD modeling in conjunction with scaling principles enables the interspecies translation of pharmacological CNS effects
3. Pharmacometabolomics provides a mean to compare the system-wide pharmacological CNS effects in multiple species
4. An integrated PK/PD-metabolomics is envisioned to increase understanding of CNS drug effects and improve translational CNS drug development
5. To achieve an integrated PK/PD-metabolomics approach, we need multi-level biomarker evaluations, to study a large dose range, and longitudinal sampling from the brain, plasma, and CSF.

# 1. Introduction

Central nervous system (CNS) diseases affect millions of people worldwide, and the number of people with such disease is quickly growing [1]. They are characterized by their high complexity as multiple neurotransmitter systems and biochemical pathways are involved [2–4]. It is therefore not surprising that CNS drug development suffers from low success rates (< 10%) and long duration (~12.6 years) [5,6]. Moreover, it is hampered by CNS mediated side effects (e.g. nausea, dizziness), the presence of the BBB, lack of effective animal models and/or lack of integrative investigations in animals to investigate the mechanisms of CNS pathology and pharmacology, and the lack of biomarkers representing these mechanisms [6–9]. In particular, the translation from preclinical to early clinical studies is difficult.

Clearly, there is a need to improve the current methodologies within CNS drug development. Two promising methods in this regard are pharmacokinetic/pharmacodynamic (PK/PD) modeling and pharmacometabolomics [10–12]. PK/PD modeling allows to "characterize and predict the time course of drug effects under (patho)physiological conditions" [13]. Pharmacometabolomics involves the "determination of the metabolic state to define signatures before and after drug exposure that might inform treatment outcomes" [14]. This review discusses how translational CNS drug development can be improved by the integrated application of PK/PD modeling and pharmacometabolomics. An overview will be provided of the role of both fields in translational CNS drug development, after which the opportunities and challenges of an integrated approach will be discussed.

# 2. Biomarker-driven development of central nervous system drugs

Current translational CNS drug development highly relies on behavioral endpoints, such as the 5-choice serial reaction time task. While these endpoints may provide reasonable construct validity, their predictive validity is low [15,16]. Predictive validity, which includes a mechanistic rationale between the drug effect and the endpoint, is important to translate the preclinical to the clinical pharmacology [17]. It is therefore that biomarkers are increasingly recognized as an essential element of CNS drug development [7,18–20]. Indeed, biomarkers have been defined as indicators of specific pharmacological or physiological processes [21,22]. Current biomarker strategies include receptor occupancy [23–25], functional imaging [26,27], biochemical measures in CSF [20], EEG [28,29], or physiological measures such as hormone release [30]. Biomarkers have been classified into multiple pharmacological levels following the causal relation of the drug dose to the clinical effect [31]. These are i) genotype or phenotype, ii) drug exposure, iii) target occupancy, iv) target activation, v) physiological/laboratory measures, vi) disease processes, vii) clinical scales.

Such classification provides a framework for rational drug development. In particular, as depicted in Figure 1, confidence in the drug exposure, target binding, and target activation are key components to guarantee successful translational drug development [12].

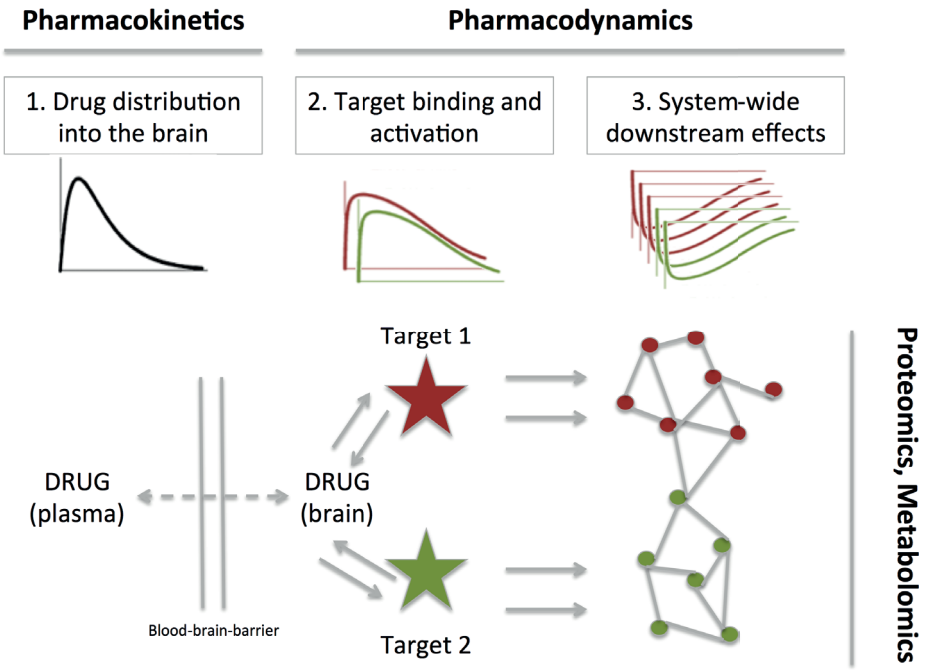



**Figure 1. The conceptualization of an integrative approach. The plasma and brain drug exposure profile are determined by the pharmacokinetics, to drive the target binding and activation of potentially multiple targets. The activation (or inhibition) of these targets elicits multiple downstream biochemical effects, which can be evaluated by proteomics or metabolomics. These processes are described by mathematical expressions as developed in the field of PK/PD modeling.**

## 3. PK/PD modeling in biomarker-driven CNS drug development

Not only the measurement of biomarkers is important for prediction of the dose-effect relation. It is also important to quantify the non-linear and time-dependent relations between the biomarkers to obtain insight into the dynamics of the pharmacological processes. PK/PD modeling is used to mathematically describe these processes in terms of PK and PD parameters, for example, clearance, volume of distribution, maximal drug effect or *in vivo* potency. Biomarkers thus enable the quantitative characterization of the processes that are on the causal path between dose and effect. More specifically, biomarker data gives insight into pharmacokinetic (PK) parameters such as clearance and volume of distri-

bution, or pharmacodynamic (PD) parameters such as maximal effect and *in vivo* potency. As such, PK/PD parameters provide a quantitative and scalable perspective on interspecies differences, thereby allowing the prediction of the first-in-human dose [17,30,32]. The components of a PK/PD model are the i) PK model that describes the exposure of the drug in the body; ii) the PD model that captures the relation between the drug concentration and the effect and iii) the link model that accounts for the possible delay between the concentration-time and the effect-time profile [13]. These components are further described in the next section.

## *3.1 PK/PD models*

### *3.1.1 PK models*

A crucial aspect of successful CNS drug development is the understanding of the distribution of the drug into the brain [33–35]. The intensity, onset, and duration of CNS drug effects depend on the concentration-time profile at the site of drug action. This brain is separated from the plasma by the blood-brain-barrier (BBB), which often influences the rate and extent of drug distribution into the brain. The transport over this barrier may be passive (driven by concentration gradient) and active (driven by transporters). In addition to the BBB, other factors, such as plasma protein binding, brain tissue binding, cellular uptake, brain metabolism, CSF flow, and physicochemical properties of the drug influence the drug exposure profile in the brain (for reviews and key research on this topic see references [36–41]). Although classical PK modeling still often is used, physiology-based PK (PBPK) modeling is increasingly applied to predict the time course of drug concentrations at the site of drug action.

### *3.1.2. PD models*

Whereas the understanding of the drug exposure at the target site is a crucial aspect in CNS drug development, the subsequent linkage to the PD (i.e. target binding and activation, and downstream physiological responses) is equally important for understanding drug effects [11,12]. Among others, receptor occupancy [23,25], EEG measures [28,42], hormone release [30] have been used to characterize the pharmacodynamic response of CNS drugs. The mathematical linkage of PD responses to the drug exposure has been extensively reviewed by Danhof et al. (2007) [43]. Still, in practice, an integrative approach including PK and PD in one study is often lacking. A widely used equation to link PK to PD is the empirical sigmoid $E_{max}$ equation:

$$E = \frac{E_{max} \cdot C}{EC_{50} + C}, \quad (1)$$

where $E_{max}$ is the maximal observed drug effect, $EC_{50}$ is the *in vivo* potency and $C$ is the concentration around the target (e.g. $brain_{ECF}$).

*3.1.3 Link models*

The effect-time profile is often delayed as compared to the drug concentration-time profile. If only plasma drug concentrations are known, one may assume that the delay is caused by slow distribution from plasma to the site of drug action. In such case, an effect compartment model is used to account for the delay [44]. Slow target binding kinetics may also cause a delay between PK and PD, and in such case, these can be explicitly included in the model [45]. Finally, downstream signal transduction may be relatively slow compared to the plasma PK, drug distribution, and the target binding kinetics, being responsible for the delay of the effect-time profile. This is often accounted for by a turnover model [46]. It assumes a continuous process of production and degradation (turnover) that drives the basal biomarker levels. The drug effect influences either the production or the degradation rates through inhibition or stimulation, thereby causing an increase or a decrease of the biomarkers levels.

## *3.2 Interspecies scaling*

PK/PD modeling enables the rational extrapolation of drug effects between animal and men [47]. It does so by explicitly distinguishing the drug- and system-specific parameters [17,32]. Typical drug-specific parameters are plasma protein binding, target-binding affinity, and intrinsic efficacy, while examples of system-specific parameters are tissue volumes, clearances, receptor expression, and turnover rate constants. While drug-specific parameters can be obtained from *in vitro* experiments, system-specific parameters can only be estimated from *in vivo* data and may be species-dependent. The interspecies scaling of these parameters follows two principles: allometric scaling and physiology-based scaling. With allometric scaling it is assumed that the parameters are dependent on bodyweight following a power function [48,49]:

$$P_{hum} = P_{animal} \cdot \left( \frac{BW_{human}}{BW_{animal}} \right)^{b}, \tag{2}$$

where $P$ is the mathematical model parameter, $BW$ is the bodyweight, and $b$ is the species-independent scaling exponent. Typically, allometric scaling is applied to clearance, volume of distribution, and turnover rate constants. The scaling exponent generally is 0.75 for the clearance, 1 for the drug distribution volume and -0.25 for the turnover rate constant [50,51]. As an illustrative example, the acetaminophen clearance extrapolates over a large range of species, including zebrafish larvae, rat and human, using allometric scaling [52].

In another study, the prolactin effects of remoxipride were successfully scaled from rat to man, by applying allometric scaling on the turnover rate of prolactin in plasma [30].

The principle of physiology-based scaling is to replace the animal parameters by the human parameters [53]. While the physiology-based scaling of CNS PK is well developed, for example to predict the human CSF drug concentrations of acetaminophen and morphine [37,39,54,55], it has only started to emerge for PD. Some studies have shown that PD parameters such as $E_{max}$ and $EC_{50}$ may be similar across species for a series of drugs, for example for opioids and their effect on electrocardiogram output [29,56]. In contrast, other studies showed species-dependent PD parameters. A recent evaluation of a Transient Receptor Potential Melastatin-8 blocker showed 3-fold cross-species (mouse versus dog) differences in its potency, resulting in clinically important differences in core body temperature predictions [57]. In another study, the $E_{max}$ and $EC_{50}$ for prolonging the QT-interval were found to differ between humans and dogs [58]. A third publication showed that the affinity of psychoactive drugs differed significantly between, for example, the $D1_{rat}$ and $5HT2_{rat}$, and $D1_{human}$ and $5HT2_{human}$ receptors [9]. Also, the $E_{max}$ and the $EC_{50}$ of prolactin to control its own release was found different between rats and humans [30].

Overall, these examples show that the interspecies translation of CNS drug effects needs to be driven by the mechanistic understanding of drug- and system-specific properties at the level of PK and PD. Both allometric scaling and physiology-based scaling of PK/PD parameters can be used to support interspecies translation on basis of *in vitro* (drug-specific) and *in vivo* (system-specific) parameters. If clinical data is not available from same-in-class drugs, multiple species can be evaluated for these properties and simulations of worst-to-best case scenarios can be used to guide the dosing strategies during early clinical development [57].

## 4. Pharmacometabolomics in biomarker-driven CNS drug development

Although PK/PD modeling aims to predict single biomarker time courses, it appears that CNS drugs typically affect multiple biochemical pathways [59,60]. For example, risperidone affected multiple pathways including energy metabolism, antioxidant defense systems, neurotransmitter metabolism, fatty acid biosynthesis, and phospholipid metabolism [61]. In fact, many successful CNS drugs were identified by serendipity on basis of phenotypic changes *in vivo* [62]. Indeed, the efficacy of neurological drugs is associated with multi-target affinity [63–65]. As an example, antipsychotics typically have interactions with multiple targets (up to 26 for clozapine and quetiapine) [63,64]. A comparison of haloperidol and clozapine showed that they caused a different biochemical phenotype,

that of clozapine close to that of the 5-$HT_{2A}$ antagonist M100907 [66]. However, although multi-target pharmacology may be related to the efficacy of e.g. clozapine, it is also associated with unwanted effects, for example cardiovascular disease [63]. Good insight into the systems behavior of multi-target drugs is essential to anticipate the (post-)clinical benefit-risk balance of drugs during early development. As such, pharmacometabolomics is suggested as an important method in drug development to biochemically understand *in vivo* neuropharmacological effects [67–69]. For example, using lipidomics, the underlying pathways were identified that may explain antipsychotic-induced weight gain [70]. Metabolomics analyzes hundreds of biochemical molecules in biological samples, and as such, it can provide system-wide pharmacological biomarkers [14]. By measuring the biochemical end-products of cellular reactions it provides an intermediate metabolic phenotype between gene expression and drug effects on one hand, and clinical outcome on the other hand. In other words, it fulfills the definition of a type 4 biomarker [31] and can provide insights into the pharmacological pathways relevant to the clinical outcome. For example, a urinary metabolomics fingerprint could be used to predict the Kellgren-Lawrence grade as a clinical endpoint for osteoarthritis [71]. As compared to other biomarker types, such as functional imaging, pharmacometabolomics is relatively cheap and easy to apply in preclinical and early clinical studies. Moreover, biochemical pathways are relatively similar across mammalian species, suggesting potential for applying pharmacometabolomics in translational drug development [72,73]. The main analytical tools that are used for metabolomics are nuclear magnetic resonance (NMR) technology and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Both technologies have the advantage that they can identify a wide range of small molecules, providing a comprehensive picture of the metabolome. The metabolome contains more than 40000 molecules, which typically have a molecular weight below 2 kD [74].

Of interest for the CNS-pharmacology are the energy substrates, neurotransmitters, amino acids and structural lipids, all of which are involved in cell viability, signaling, and cell membrane function [2]. It was specifically observed that the corresponding pathways were overlapping among CNS drugs and diseases, indicating that multi-biomarker approaches are important for the evaluation of drug effects [2,59]. Several clinical studies have been performed utilizing pharmacometabolomics for the study of CNS drug effects, although the main focus has been on the disease rather than on the treatment [75]. These studies showed that pharmacometabolomics has the potential to reveal new insights into lipid-related side effects of antipsychotics [70,61], enable the early prediction of antidepressant effects on multiple biochemical pathways [76], or identify systems biomarkers of motor neuron disease treatment [77] and antiparkinson drugs [78].

### *4.1 Multivariate analysis of pharmacometabolomics data*

The endogenous metabolites are members of biologically highly connected pathways. Pharmacometabolomics data is therefore often evaluated by multivariate data analysis, which takes into account the connectivity among the individual metabolites. The purpose is to identify biomarkers that classify subgroups (e.g. treated vs. non-treated), and to elucidate the biochemical pathways that are perturbed with drug treatment. There are roughly three types of multivariate analyses: descriptive analyses (e.g. correlations), unsupervised methods, and supervised methods. (for review see [79]). An example of descriptive analysis is correlations between metabolite levels. These can be used to define a network with metabolites as nodes, while edges are drawn if the correlation coefficient exceeds a certain threshold (e.g. 0.8). In addition to correlation-based networks, more sophisticated methods have been developed, such as Gaussian graphical networks. These networks eliminate the direct correlations that are explained by indirect correlations, providing a much cleaner network [80,81]. The power of network analysis is that it shows a clear picture of the multifactorial changes under particular conditions, for example, treated vs. non-treated. In particular, it can identify the key metabolite pathways that underlie the pharmacological effects [82], as well as their synergistic or resilient characteristics [83]. A network approach was, for example, used to understand the systems-wide effects of sertraline, showing that the tricarboxylic acid and the urea cycle, fatty acids and intermediates of lipid biosynthesis, amino acids, sugars and gut-derived metabolites were changed with four-week treatment [76]. A well-known unsupervised method is cluster analysis, which classifies samples or metabolites on basis of the proximity to each other with regard to, for example, the metabolite levels or the chemical similarity. This can reveal interesting patterns in the data, such as clusters of genes or metabolites that have similar biological functions [84]. Another well-known unsupervised method is principal component analysis (PCA), which identifies the latent variables (principal components) underlying pharmacometabolomics data [85]. These latent variables then represent the 'overall' effect of a treatment in case of a pharmacometabolomics study. Closely related to PCA is the supervised partial least squares regression (PLS). This method optimizes a model to predict a certain output variable, for example, disease status or dose [77]. Both PCA and PLS elucidate which metabolites are most influential in explaining the variation between the subgroups.

### *4.2 Translational pharmacometabolomics to study CNS drug effects*

The specific application of metabolomics in translational drug development has gained attention more than 10 years ago [72]. Metabolomics has an advantage over other 'omics' approaches with regard to interspecies translation. Indeed, endogenous metabolite pathways are highly identical among mammalian species. A recent study thoroughly compared the biochemical reaction network of rat and human, showing a strong overlap [73]. There

are, however, only a few studies that applied metabolomics *in vivo* to compare different species. Some studies showed how the metabolic phenotype of animal disease models for osteoarthritis and multiple sclerosis overlapped with the patients' metabolic phenotype, indicating the potential of metabolomics for interspecies translation [72,86,71,87]. Although no efforts have yet been made to compare the animal and human metabolic phenotypes after drug treatment *in vivo*, the rat and mouse metabolic phenotypes were compared to study their differential sensitivity to cocaine [88]. It was found that the aryl hydroxylation pathway was dominant in rats, causing increased excretion of cocaine, which was not the case in mice. Interestingly, when comparing microsomes of humans versus these two species, the human cocaine metabolism showed a closer resemblance to the mice cocaine metabolism, indicating that the mouse is a better animal model for evaluation of cocaine sensitivity in humans. This study shows how pharmacometabolomics could be used to guide interspecies translation of CNS drug effects. Nevertheless, care should be taken with regard to the assumption that the biochemical reaction networks are species independent. The bile acid, carbohydrate, glycine-serine-threonine, purine and ascorbic acid pathways were found to have reactions specific for rats, while the glycan and sphingolipid pathways included human specific reactions, as measured in hepatic cells. These species differences may result in large differences in even opposite effects on certain endogenous metabolites [73]. In such case, further information on the pathway is important to extrapolate the preclinical findings. The ascorbic acid change in rats, for example, reflects a change in the glucuronic acid metabolism, which is also present in humans [89]. This information can then be used for the interspecies translation.

## 5. The integration of pharmacometabolomics and PK/PD modeling in translational CNS drug development

Translational CNS drug development can thus potentially profit from both PK/PD modeling and pharmacometabolomics; both are envisioned to contribute to biomarker-driven development. An integration of PK/PD modeling and pharmacometabolomics is envisioned to provide scalable system-specific parameters for multiple biochemical pathways that are potentially relevant for the clinical drug effects. A conceptual workflow of such translational approach is depicted in Figure 2. Recent suggestions have been made to use pharmacometabolomics in PK/PD frameworks as static or dynamic markers [90,91]. While static metabolic phenotypes can be used as a predictor for treatment responses, dynamic metabolic phenotypes allow to follow the treatment effect over time to evaluate the system-wide dynamics [90,92,93].

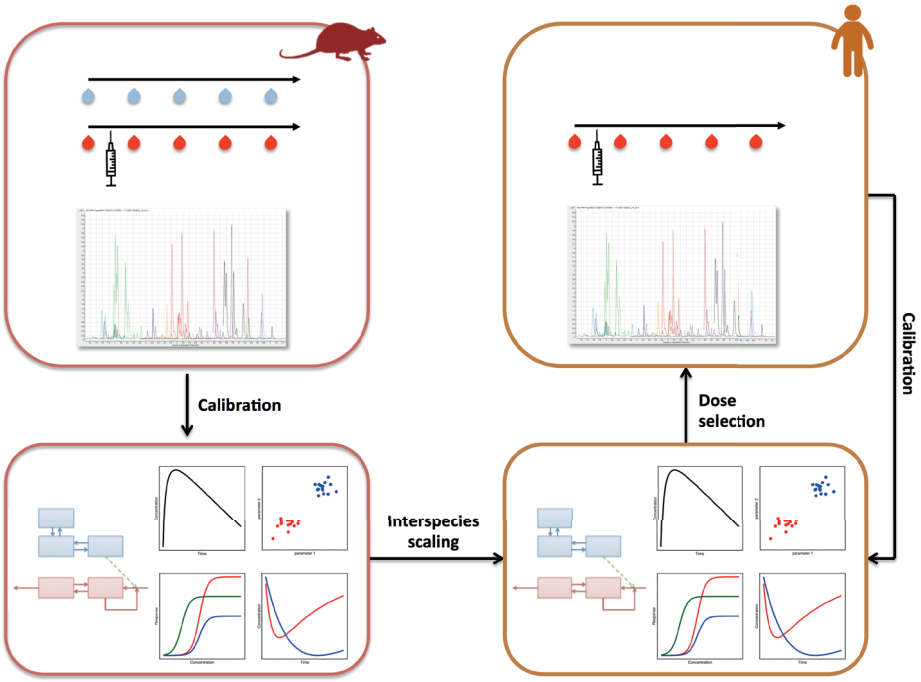



**Figure 2. The integrative approach of metabolomics and PK/PD modeling as applied to interspecies scaling in CNS drug development. Such approach starts with animal experiments to collect longitudinal brain$_{ECF}$ and plasma samples during treatment with a CNS drug. These samples are analyzed for drug concentrations and metabolomics to subsequently develop a multivariate PK/PD model. By applying the principles of interspecies scaling a humanized model is defined to select doses for the clinical study. Plasma drug concentrations and metabolomics data of the clinical study will be used to recalibrate the model and increase the understanding of interspecies differences.**

### *5.1 Longitudinal analysis of pharmacometabolomics responses*

A longitudinal multivariate evaluation of pharmacometabolomics data was performed by Rasmussen and colleagues, who were one of the first doing that in the field of clinical pharmacology [94]. This multivariate fingerprint was suitable for guiding dose selection of recombinant interleukin-21 in patients with metastatic melanoma.

### *5.2 PK/PD based analysis of pharmacometabolomics responses*

In addition to longitudinal evaluation of the pharmacometabolomics response, the integration with PK/PD modeling has been shown in a few studies. Clustering of longitudinal transcriptomics data formed the basis for the 6 turnover models in one study. Together, these turnover models formed a complex PK/PD model that described the gene-expression signaling cascade in the rat liver after corticosteroid treatment [84]. In another study, clustering was applied to the PK/PD parameters identified from pharmacometabolomics data

in rats after remoxipride treatment [95]. This analysis revealed 6 unique PK/PD relations, 18 potential biomarkers and two perturbed pathways (Figure 3). It has the potential to define a therapeutic window on basis of multiple biomarkers, provides a list of biomarkers to take into account in additional studies, and gives insight into biological effects of remoxipride. The application of such analysis in multiple species will give insights into species-differences on the PK/PD parameters that describe the longitudinal pharmacometabolomics response. Depending on the differences in parameters, dosing strategies can be defined following simulation of worst-to-best case scenarios as was performed by Gosset *et al.* [57] for the effect of a Transient Receptor Potential Melastatin-8 (TRPM8) blocker on a single marker (core body temperature). Eventually, pharmacometabolomics data analysis methods can aid the development of quantitative systems pharmacology (QSP) models which aim to mathematically describe the interactions between multiple elements of the biological system (e.g. biomolecules, cells, tissues) in order to understand the impact of drugs on the system as a whole [91,96,97]. Quantitative metabolic networks can provide a topological basis of QSP models to be integrated with organ-level networks, receptor binding kinetics and PK [91,97]. QSP models are promising for interspecies translation by humanizing the animal-based model parameters [9,98,99].

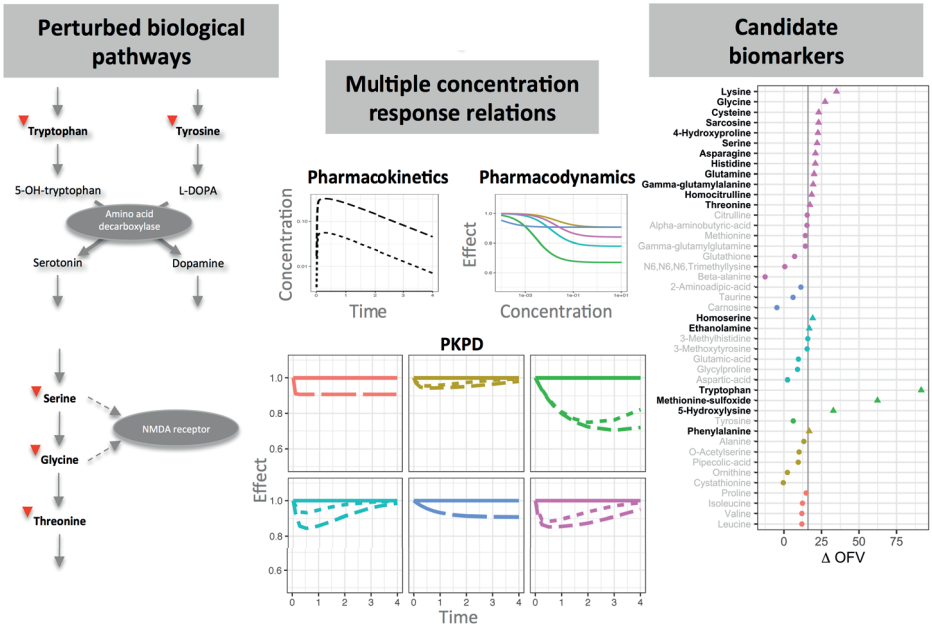



**Figure 3. A metabolomics study combined with multivariate PK/PD modeling revealed 6 diverse response patterns (middle) for remoxipride in rats. These response patterns were represented by 18 metabolites that could potentially function as biomarker (right), rendering further validation. The response clusters were associated with 2 known biological pathways (left). Modified from reference 95.**

### *5.3 Prediction of the human brain pharmacometabolomics responses*

*In vivo* pharmacometabolomics studies typically use plasma samples to characterize the system-wide drug effects. The plasma metabolic phenotype is a composite extraction of all individual tissue metabolic phenotypes. Although this provides the opportunity to evaluate whole-body treatment effects in an easily accessible body fluid, it can limit the quantitative interpretation of the treatment response that originates in a specific tissue. This is particularly true for CNS treatments, for which the metabolic biomarkers have to distribute over the BBB (Figure 4). This was illustrated by the fact that plasma monoamine levels were decreased with CNS drug treatment, whereas CSF levels were not affected [20,78]. Likely, the effects were caused in the periphery, and did not provide information on the central brain effects of these drugs.

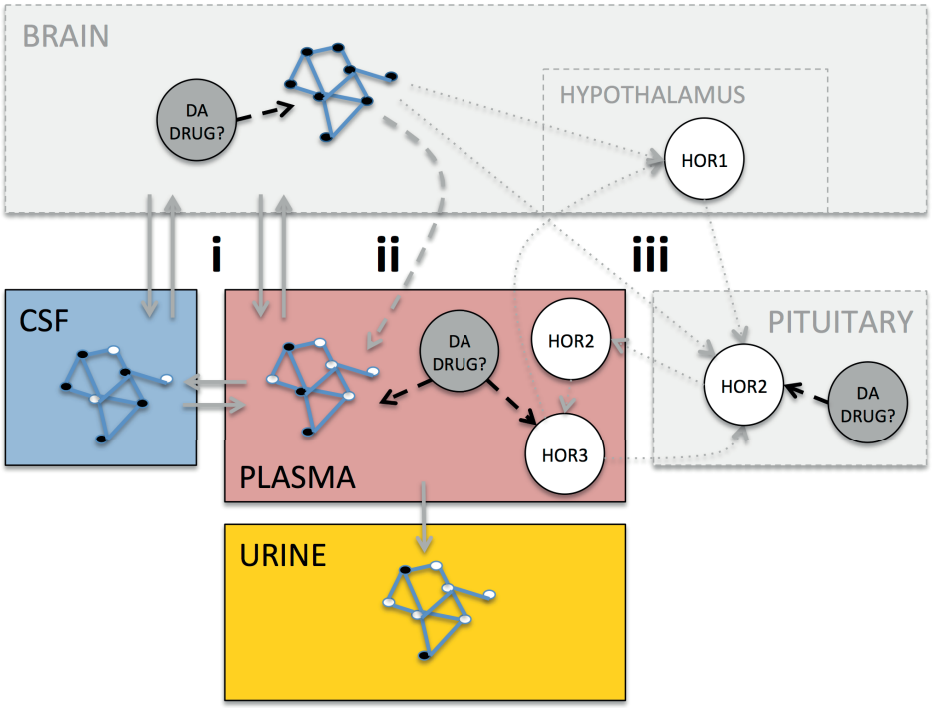



**Figure 4. Brain metabolic phenotypes are reflected in the periphery via three mechanisms: i) individual metabolites distribute to CSF, plasma and urine, and become integrated in the peripheral metabolic phenotype; ii) the brain metabolic phenotype affects the peripheral nervous signaling, thereby controlling the release of peripheral metabolites, such as acetylcholine or norepinephrine; iii) the brain metabolic phenotype influences the neuroendocrine system via the hypothalamus, modifying the pituitary hormone release. Modified from reference 59.**

A useful technique that has been used to study CNS drug PK and PD is intracerebral microdialysis [100–103]. It allows longitudinal sampling within a single individual to follow the treatment response over time. Moreover, since microdialysis allows the collection of molecules with a molecular weight below 20 kD, it is highly suitable for pharmacometabolomics analysis [104,105]. Notably, microdialysis, for ethical reasons, is limited in humans. Animals are therefore typically used to characterize the relation between the brain- the CSF- and the plasma metabolic phenotypes. Following the translation PK/PD-metabolomics workflow depicted in Figure 2, the human brain metabolic phenotype can subsequently be predicted using the principles of interspecies scaling and calibrated with the human plasma and CSF metabolic phenotypes.

### *5.4 Disease dependent PK/PD-metabolomics approach*

This review has mainly focused on the treatment, rather than on the disease. Here, we would like to spend a few words on the influence of pathology on the pharmacology; a patient may respond differently to a treatment than a healthy individual. Both the CNS drug PK and PD can be affected by the disease, and this influence is drug-specific. For example, the morphine PK changed with traumatic brain injury [39], and the rate of dopamine metabolism was higher in a rotenone rat model of Parkinson's Disease as compared to control [103]. Thus, the understanding of the two-way interaction between pathology and pharmacology in the context of translational CNS drug development is important. Metabolomics was found useful to understand species differences with regard to pathology [72]. As such, it has potential to translate the pathology-dependent pharmacology from animal to men [106].

## 6. Conclusion

This review discussed the merits of PK/PD modeling and pharmacometabolomics in the field of translational CNS drug development. PK/PD models can predict human biomarker time courses on basis of animal data using the principles of interspecies scaling. Pharmacometabolomics can measure the biochemical responses to evaluate the system-wide CNS drug effects among species. The integration of PK/PD modeling and pharmacometabolomics studies is envisioned to enable the prediction of longitudinal, dose-dependent system-wide responses, and has begun to receive attention [90,91,95]. The opportunities and challenges of such integration were discussed with regard to translational CNS drug development. Although we are still at the stage of early conceptual development, such integration is envisioned to increase understanding of system-wide pharmacology and to improve the interspecies translation of CNS drug effects.

# 7. Expert opinion

## *7.1 The potential of integrated PK/PD and pharmacometabolomics in translational CNS drug development*

CNS drug development is suffering from low success rates, which, for a large part, can be attributed to the empirical approach in translational development [6,12]. This led to the realization to shift towards mechanism-based prediction of clinical on basis of preclinical pharmacology. In particular, PBPK and PK/PD modeling are increasingly applied in drug development to guide dosing strategies for early clinical studies [39,47,57,107]. The strength of these models is that they describe the dynamics of pharmacological processes, which can be scaled from animals to humans. While the PD models typically describe the drug effect on a single biological pathway, pharmacometabolomics provides a means to evaluate multiple pathways obtaining a comprehensive insight into the system-wide pharmacology of a CNS drug [69,108]. Interestingly, the metabolome is structurally very similar among mammalian species, enabling a direct comparison of their metabolic phenotypes, although there are a few differences that need caution (e.g. ascorbic acid production in rats, but not in humans) [72,73]. At this moment, only very few studies have been performed to investigate the interspecies correlations of metabolic phenotypes. Moreover, pharmacometabolomics is mostly applied in a static manner, although dynamic approaches are emerging [90,94,95].

PK/PD modeling and pharmacometabolomics are thus complementary to each other. Since both fields have a potential for translational CNS drug development, their integration is promising. It has the potential to identify the pharmacologically relevant parameters of the system-wide drug effects [95]. Using the principles of interspecies scaling, these parameters can be humanized, and predict the clinical on basis of the preclinical pharmacology. The model can subsequently be validated on basis of the clinical metabolic phenotype (Figure 2).

## *7.2 Challenges and recommendations for the integration of PK/PD modeling and pharmacometabolomics*

Several aspects of study design and data analysis need consideration to achieve an integration of PK/PD modeling and pharmacometabolomics.

### *7.2.1 Multi-level biomarker evaluation*

To achieve an integrative understanding of the pharmacological action, multi-level biomarker data needs to be collected, for example, plasma drug concentrations, brain drug concentrations, (multiple) target occupancies, biochemical biomarkers. Eventually, these biomarkers will be linked to physiological measures and clinical outcome during clinical development.

*7.2.2 Longitudinal sampling over a large dose range*

To capture the dynamics of the PK/PD response, longitudinal data is essential. Serial plasma sampling and intracerebral microdialysis are useful methods to obtain time courses of CNS drug concentrations, as well as biochemical markers, in plasma and brain. Of interest, isotope-labeling based metabolomics (also called flux-based metabolomics) is an emerging discipline that enables the capturing of network dynamics when applied in combination with longitudinal sampling [109,110]. Additionally, a large drug concentration range is needed to have information on all parts of the non-linear concentration-response curve. This is particularly important with a comprehensive pharmacometabolomics evaluation, since individual metabolites may have a different position on the concentration-response curve [95].

*7.2.3 Integrated PK/PD-metabolomics analysis*

Longitudinal pharmacometabolomics data in conjunction with drug concentration data needs to be described using PK/PD modeling in order to identify a fingerprint of pharmacologically relevant parameters such as the *in vivo* potencies, the maximal drug effects or the turnover rates [90,95] (Figure 2, 3).

*7.2.4 Multi-tissue biomarker evaluation*

Drug concentrations and endogenous metabolites must be analyzed in multiple biofluids, such as plasma, $brain_{ECF}$, and CSF to understand how the plasma metabolic phenotype relates to the target site effect (Figure 4).

*7.2.5 Generate proof of principle for an integrated PK/PD-metabolomics approach in translational CNS drug development*

A primary challenge will be the generation of proof of principle for the integrated PK/PD-metabolomics approach.

First of all, multiple same-in-class drugs are to be compared biochemically using a pharmacometabolomics approach. Haloperidol and clozapine showed different efficacy on basis of a multivariate analysis with 58 different components of movement, as well as a multivariate evaluation with monoamines [66]. Although both analyses marked the fact that haloperidol and clozapine showed different efficacy, the pattern was not similar for the behavioral and the monoamine analysis. This indicates two things: 1) a multivariate biochemical is promising with regard to understanding differences between same-in-class drugs. 2) The abovementioned analysis showed that the monoamine based evaluation, although recognizing the pharmacological complexity, still is oversimplified to explain the behavioral outcome.

A second aspect that needs to be included to provide proof of principle is the application of longitudinal metabolomics in multiple species, including humans. Taking into account the known species-differences, the interspecies metabolomes should be compared to understand and map species differences and evaluate applicability of pharmacometabolomics in translational CNS drug development [73]. In particular, it will be important to validate the scaled PK/PD models in humans.

A third aspect is to relate the metabolic fingerprint to relevant clinical (side) effects. Kaddurah-Daouk *et al.* [70] nicely showed this for risperidone, olanzapine and aripiprazole, comparing their lipidomic profiles. Interestingly, aripiprazole showed less impact on lipids, which was associated with the absence of weight gain as a side effect. Further studies will indicate whether such approach is generally applicable in drug development.

### *7.3 The future of translational CNS drug development with an integrated PK/PD-metabolomics approach*

It is envisioned that the integration of PK/PD and pharmacometabolomics will increase the understanding of system-wide pharmacology and improve the interspecies translation of CNS drugs. Specifically, it is envisioned to enable the extraction of system-wide pharmacologically relevant parameters that can be scaled to humans. Additionally, information on biomarkers and pathways is obtained. This advancement must be seen together with the developments in the field of QSP [91,96,97]. The integrated PK/PD-metabolomics approach reveals a PK/PD fingerprint biomarker representing the dynamics of known and unknown pathways. QSP aims to connect the cellular pathway response with the organ- or system-level response. On one hand, the integrated PK/PD-metabolomics approach can thus inform QSP models on relevant pharmacological pathways. On the other hand, QSP models can identify the mechanistic relationship between the single metabolites described by an integrated PK/PD-metabolomics model.

Altogether, an integrated PK/PD-metabolomics approach is envisioned to have a promising role in translational CNS drug development by providing a method to scale system-wide effects from animal to men (Figure 2).

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# CHAPTER 3

## ACCESS TO THE CNS: BIOMARKER STRATEGIES FOR DOPAMINERGIC TREATMENTS

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## Abstract

Despite substantial research carried out over the last decades, it remains difficult to understand the wide range of pharmacological effects of dopaminergic agents. The dopaminergic system is involved in several neurological disorders, such as Parkinson's disease and schizophrenia. This complex system features multiple pathways implicated in emotion and cognition, psychomotor functions and endocrine control through activation of G protein-coupled dopamine receptors. This review focuses on the system-wide effects of dopaminergic agents on the multiple biochemical and endocrine pathways, in particular the biomarkers (i.e., indicators of a pharmacological process) that reflect these effects. Dopaminergic treatments developed over the last decades were found to be associated with numerous biochemical pathways in the brain, including the norepinephrine and the kynurenine pathway. Additionally, they have shown to affect peripheral systems, for example the hypothalamus-pituitary-adrenal (HPA) axis. Dopaminergic agents thus have a complex and broad pharmacological profile, rendering drug development challenging.

Considering the complex system-wide pharmacological profile of dopaminergic agents, this review underlines the needs for systems pharmacology studies that include: i) proteomics and metabolomics analysis; ii) longitudinal data evaluation and mathematical modeling; iii) pharmacokinetics-based interpretation of drug effects; iv) simultaneous biomarker evaluation in the brain, the cerebrospinal fluid (CSF) and plasma; and v) specific attention to condition-dependent (e.g., disease) pharmacology. Such approach is considered essential to increase our understanding of central nervous system (CNS) drug effects and substantially improve CNS drug development.


**Keywords:** dopaminergic agents; biomarkers; systems pharmacology; CNS drug development

### Abbreviations

3-MT: 3-methoxytyramine; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; α-MSH: alpha-melanocyte stimulating hormone; ACh: acetylcholine; ACTH: adenocorticotropic hormone; BBB: blood-brain-barrier; $Brain_{ECF}$: brain extracellular fluid;; CNS: central nervous system; CRH: corticotropin releasing hormone; CSF: cerebrospinal fluid; DOPAC: 3,4-dihydroxyphenylacetic acid; DRN: dorse raphe nucleus; EPN: entopeduncular nucleus; EPS: extrapyramidal symptom; FSH: follicle stimulating hormone; GABA: gamma-aminobutyric acid; GnRH: gonadotropin releasing hormone; GPe: external globus pallidum; GPi: internal globus pallidum; HPA: hypothalamic-pituitary-axis; HVA: homovanillic acid; LH: luteinizing hormone; MSN: medium spiny neuron; NAc: nucleus accumbens; NMDA: N-methyl-D-aspartate; NOS: nitric oxide synthase; PFC: prefrontal cortex; PNS: peripheral nervous system; PVN: paraventricular nucleus; SN: substantia nigra; VMAT: vesicular monoamine transporter; VTA: ventral tegmental area

# Introduction

Over the last decades, the development of therapies targeting diseases affecting the central nervous system (CNS) has been facing numerous challenges while the number of people suffering from CNS disorders has tremendously grown, exceeding one billion worldwide nowadays [1,2]. The challenges mostly rely on the insufficient knowledge of biomolecular mechanisms underlying many CNS-related diseases, as well as the poor understanding of mechanisms of action of many CNS drugs. In order to improve drug efficacy, both pharmaceutical industry and academic community have fostered the implementation of biomarker-based approaches for translational pharmacology and dose decision-making in clinical settings. A biological or biochemical marker represents a measurable sign with regard to a pharmacological or pathological process, providing a clinically meaningful endpoint in predicting the effect of a chosen treatment [3–5]. Biological markers are recognized as a valuable tool in drug development, allowing for further elucidation of both drug efficacy and side effects. CNS drug discovery and development faces multiple challenges, including the large number of drugs that fail in late phases of clinical trials due to poor understanding of processes underlying the dose response relation [6]. In this context, biomarkers represent an attractive alternative approach to support identification of most promising compounds, guide the dosing strategies in early clinical trials, and help recognizing a patient population that is most likely to benefit from a specific treatment.

This systematic and exhaustive review presents all biochemical indicators that have been previously reported as being related to dopaminergic drug effects, as well as their potential role in biomarker-driven CNS drug development, focusing on biomarkers in rodents biofluids, specifically brain extracellular fluid ($brain_{ECF}$), cerebrospinal fluid (CSF), plasma and urine.

## *Anatomy and physiology of the dopaminergic system*

Dopamine is a neurotransmitter that belongs to the catecholamine family and is primarily synthesized in the brain and the kidneys. In the brain, dopamine is produced in the cell bodies of dopaminergic neurons located in the substantia nigra (SN), the ventral tegmental area (VTA) and the hypothalamus. These neurons send projections to multiple brain areas where dopamine is stored and released, including the striatum (nigrostriatal pathway), the prefrontal cortex (PFC) (mesocortical pathway), the nucleus accumbens (NAc) (mesolimbic pathway) and the pituitary gland (tuberoinfundibular pathway), as illustrated in Figure 1. It should be noted that these pathways do not represent all dopamine systems in the brain. Other systems, such as the thalamic dopamine system, are increasingly recognized as important additional components of the brain dopamine pathways [7]. The presence of dopamine in the mesolimbic pathway is related to positive reinforcement, reward and/or pleasure, while in mesocortical pathway it is involved in cognitive control of behavior.

Furthermore, the role of dopamine in the nigrostriatal pathway, transmitted from the SN (midbrain) to the putamen in the dorsal striatum, is to simulate reward-related cognitive processes as well as psychomotor function. The tuberoinfundibular pathway projects dopaminergic neurons from the hypothalamus to the pituitary gland to modulate secretion of hormones, including prolactin. Dopaminergic pathways also project from the VTA (midbrain) to the amygdala, the hippocampus, and the cingulate cortex. As such, dopamine is simultaneously involved in both emotional and memory processing. Dopaminergic neurons form a tight network with a number of other neuronal pathways, including choline, glutamate and gamma-aminobutyric acid (GABA) systems, showing its possible role in multiple complex processes. Therefore, any drug targeting the dopaminergic neurons may influence multiple transduction pathways including both the dopaminergic and other systems.

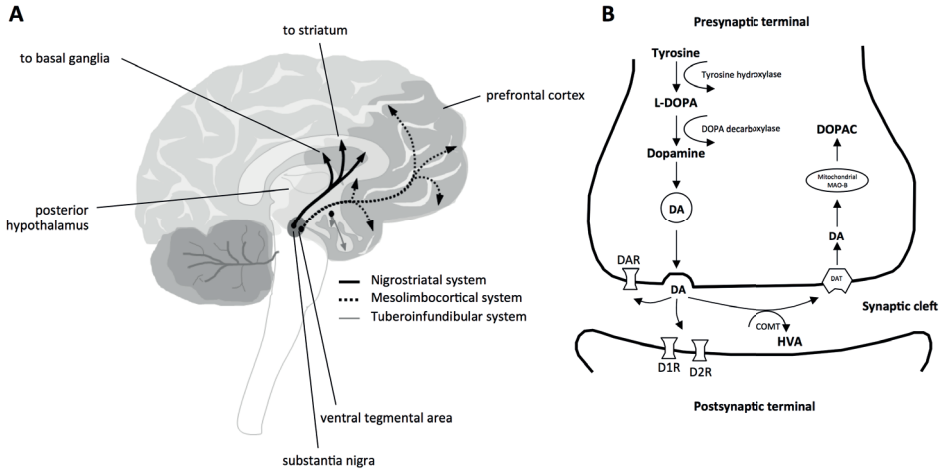



**Figure 1. Overview of the dopaminergic system. A. Representation of the dopamine pathway architecture in the brain. B. Illustration of the dopamine production and degradation, as well as the synaptic signaling.**

Five dopamine receptor subtypes, often referred to as $D_{1–5}$ receptors, have been reported in the CNS, all being G-protein coupled receptors that may function independently but of which the downstream pathways may also interact [8]. Dopamine receptors are divided into $D_1$- and $D_2$-like receptor classes, the $D_1$ receptor class including $D_1$ and $D_5$ receptors while $D_2$ receptor class includes $D_2$, $D_3$, and $D_4$ receptors. $D_1$ receptor and $D_2$ receptor classes have opposing effects with regard to adenylyl cyclase activity, cAMP concentrations, as well as phosphorylation of proteins, resulting in either stimulatory or inhibitory action on voltage-gated and ion channels in synapses [9]. $D_1$ receptor are highly expressed in the striatum, NAc, SN, frontal cortex and amygdala, while lower expression of $D_1$ receptor is

found in the hippocampus, thalamus, and cerebellum. $D_2$ receptor are mainly localized in the striatum, NAc, SN, hypothalamus, cortical areas, amygdala and hippocampus. Although dopamine receptors are most densely expressed in the brain, they are also found in the periphery in different patterns of expression [10], highlighting the system-wide effects of dopamine that are crucial in maintaining homeostasis.

## *Dopaminergic agents for treatment of neurological disorders*

The dopaminergic system has been exploited for treatment opportunities in a large variety of disorders. Due to its broad implication in pathophysiology, the current pharmacological efforts mostly focus on targeting both the dopamine receptors and subsequent post-receptor mechanisms. Different types of dopaminergic drugs have been developed so far, primarily dopamine agonists and dopamine antagonists.

### *Dopamine agonists*

dopamine agonists have been developed for treating Parkinson's disease, a progressive neurodegenerative disorder presenting both motor and non-motor symptoms. The pathology of the Parkinson's disease is characterized with an extensive loss of dopamine neurons in the SN and accumulation of the protein α-synuclein in Lewy bodies within nerve cells in specific brain regions [11]. Although the underlying mechanisms leading to Parkinson's disease remain poorly understood, a strong association between low dopamine brain levels and Parkinson's disease symptoms has been frequently reported [12]. Dopamine receptor agonists, introduced first in 1970 for the treatment of Parkinson's disease, act directly on dopamine receptors to mimic endogenous neurotransmission. Levodopa (L-DOPA), a pro-drug crossing the blood-brain barrier (BBB), was the first therapeutic option available for treating Parkinson's disease. Various other agonists, e.g., apomorphine, bromocriptine and pramipexole, have been later developed and commercialized, showing comparable effectiveness [13].

### *Dopamine antagonists*

While most of the currently available dopamine agonists are used for Parkinson's disease, the vast majority of dopamine antagonists have been developed for the treatment of schizophrenia. Multiple studies using animal models of schizophrenia have elucidated a pattern of persistent hyperdopaminergic state, accompanied with altered stimulus recruits of dopamine in different brain regions. Cognitive impairments during psychosis might thus be explained by a rapid release of dopamine into the mesolimbic and the nigrostriatal regions [14]. Chlorpromazine was the first and extremely potent antagonist of $D_2$ receptor discovered, which considerably fostered antipsychotic drug development. Nevertheless, chlorpromazine treatment is accompanied with pronounced adverse effects, including neuroleptic malignant syndrome and extrapyramidal symptoms (EPS) such as tardive

dyskinesia. Other $D_2$ receptor antagonists, e.g., haloperidol, risperidone and clozapine, have been developed to exhibit comparable or greater effectiveness with fewer of these side effects, in particular EPS [15,16].

Many of dopaminergic agents were discovered with incomplete understanding of their modes of action, often resulting in unpredictable side effects and/or off-target effects. It is only after having been introduced to market that studies were conducted to elucidate their modes of actions, which revealed multiple pathways affected [17–19].

*Selectivity of dopaminergic drugs*

Clozapine is currently the "gold standard" for the treatment of schizophrenia[15]. Interestingly, this is one of the least selective $D_2$ receptor antagonists [16,20]. Indeed, schizophrenia is a polygenic disease, and therefore a 'shotgun-approach' may be more successful than a 'magic-bullet approach' [16]. Many $D_2$ receptor antagonists have therefore affinity for more receptors, including serotoninergic, adrenergic, muscarinic, and histaminergic receptors [16,20]. Also many $D_2$ receptor agonists were found non-selective, with affinity for other dopaminergic, serotonergic, adrenergic and histaminergic receptors [21]. This should be taken into consideration when evaluating the effects of these agents on the system-wide biochemical pathways.

This review aims to further improve the understanding of mechanisms of action by providing an extensive overview of the pathways that are affected by dopaminergic agents, with the hope to increase our understanding of system-wide dopaminergic pharmacology, as well as to provide directions on how to improve pharmacological biomarker strategies during early drug development.

## Methods

A systematic overview of literature over the past 25 years has been built, focusing on dopaminergic treatment effects on central and peripheral biomolecular pathways in rats. A search of the PubMed database was conducted in September 2017 by using the following key words: *dopamine antagonists, dopamine agonists, biogenic amine, amino acid, hormone, cytokine, lipid, neurotransmitter, cerebrospinal fluid, intracerebral microdialysate, plasma, urine, rat* (see Supplementary Data S1 for the exact search code), yielding to 1058 articles (English only). Only studies describing the effects of dopaminergic agents and elucidating a potential biochemical indicator of drug action in rats were included. *In vitro* studies, experimental studies focusing only on behavioral changes and/or reactions, studies of cognition patterns or event-related potentials, and studies that only included pharmacokinetic information were excluded. Furthermore, studies including functional

imaging techniques or electroencephalography, investigating dopamine receptor affinities, functions, and synthesis, exploring the effect of dopaminergic agents in combination with other pharmacological agents, under pathological conditions, after surgical procedures such as adrenalectomy or ovariectomy, with pregnant or lactating animals, and with animals under long-term food restriction were excluded as well. Finally, prolactin, being considered a standard marker of dopaminergic activity with well-explored functions and relationship with dopamine [22–24], has been excluded. After selection, 260 articles were included.

## *Dopaminergic treatment effects on endogenous metabolites levels in the CNS*

The CNS-wide effects of dopamine receptor agonists and antagonists reported in the selected studies are shown in Table I and Figure 2. Although information was also gathered from studies involving intracerebral administration, only data after systemic administration is presented to obtain insights into clinically relevant effects. Moreover, a distinction is made between short-term and long-term treatment effects. Most of the effects reported in the CNS have been mainly observed in $brain_{ECF}$, using microdialysis, leading to deeper insights into neurotransmitter pathways. Overall, the reported literature emphasizes the CNS-wide effects of dopaminergic agents, including dopamine pathway but also norepinephrine, cholinergic, GABA-glutamate, serotonin, kynurenine, nitric oxide and endocannabinoid pathways.

Several considerations have to be taken into account for the discovery of easily accessible biomarkers that reflect these systematic effects, notably (Figure 3):

i) detectability in CSF, plasma or/and urine;
ii) simultaneous evaluation together with other markers of the pathway of interest to understand the dynamics between the drug and the pathway;
iii) Sufficient understanding of central and peripheral response
iv) Identification of distribution rates between brain, CSF, plasma and urine to understand the temporal relation between the biomarker peripheral concentration and effects in the brain.

## *Effects on the dopamine pathway*

### *Metabolism and signaling of the dopamine pathway*

The synthesis of dopamine involves the conversion of tyrosine into L-DOPA, the precursor of dopamine. It is stored into vesicles in the presynaptic neuron, following uptake via the vesicular monoamine transporter (VMAT). These vesicles release dopamine into the synaptic cleft, where it may bind to pre- or postsynaptic dopamine receptors to pass on neuronal signals to the post-synaptic neuron. The dopamine present in the synaptic cleft is eliminated through conversion to its metabolites homovanillic acid (HVA), 3,4-di-

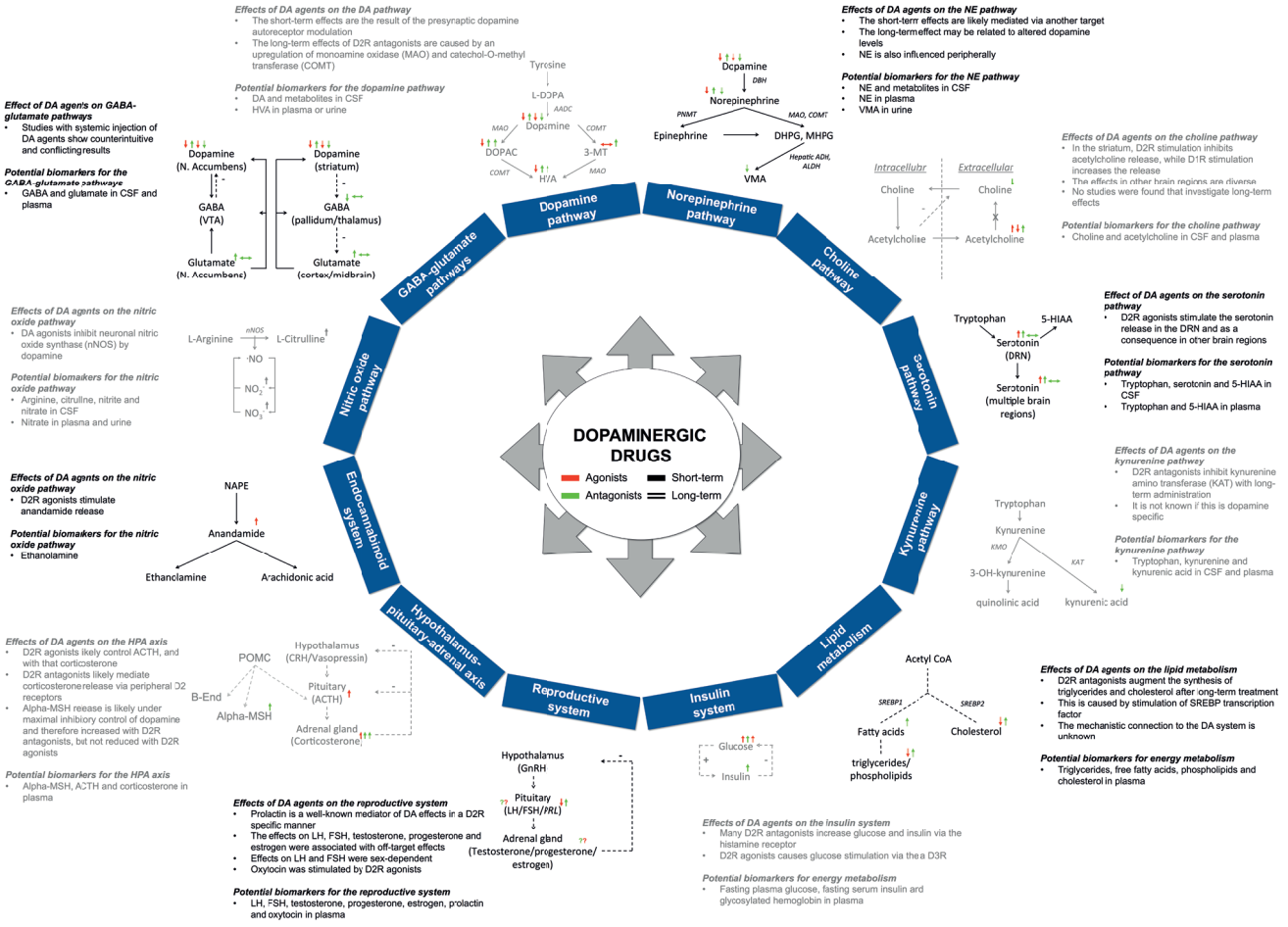

DOPAMINERGIC DRUGS
Agonists
Antagonists
Short-term
Long-term
Dopamine pathway
Norepinephrine pathway
Choline pathway
Serotonin pathway
Kynurenine pathway
Lipid metabolism
Insulin system
Reproductive system
Hypothalamus-pituitary-adrenal axis
Endocannabinoid system
Nitric oxide pathway
GABA-glutamate pathways
Effects of DA agents on the DA pathway
The short-term effects are the result of the presynaptic dopamine autoreceptor modulation
The long-term effects of D2R antagonists are caused by an upregulation of monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT)
Potential biomarkers for the dopamine pathway
DA and metabolites in CSF
HVA in plasma or urine
Tyrosine
L-DOPA
AADC
Dopamine
MAO
COMT
DOPAC
3-MT
HVA
Effects of DA agents on the NE pathway
The short-term effects are likely mediated via another target
The long-term effect may be related to altered dopamine levels
NE is also influenced peripherally
Potential biomarkers for the NE pathway
NE and metabolites in CSF
NE in plasma
VMA in urine
Dopamine
DBH
Norepinephrine
PNMT
MAO, COMT
Epinephrine
DHPG, MHPG
Hepatic ADH, ALDH
VMA
Effect of DA agents on GABA-glutamate pathways
Studies with systemic injection of DA agents show counterintuitive and conflicting results
Potential biomarkers for the GABA-glutamate pathways
GABA and glutamate in CSF and plasma
Dopamine (N. Accumbens)
GABA (VTA)
Glutamate (N. Accumbens)
Dopamine (striatum)
GABA (pallidum/thalamus)
Glutamate (cortex/midbrain)
Effects of DA agents on the choline pathway
In the striatum, D2R stimulation inhibits acetylcholine release, while D1R stimulation increases the release
The effects in other brain regions are diverse
No studies were found that investigate long-term effects
Potential biomarkers for the choline pathway
Choline and acetylcholine in CSF and plasma
Intracellular
Extracellular
Choline
Acetylcholine
Effects of DA agents on the nitric oxide pathway
DA agonists inhibit neuronal nitric oxide synthase (nNOS) by dopamine
Potential biomarkers for the nitric oxide pathway
Arginine, citrulline, nitrite and nitrate in CSF
Nitrate in plasma and urine
L-Arginine
nNOS
L-Citrulline
·NO
NO2
NO3
Effect of DA agents on the serotonin pathway
D2R agonists stimulate the serotonin release in the DRN and as a consequence in other brain regions
Potential biomarkers for the serotonin pathway
Tryptophan, serotonin and 5-HIAA in CSF
Tryptophan and 5-HIAA in plasma
Tryptophan
5-HIAA
Serotonin (DRN)
Serotonin (multiple brain regions)
Effects of DA agents on the nitric oxide pathway
D2R agonists stimulate anandamide release
Potential biomarkers for the nitric oxide pathway
Ethanolamine
NAPE
Anandamide
Ethanclamine
Arachidonic acid
Effects of DA agents on the kynurenine pathway
D2R antagonists inhibit kynurenine amino transferase (KAT) with long-term administration
It is not known if this is dopamine specific
Potential biomarkers for the kynurenine pathway
Tryptophan, kynurenine and kynurenic acid in CSF and plasma
Tryptophan
Kynurenine
KMO
KAT
3-OH-kynurenine
quinolinic acid
kynurenic acid
Effects of DA agents on the HPA axis
D2R agonists likely control ACTH, and with that corticosterone
D2R antagonists likely mediate corticosterone release via peripheral D2 receptors
Alpha-MSH reease is likely under maximal inhibiory control of dopamine and therefore increased with D2R antagonists, but not reduced with D2R agonists
Potential biomarkers for the HPA axis
Alpha-MSH, ACTH and corticosterone in plasma
POMC
Hypothalamus (CRH/Vasopressin)
Pituitary (ACTH)
B-End
Alpha-MSH
Adrenal gland (Corticosterone)
Effects of DA agents on the lipid metabolism
D2R antagonists augment the synthesis of triglycerides and cholesterol after ong-term treatment
This is caused by stimulation of SREBP transcription factor
The mechanistic connection to the DA system is unknown
Potential biomarkers for energy metabolism
Triglycerides, free fatty acids, phospholipids and cholesterol in plasma
Acetyl CoA
SREBP1
SREBP2
Fatty acids
Cholesterol
triglycerides/ phospholipids
Effects of DA agents on the reproductive system
Prolactin is a well-known mediator of DA effects in a D2R specific manner
The effects on LH, FSH, testosterone, progesterone and estrogen were associated with off-target effects
Effects on LH and FSH were sex-dependent
Oxytocin was stimulated by D2R agonists
Potential biomarkers for the reproductive system
LH, FSH, testosterone, progesterone, estrogen, prolactin and oxytocin in plasma
Hypothalamus (GnRH
Pituitary (LH/FSH/PRL)
Adrenal gland (Testosterone/progesterone/ estrogen)
Effects of DA agents on the insulin system
Many D2R antagonists increase glucose and insulin via the histamine receptor
D2R agonists causes glucose stimulation via the a D3R
Potential biomarkers for energy metabolism
Fasting plasma glucose, fasting serum insulin ard glycosylated hemoglobin in plasma
Glucose
Insulin

**Figure 2. Effects of dopamine drugs on 12 biochemical or endocrine pathways. Potential biomarkers are mentioned for each pathway. The reader is referred to the text for detailed discussion of the interaction between dopamine drugs and each pathway. 5-HIAA: 5-hydroxyindoleacetic acid; ACTH: adenocorticotropic hormone; Alpha-MSH: alpha melanocyte stimulating hormone; B-end: beta-endorphin; COMT: catechol-O-methyl transferase; CSF: cerebrospinal fluid; D1R: dopamine 1-like receptor; D2R: dopamine 2-like receptor; DA: dopamine; DHPG: dihydroxyphenylglycol; DOPAC: 3,4-dihydroxyphenylacetic acid; DRN: dorse raphe nucleus; FSH: follicle stimulating hormone; GABA: gamma-aminobutyric acid; HVA: homovanillic acid; L-DOPA: levodopa; LH: luteinizing hormone; MAO: monoamine oxidase; MHPG: 3-methoxy-4-hydroxyphenylglycol; N. Accumbens: nucleus accumbens; NE: norepinephrine; NO: nitric oxide; NOS: nitric oxide synthase; prolactin: prolactin; VMA: vanillylmandelic acid; VTA: ventral tegmental area**

hydroxyphenylacetic acid (DOPAC) or 3-methoxytyramine (3-MT), or by uptake into the presynaptic neuron via the dopamine transporter. In the latter case, dopamine is stored into vesicles, or degraded to HVA or DOPAC.

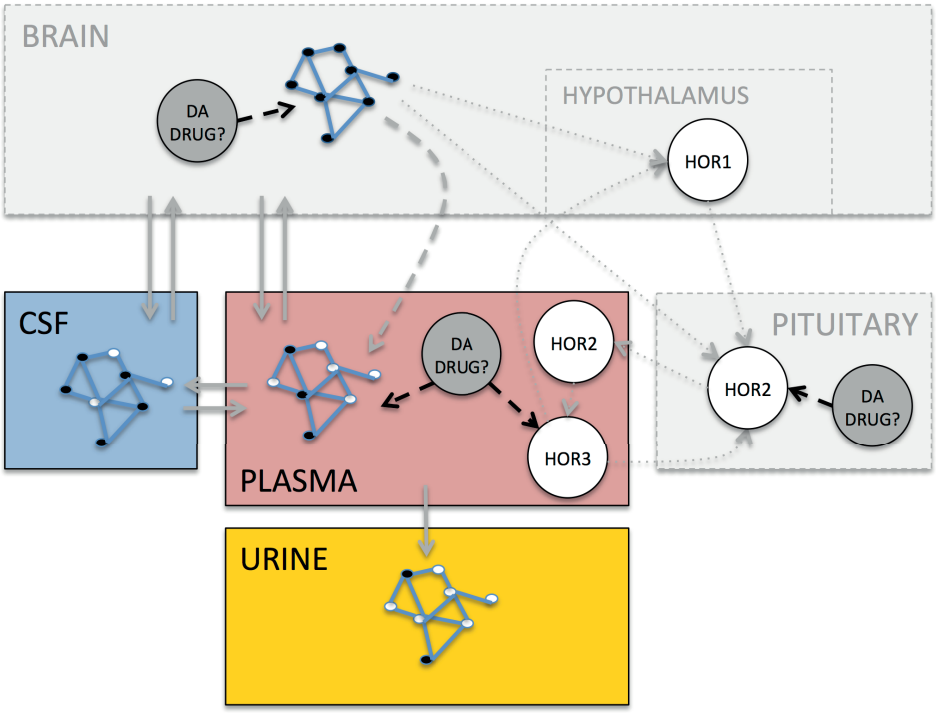



**Figure 3. Conceptual considerations for the use of accessible biomarkers in CSF, plasma or urine to reflect dopamine drug effects in the brain. The grey solid lines represent the distribution of biochemical pathway components to CSF, plasma and urine. Since only part of the pathway components may distribute to these biofluids, some of the nodes are filled blank. The grey dashed line represents the peripheral nervous system (PNS) that may influence the peripheral release of biochemical markers through electrical signaling. The grey dotted lines represent the neuroendocrine system (NES), which is electrically controlled at the level of the hypothalamus and the pituitary, causing the release of hormones into plasma. Feedback mechanisms of these hormones on their own release may complicate the interpretation of their responses in plasma. The black dashed lines represent the levels at which dopamine drugs may interact with these systems.**

*Effects of dopaminergic agents on the dopamine pathway*

Dopamine receptors are located pre- and postsynaptically, thereby influencing local concentrations of dopamine and its metabolites upon the presence of agonists and antagonists (Table I, Figure 2). Short-term treatments with $D_2$ receptor antagonists such as haloperidol, sulpiride, risperidone, olanzapine and clozapine have shown to stimulate the dopamine pathway [25,26], whereas administration of $D_2$ receptor agonists like quinpirole,

quinelorane, 7-OH-DPAT, and apomorphine inhibit this pathway [27–29]. This has been observed in $brain_{ECF}$ for dopamine as well as for its major metabolites DOPAC, HVA, 3-MT (Table I). The influence of $D_1$ receptor agents on the dopamine pathway remains poorly investigated. Only one study was identified, showing an increase in dopamine levels after intraperitoneal treatment with the $D_1$ receptor antagonist SCH23390 [30], while no studies reported the effects after systemically injected $D_1$ receptor agonists. The effects of $D_2$ receptor antagonists and agonists on the dopamine pathway may be explained by the modulation of presynaptic $D_2$ autoreceptors that provide a negative feedback function on dopamine release [31]. Moreover, many of these drugs have affinity for 5-HT receptors [16,21], which also contribute to the control of dopamine release [32,33].

After long-term treatment with $D_2$ receptor agonists, the basal dopamine pathway activity is decreased, similar to the effect observed after short-term treatment [27,34]. Interestingly, $D_2$ receptor antagonists inhibit the dopamine levels after long-term treatment, while the levels of the dopamine metabolites are increased [35–37]. This may, first of all, be explained by the upregulation of $D_2$ receptor expression after long-term treatment [38], thereby leading to an enhanced inhibition of dopamine release via the $D_2$ autoreceptor. Second, the monoamine oxidase (MAO) and the catechol-O-methyl transferase (COMT), that metabolize dopamine into DOPAC, HVA and 3-MT, were upregulated [39], providing another explanation, also supporting the increased concentrations of dopamine metabolites that are observed with long-term treatment.

*Biomarkers for the dopamine pathway*

dopamine and its metabolites can be detected in CSF, plasma and urine [40,41]. In contrast to dopamine, HVA is able to cross the BBB, providing a way to evaluate central dopaminergic activity in plasma. The difficult aspect is to distinguish between the central and the peripheral effects, since the dopaminergic system is also peripherally active in, for example, the kidney and the adrenal glands. The origin of the HVA response in urine after long-term treatment with haloperidol and clozapine [40,42] is therefore not known. Surprisingly, no further studies were identified that investigated CSF, plasma or urine biomarkers of the dopamine pathway after dopaminergic treatment.

## *Effects on the norepinephrine pathway*

*Metabolism and signaling of the norepinephrine pathway*

The largest concentrations of norepinephrine in the brain are found in neurons in the locus coeruleus. Outside the brain, it is found in the postganglionic sympathetic adrenal fibers and the chromaffin cells in the adrenal glands. Within the norepinephrine neurons, VMAT stores dopamine into synaptic vesicles, where it is converted to norepinephrine through dopamine beta-hydroxylase, and released into the synaptic cleft. Norepinephrine

may bind to alpha- or beta-adrenergic receptors, the former being mostly inhibitory and located presynaptically, while the latter are stimulatory and located postsynaptically. From the synaptic cleft, norepinephrine undergoes reuptake into the presynaptic neuron via the norepinephrine transporter, or is metabolized to epinephrine, dihydroxyphenylglycine and methoxyhydroxyphenylglycol. In the presynaptic neuron, it may be stored into vesicles, or degraded into its metabolites.

*Effects of dopaminergic agents on the norepinephrine pathway*

Norepinephrine release is stimulated by $D_2$ receptor antagonists such as clozapine, olanzapine and risperidone, although this has not been reported for haloperidol [43,44] (Table I, Figure 2). While this may be explained by dopaminergic modulation of norepinephrine release [45], these drugs also exhibit affinity for the adrenergic receptors [16]. Interestingly, in contrast to haloperidol, the other $D_2$ receptor antagonists showed affinity for the $\alpha_2$ adrenergic receptor. After long-term treatment, haloperidol caused a reduction of norepinephrine levels in the striatum [46], which may be explained by reduced conversion from dopamine to norepinephrine, since long-term $D_2$ receptor antagonist treatment decreased dopamine levels (Table I, Figure 2).

Plasma norepinephrine concentrations were decreased after $D_2$ receptor stimulation with the agonist bromocriptine [47]. This effect was blocked by administration of the $D_2$ receptor antagonist domperidone, which does not cross the BBB, suggesting the effect to be peripheral [48]. Furthermore, plasma levels of epinephrine were increased upon stimulation of $D_2$ receptor, although likely elicited through direct peripheral action on the adrenal gland and independent of the effect on norepinephrine [47,49].

*Biomarkers for the norepinephrine pathway*

norepinephrine and its metabolites have been already analyzed in CSF, plasma and urine [40,41,47], indicating that the latter biofluids can be used to estimate the central norepinephrine pathway activity. Indeed, reduced levels of the most downstream norepinephrine metabolite vanillylmandelic acid were found in urine after long-term treatment with haloperidol or clozapine [40,42]. However, as discussed in the previous paragraph, the effect on plasma (and thus also urine) norepinephrine concentrations are at least partly caused by peripheral effects. Further understanding of the relative central and peripheral effects of dopaminergic agents on the plasma or urine norepinephrine pathway responses is needed to conclude whether they can be used as biomarker for central activity. The CSF levels are likely more representative; however, the evaluation of longitudinal norepinephrine pathway responses upon dopaminergic treatment is still lacking.

## *Effects on the acetylcholine pathway*

### *Metabolism and signaling of the acetylcholine pathway*

Acetylcholine (ACh) is produced from choline in the presynaptic neurons and stored into vesicles via the vesicular acetylcholine transporter. These vesicles release ACh into the synaptic cleft where it binds to the postsynaptic ACh receptors, which are subclassified into nicotinic receptors that modulate neuronal activity and muscarinic receptors that elicit G-protein dependent signaling. ACh is degraded to choline and acetate, the former being recycled into the presynaptic neuron by the sodium-dependent choline transporter. Interestingly, anticholinergic drugs are typically prescribed to decrease the EPS accompanying antipsychotic treatments, suggesting that the dopaminergic and the cholinergic system are tightly connected. Cholinergic interneurons in the striatum represent only 1-2% of all neurons, yet they play an important role in the integration of multiple neurotransmitter signals [50], thereby contributing to the stabilization of dopaminergic signaling in the psychomotor circuit (also cortico-basal ganglionic system) [51].

### *Effects of dopaminergic agents on the acetylcholine pathway*

As listed in Table I and Figure 2, ACh release from cholinergic interneurons in the striatum is inversely related to $D_2$ receptor stimulation or inhibition. On the other hand, choline, the precursor of ACh, was reduced after $D_2$ receptor antagonist treatment, probably as a consequence of ACh release, since the uptake of choline was increased to support ACh production [52,53].

Contrary to their effect in the striatum, $D_2$ receptor agonists increased ACh levels in the hippocampus and the frontal cortex [54–57]. Furthermore, ACh in the PFC and the hippocampus was increased after treatment with second-generation $D_2$ receptor antagonists, which was not the case for first-generation $D_2$ receptor antagonists [26,56,58–61]. ACh levels in the NAc were not affected by $D_2$ receptor antagonism [26]. Overall, this indicates that the relation between the dopaminergic system and cholinergic signaling is region-specific. Indeed, there is evidence for $D_2$ receptor specific regulation of ACh in the striatum, while for other regions the results are conflicting. $D_1$ and $D_2$ receptors are certainly involved, taking into account that several of the $D_2$ receptor binding drugs discussed here also exhibit affinity for the muscarinic receptors [16,56,59].

$D_1$ receptor agonists have consistently been reported to lead to increased ACh levels in several brain regions, including the striatum [55,62–65], while $D_1$ receptor antagonism led to decreased ACh concentrations [63], or had no effect [55,56]. Cholinergic neurons indeed express the $D_1$ , mostly the $D_5$ receptor, increasing excitability after receptor stimulation [50].

**Table I. CNS-wide effects on endogenous metabolites by dopamine receptor agonists and antagonists**

| | | D1-like receptor | | D2-like receptor | | Dosing period | | |
|---|---|---|---|---|---|---|---|---|
| **Pathway** | **Marker** | **Agonist** | **Antagonist** | **Agonist** | **Antagonist** | | **Matrix** | **References** |
| **Dopamine metabolism** | DA | | + | - | + | Short-term | $Brain_{ECF}$ | (25–33) |
| | DOPAC | | | - | + | Short-term | $Brain_{ECF}$ | (28,29,34–40) |
| | HVA | | | - | + | Short-term | $Brain_{ECF}$ | (28,34–36,40–43) |
| | 3-MT | | | 0 | + | Short-term | $Brain_{ECF}$ | (36,42,43) |
| | DA | | | - | - | Long-term | $Brain_{ECF}$ | (25,44–47) |
| | DOPAC | | | | + | Long-term | $Brain_{ECF}$ | (48–51) |
| | HVA | | | | + | Long-term | $Brain_{ECF}$ | (48–51) |
| | HVA | | | | + | Long-term | Urine | (41,52) |
| **Norepinephrine metabolism** | NE | - | | | + | Short-term | $Brain_{ECF}$ | (27,53–56) |
| | NE | | | - | | Short-term | Plasma | (57) |
| | E | | | + | 0 | Short-term | Plasma | (57,58) |
| | NE | | | | - | Long-term | $Brain_{ECF}$ | (59)116 |
| | VMA | | | | + | Long-term | Urine | (41,52) |
| **Cholinergic signaling** | Choline[a] | | | | - | Short-term | $Brain_{ECF}$ | (60,61) |
| | Acetylcholine[b] | + | 0 | - | + | Short-term | $Brain_{ECF}$ | (60–68) |
| **GABA-glutamate circuits** | GABA[c] | | | +[d] | 0/- | Short-term | $Brain_{ECF}$ | (28,53,69–71) |
| | Glutamate[c] | | 0 | - | 0/+ | Short-term | $Brain_{ECF}$ | (25,28,53,72–74) |
| | GABA[c] | | | | 0 | Long-term | $Brain_{ECF}$ | (75–78) |
| | Glutamate[c] | | | - | 0/+ | Long-term | $Brain_{ECF}$ | (25,76,77,79) |
| **Serotonin signaling** | 5-HT | | | + | 0 | Short-term | $Brain_{ECF}$ | (28,32,80–82) |
| **Kynurenine metabolism** | Kynurenic acid | | | | - | Long-term | $Brain_{ECF}$ | (83) |
| **Nitric oxide cycle** | | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Nitric oxide cycle** | Citrulline[b] | + | | + | | Short-term | $Brain_{ECF}$ | (84,85) |
| | Nitrite | | | + | | Short-term | $Brain_{ECF}$ | (86) |
| | Nitrate | | | + | | Short-term | $Brain_{ECF}$ | (86) |
| | Nitrate | + | | | | Short-term | Urine | (87) |
| **Endocannabinoid system** | Anandamide | 0 | | + | | Short-term | $Brain_{ECF}$ | (88) |

**In case multiple studies were identified for the effects of a particular drug class on a particular marker, only the 4 most recent publications were reported. [a]Only in striatum; [b]Only observations after intracerebral administration; [c]Few and/or conflicting data; [d]Measured in the prefrontal cortex.**

**DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; 3-MT: 3-methoxytyramine; NE: norepinephrine; E: epinephrine; VMA: vanillylmandelic acid; GABA: gamma-aminobutyric acid; 5-HT: serotonin; $brain_{ECF}$: brain extracellular fluid**

*Biomarkers for the acetylcholine pathway*

Both ACh and choline can be detected in CSF and plasma with state-of-the-art analytical methods [66–69]. Furthermore, the plasma levels of these molecules may reflect central cholinergic activity, since they both can cross the BBB [70]. However, ACh is an important neurotransmitter of the PNS, sending signals from neural endfeet to muscle cells. This might confound the plasma levels as a marker of central activity. Quantitative understanding of the BBB distribution relative to the PNS response is essential to be able to interpret the plasma levels. Moreover, the relation between dopamine treatment and the cholinergic system appeared brain region specific, which may limit the usefulness of CSF and plasma for cholinergic biomarker detection. No studies have investigated cholinergic CSF and plasma in relation to dopaminergic treatment so far. Therefore, it is not possible to conclude whether it is possible to use these biofluids for biomarker evaluation.

## *Effects on the GABA-glutamate pathways*

*Metabolism and signaling of the GABA-glutamate pathways*

GABA and glutamate are the main inhibitory and excitatory neurotransmitters, respectively, in the brain. Glutamate is synthesized from glutamine by the enzyme glutaminase and is stored in vesicles in glutamatergic neurons via the action of vesicular glutamate transporters. These vesicles release glutamate into the synaptic cleft where it binds to the glutamate receptors, i.e., metabotropic receptor and ionotropic receptors (NMDA, kainate,and AMPA receptors). From the synaptic cleft, glutamate distributes into glial cells, using the glutamate transporter 1 or the glutamate aspartate transporter, where it is metabolized into glutamine. Glutamine is subsequently released from the glial cells and recycled into glutamatergic neurons. Also in GABAergic neurons, glutamate is produced from glutamine. However, these neurons also contain the enzyme glutamate decarboxylase that converts glutamate into GABA. Vesicular GABA transporters store GABA into vesicles which release it into the synaptic cleft. There, it binds to the GABA receptors to inhibit the activity of the postsynaptic neuron. GABA diffuses to the glial cells via the GABA transporter where it is metabolized to glutamate via the Krebs cycle, and subsequently converted to glutamine. Glutamine is recycled into the presynaptic GABAergic neurons. Although glutamate and GABA have many roles in the brain and are distinct neurotransmitters, we discuss here their interconnection in relation to two dopaminergic pathways: the nigrostriatal pathway and the mesocorticolimbic pathway. These pathways belong to the so-called circuits that connect multiple brain regions by neuronal fibers. Concretely, in the nigrostriatal pathway, activation of the striatal $D_1$ receptor leads to release of GABA into the internal globus pallidum (GPi) and the substantia nigra reticula (SNr). This subsequently reduces the release of GABA into the thalamus. Activation of the striatal $D_2$ receptor inhibits the release of GABA into the external globus pallidum (GPe), which then stimulates the release of GABA into the subthalamic nucleus and the GPi. This also reduces

the release of GABA into the thalamus. As such, these two pathways, also referred to direct and indirect pathway, enhance the thalamic release of glutamate into the PFC. Since cortical glutamatergic neurons project to multiple regions in the midbrain, amongst which the striatum and the substantia nigra, many functionalities are stimulated. In the meso-corticolimbic pathway, activation of $D_2$ receptors in the VTA stimulates GABAergic neurons in the NAc. This leads to enhancement of GABA release into the other brain regions such as VTA and ventral pallidum. Additionally, $D_2$ receptor activation in the VTA stimulates the release of dopamine into the PFC. This enhances the activity of the pyramidal neurons that release glutamate into other brain regions, including NAc and VTA.

*Effects of dopaminergic agents on the GABA-glutamate pathways*

While these circuits for a large part were unraveled by local injection of dopaminergic, GABAergic and glutamatergic agents [71–73], not many studies have been performed showing the effect of systemically injected dopaminergic agents (Table I, Figure 2). Only one $D_1$ receptor agent, an antagonist, was systemically injected to show no effect on glutamate levels in the entopeduncular nucleus (EPN) [74]. The cortical GABA levels were increased with systemic injection of $D_2$ receptor agonists, while glutamate levels in the NAc or EPN were decreased [27,74,75], contrasting the response expected from the above-described circuits. $D_2$ receptor antagonists typically did not show an effect on GABA levels in the ST, the GPe, the PFC and the NAc [26,76–78], or glutamate levels in the ST, EPN, PFC or NAc [26,74,77,79–81]. It should be noted that the results are not always consistent, since some studies with $D_2$ receptor antagonists found reduced GABA levels in the GP, NAc or PFC [76,78,82,83], increased GABA concentrations in the GP or the striatum [84,85], or increased glutamate levels in the SN, ST, EPN, PFC, or NAc [79,81,86,87]. These contradictions highlight the delicate balance of this circuit, which is affected by multiple factors (e.g., target site exposure, experiment time, off-target effects, etc.) that can cause concentration-, time-, or drug-dependent differences among the studies. Moreover, with systemic injection, these circuits are perturbed at multiple regions, rendering its pharmacological interpretation non-intuitive. Systematic studies that account for these factors, and that evaluate glutamate, GABA and dopamine in multiple brain regions simultaneously, are warranted to obtain a deeper insight into the effects of systemic administration of dopaminergic agents on such circuits.

*Biomarkers for the GABA-glutamate pathways*

Although GABA and glutamate concentrations are well measurable with modern analytical approaches [88], it is not known how the levels relate to dopaminergic treatment. GABA and glutamate responses have shown to be region-dependent, which may confound the CSF and plasma response. Further experimental evidence needs to be collected to

evaluate the potential of CSF and plasma to assess the GABA-glutamate pathway activity in relation to dopaminergic agents.

## *Effects on the serotonin pathway*

### *Metabolism and signaling of the serotonin pathway*

Serotonin is produced from the amino acid tryptophan via 5-hydroxytryptophan and stored into vesicles by VMAT. When it is released from these vesicles into the synaptic cleft, it binds to different classes of 5-HT receptors (5-$HT_1$ – 5-$HT_7$). It is recycled into the presynaptic neuron by the serotonin transporter, where it is stored into vesicles or metabolized to 5-hydroxyindoleacetic acid (5-HIAA).

### *Effects of dopaminergic agents on the serotonin pathway*

In contrast, the modulation of serotonin circuits by dopamine is mainly restricted to $D_2$ receptor mediated stimulation of serotonin neuron cell bodies in the dorsal raphe nucleus (DRN) that control motor activity. This leads to increased serotonin release in the DRN and other regions such as the striatum [32], as identified with systemic administration of $D_2$ receptor agonists [89,90] (Table I, Figure 2). No effects of dopamine agonists were found on the levels of the metabolite 5-HIAA [91,92]. Additionally, it was suggested that $D_2$ receptor agonists modulate serotonin afferents presynaptically in the hippocampus [93] or the SN [94]. $D_2$ receptor antagonists did not show an effect on serotonin levels [26,95,96], except for atypical antipsychotics such as risperidone and clozapine, likely elicited through presynaptic serotonin receptors [16,20,95–97]. Moreover, 5-HIAA was found increased after risperidone in but not all studies [83,98–102].

### *Biomarkers for the serotonin pathway*

The serotonin metabolite 5-HIAA, but not serotonin itself, has been already detected in CSF [41]. serotonin, 5-HIAA and the precursor tryptophan can be also detected in plasma. Although serotonin cannot pass the BBB, the central serotonin pathway activity may be inferred from the tryptophan and 5-HIAA responses. It is, however, important to realize that the serotonin pathway is also present in peripheral systems, for example in platelets. Moreover, tryptophan is provided via food intake. These factors may confound the plasma biomarker response to reflect central activity. Experimental evidence is further needed to investigate the relation between dopaminergic treatments, central serotonin activity and CSF or plasma biomarker responses.

## *Interactions among neurotransmitter systems*

The above-described effects of dopaminergic agents clearly show that the neurotransmitter systems of dopamine, norepinephrine, GABA, serotonin, glutamate and ACh are highly interconnected. Moreover, many of these agents also influence these neurotransmitter

systems via binding to other receptors, such as serotonineric and adrenergic receptors. Therefore, in order to understand the effects of these agents, neurotransmitter responses should be evaluated altogether. Qi et al. (2016) established a network of the connections between these neurotransmitters, taking into account the spatial and functional organization of their neurons and interactions [103] (Figure 4). This network was used to understand the neurotransmitter disbalances in schizophrenia and their normalization upon antipsychotic treatment. Indeed, disease pathology and drug action must understood in terms of a disbalance among multiple signaling pathways, rather than describing pathology and pharmacology as a single pathway disruption.

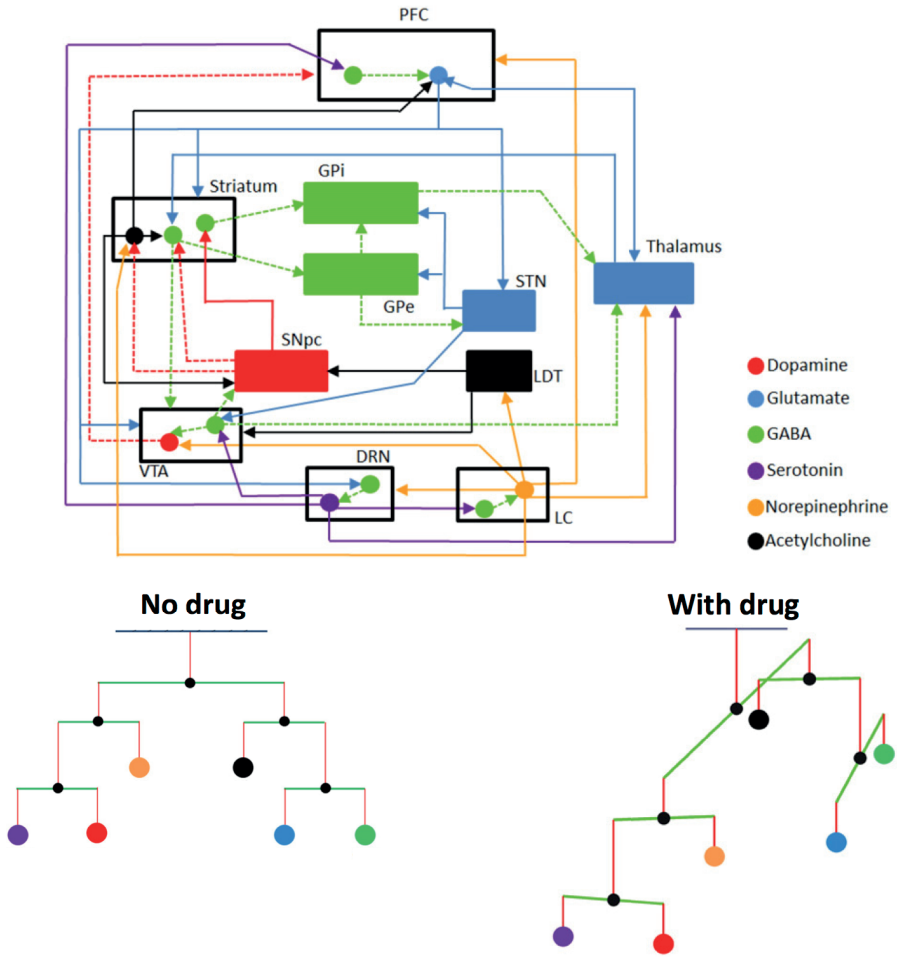



**Figure 4. Mathematical model containing expressions for the interactions between the different neurotransmitter systems in multiple brain regions. Rather than looking at single biomarkers, this model enables the prediction of disbalances among the neurotransmitter systems under conditions of drug administration. Adapted from reference (168) with permission.**

*Biomarkers that reflect the balance among the neurotransmitter systems*

It will become important to identify accessible biomarkers in CSF, plasma or urine that can reflect the balance among the neurotransmitter systems. While such approach has been followed for a glutamate receptor agonist, identifying the turnover of the dopamine, norepinephrine and serotonin pathway in CSF [41], there has not been such attempt for dopaminergic agents.

## *Effects on the kynurenine pathway*

*Metabolism and signaling of the kynurenine pathway*

Similar to serotonin, kynurenine is a metabolite of tryptophan. In fact, about 95% of tryptophan in the brain is metabolized via the kynurenine pathway, further leading to kynurenic acid, quinolinic acid and 3-OH-kynurenine [104,105]. Whereas quinolinic acid is a pro-glutamatergic molecule, kynurenic acid has several anti-glutamatergic properties, such as the antagonism of the NMDA receptor and the inhibition of glutamate release through ACh receptors. 3-OH-kynurenine is involved in the generation of free radicals, independent of the glutamate system [104]. 3-OH-kynurenine and quinolinic acid have neurotoxic properties, while kynurenic acid has proven to be neuroprotective [106]. A disbalance in the kynurenine metabolism was therefore associated with several neurological disorders, amongst which Parkinson's disease and schizophrenia [104,107,108].

*Effects of dopaminergic agents on the kynurenine pathway*

Kynurenic acid was reduced after long-term (1 – 12 months), but not after shorter-term (1 week) administration of clozapine, raclopride and haloperidol [109] (Table I, Figure 2). $D_2$ receptor antagonists may potentially interfere with the kynurenine amino transferase (KAT) enzyme, which converts kynurenine to kynurenic acid. Indeed, kynurenine and its metabolites other than kynurenic acid were not altered after treatment with $D_2$ receptor antagonists [109]. It is likely that this effect is $D_2$ receptor specific, given that raclopride is a highly selective $D_2$ receptor antagonist [110]. $D_2$ receptor antagonists thus likely inhibit the neuroprotective branch of the kynurenine metabolism, which could be a potential unwanted effect in the long term.

*Biomarkers for the kynurenine pathway*

Kynurenine and kynurenic acid are present in sufficient concentration in CSF to be quantified [107,108]. Moreover, 40% of the kynurenine synthesis occurs in the brain, while 60% takes place in the blood and is transported over the BBB. It is thus likely that kynurenine and kynurenic acid in CSF and plasma reflect the levels in the brain; however, it is not known to which extent. CSF and plasma levels changes upon dopaminergic treatment remain to be investigated.

## *Effects on the nitric oxide pathway*

### *Metabolism and signaling of the nitric oxide pathway*

Nitric oxide is generated by nitric oxide synthase (NOS) through the conversion of arginine to citrulline. Nitric oxide has a short half-life (i.e., few seconds) and is readily oxidized to nitrite and nitrate, which can then be measured as an indication of NOS activity. By binding to soluble guanylyl cyclase, nitric oxide stimulates local postsynaptic excitability via modulation of voltage-gated ion channels and possibly also presynaptic neurotransmitter release, thereby modulating synaptic plasticity [111,112]. Nitric oxide is tightly connected to glutamatergic signaling. Moreover, it contributes to gonadotrophin and oxytocin release, circadian and respiratory rhythms, locomotor and thalamocortical oscillation, as well as learning process and memory [111]. The nitric oxide pathway is downregulated in Parkinson's disease and schizophrenia, indicating a connection with dopamine [111,113,114].

### *Effects of dopaminergic agents on the nitric oxide pathway*

Citrulline, nitrite and nitrate have shown to be upregulated after short-term treatment with $D_1$ receptor and $D_2$ receptor agonists (Table I, Figure 2). Only two studies with systemic administration have been reported [115,116], while other studies focused on the effects after intracerebral injections [117,118]. A possible hypothesis for this upregulation is the stimulation of NOS activity by dopamine, thereby augmenting the production of citrulline and nitric oxide [117]. The effect on the nitric oxide pathway was proven to be $D_2$ receptor-specific in the striatum [118], while the $D_1$ receptor was involved in the NAc [117]. Although $D_2$ receptor antagonists blocked the effect of $D_2$ receptor agonists on nitric oxide concentrations [119], they did not exhibit a significant effect when administered alone [118,120]. However, long-term treatment with haloperidol led to an upregulation of neuronal NOS in the hypothalamus [38].

### *Biomarkers for the nitric oxide pathway*

Nitrite and nitrate have been measured in the CSF of patients suffering from neurological disorders [113,114], indicating their potential as easily-accessible biomarkers. Nitrate urine levels were found increased after intravenous administration of fenoldopam, a $D_1$ receptor agonist, although this effect might have been exerted via $D_1$ receptors present in the kidney, rendering difficult to discriminate between peripheral and central effects [115].

## *Effects on the endocannabinoid system*

### *Metabolism and signaling of the endocannabinoid system*

The most well-known components of the endocannabinoid system are anandamide, which is synthesized from N-arachidonoyl phosphatidylethanolamine, and 2-arachidonyl glycerol (2-AG), that is produced from phosphatidylinositol [121]. Anandamide is degraded to

ethanolamine and arachidonic acid by fatty acid amide hydrolase, while 2-AG is broken down to arachidonic acid by monoglyceride lipase [121]. Arachidonic acid is the precursor of a wide range of biologically and clinically important eicosanoids and respective metabolites, including prostaglandins and leukotrienes. The endocannabinoid system is widely distributed in the CNS where it reduces synaptic input through retrograde signaling via cannabinoid receptors, in the brain mainly the $CB_1$ receptor subclass [121].

*Effects of dopaminergic agents on the endocannabinoid system*

dopamine influences the endocannabinoid system mainly in the nigrostriatal pathway by upregulation of endocannabinoid system in the striatum and downregulation in the GPe in a $D_2$ receptor dependent manner [122]. Indeed, quinpirole stimulated the release of anandamide in the striatum [123], an effect that was blocked by raclopride (Table I, Figure 2). This provides evidence for $D_2$ receptor-dependent involvement of the dopaminergic system in endocannabinoid signaling. Furthermore, although the $D_1$ receptor agonist SKF38393 did not cause an effect on anandamide [123], it was found that, with impaired dopamine release, the striatal $D_1$ receptor may also affect the endocannabinoid system [122].

*Biomarkers for the endocannabinoid system*

Even though anandamide can be detected and quantified in the brain, its levels in CSF and plasma are very low [124], rendering its quantitation challenging. Moreover, 2-AG is chemically unstable in aqueous solution, leading to the formation of its isomer 1-AG. Nevertheless, ethanolamine levels can be measured in CSF suggesting this compound as a potential biomarker candidate to reflect the activity of the endocannabinoid system [125].

## *Dopaminergic treatment effects on the neuroendocrine and the energy systems*

Additional to its role in the CNS, the dopamine system is widely expressed in peripheral tissues [10], supporting the importance of evaluating the peripheral effects of dopaminergic agents. The CNS is connected to the periphery via the PNS and the neuroendocrine system, allowing for the opportunity to capture the consequence of central drug effects in the periphery, as done for instance with prolactin [23,24]. A significant influence on the hypothalamic-pituitary-adrenal (HPA) axis, the reproductive system, insulin signaling and the lipid metabolism has been found in this systematic review (Table II, Figure 2). With regards to biomarker discovery, two important aspects can be highlighted (Figure 3):

i) Biomarkers need to be evaluated together with other markers of the pathway of interest to understand its interaction with the drug;
ii) The connection between brain and target pathway must be quantitatively understood to allow for estimation on how the biomarker response reflects the central effect.

## *Effects on the hypothalamic-pituitary-adrenal (HPA) axis*

### *Signaling in the HPA axis*

The hypothalamo-pituitary-adrenal (HPA) axis is involved in the homeostasis of metabolic and cardiovascular systems, stress response, reproductive system, as well as immune system. It is a complex system of signals and feedback mechanisms between the hypothalamus, the pituitary gland and the adrenal glands. The hypothalamus releases corticotrophin releasing hormone (CRH) and vasopressin to modulate the secretion of adenocorticotropin hormone (ACTH) by the pituitary gland. ACTH subsequently stimulates the release of glucocorticoids (corticosterone in rodents, cortisol in humans) and catecholamines, which control CRH and ACTH release via a negative feedback loop. ACTH is cleaved from the prohormone pro-opiomelanocortin, which also yields to a number of different peptides including alpha-melanocyte stimulating hormone (α-MSH), beta-endorphin and a few other peptides that are also secreted from the pituitary gland.

### *Effects of dopaminergic agents on the HPA axis*

A wide range of neural systems influence the HPA axis [126], including dopaminergic system, both in a $D_1$ and $D_2$ receptor dependent manner (Table II, Figure 2) [127–129]. This effect is mainly observed after short-term treatment with $D_1$ and $D_2$ receptor agonists, while long-term treatment did not show a significant effect on basal ACTH levels [130].

Surprisingly, in contrast to haloperidol, the $D_2$ receptor antagonists eticlopride and remoxipride have been reported to increase ACTH plasma levels [24,131]. However, remoxipride was 40 times less potent to elicit the ACTH response than to induce the prolactin response [24], suggesting that these observations are explained by off-target effects.

Contrary to their conflicting results for ACTH release, $D_2$ receptor antagonists showed a consistent stimulation of corticosterone plasma levels (Table II, Figure 2), indicating that glucocorticoid release is not only mediated via a central mechanism of ACTH secretion. Additionally, the stimulation of the PNS was suggested to control the sensitivity of the adrenal medulla to ACTH, thereby enhancing the release of corticosterone. It is not certain whether this process is under dopaminergic control, but catecholamines certainly play a role [132]. Furthermore, $D_2$ receptor antagonists might directly modulate the release of corticosterone, given that $D_2$ receptors have been found on the adrenal cortex [133]. It is worth mentioning that investigations on dopaminergic innervation in the glucocorticoid release focused on aldosterone release from the zona glomerula, and not on corticosterone release from the zona fasciculate and reticularis [133]. Whether the effects of dopaminergic drugs are primarily mediated via dopamine receptors is not fully elucidated. While the ACTH response to $D_2$ agonist quinpirole was blocked by the $D_2$ antagonist sulpiride, indicating the involvement of the $D_2$ receptor, the corticosterone response was not evaluated by such approach [129].

**Table II. Effects of dopamine receptor agonists and antagonists on the neuroendocrine and energy system**

| | | D1-like receptor | | D2-like receptor | | Dosing period | | |
|---|---|---|---|---|---|---|---|---|
| **Pathway** | **Marker** | **Agonist** | **Antagonist** | **Agonist** | **Antagonist** | | **Matrix** | **References** |
| **HPA axis** | ACTH[a] | + | 0 | + | +/0 | Short-term | Plasma | (24,89–91) |
| | Corticosterone | + | 0 | + | + | Short-term | Plasma | (89,91–94) |
| | Alpha-MSH | | | 0 | + | Short-term | Plasma | (95,96) |
| | Corticosterone | 0 | | 0 | + | Long-term | Plasma | (97–102) |
| **Reproductive system** | LH | | | 0 | +/0 | Short-term | Plasma | (24,103) |
| | FSH | | | 0 | +/0 | Short-term | Plasma | (24,103) |
| | Progesterone | | | | + | Short-term | Plasma | (104,105) |
| | Oxytocin | | | + | | Short-term | Plasma | (106) |
| | LH | | | +/0 | -/0 | Long-term | Plasma | (99,102,107–111) |
| | FSH | | | +/0 | -/0 | Long-term | Plasma | (99,107,109–111) |
| | Testosterone[a] | | | 0 | -/0 | Long-term | Plasma | (97,101,108,110,112,113) |
| | Progesterone[a] | | | | +/- | Long-term | Plasma | (98,109–111,114) |
| | Estrogen[a] | | | | +/- | Long-term | Plasma | (98,110,111,114,115) |
| **Insulin signaling** | Glucose | | | + | + | Short-term | Plasma | (94,100,116–119) |
| | Insulin | | | 0 | + | Short-term | Plasma | (116–120) |
| | Glucagon | | | 0 | | Short-term | | (106) |
| | Glucose[a] | | | + | +/0 | Long-term | Plasma | (99,100,121–124) |
| | Insulin | | | 0 | +/0 | Long-term | Plasma | (98–100,115,121,122,125) |
| | Glucagon | | | 0 | | Long-term | | (99) |
| **Lipid metabolism** | Cholesterol[b] | | | - | +/0 | Long-term | Plasma | (98,99,121,125,126) |
| | Triglycerides | | | - | +/0 | Long-term | Plasma | (98,99,121,125,126) |

**In case multiple studies were identified for the effects of a particular drug class on a particular marker, only the 4 most recent publications were reported. [a]Few and/or conflicting data; [b]The atypical antipsychotics risperidone and clozapine showed a positive effect, whereas haloperidol showed a negative effect.**

**ACTH: adenocorticotropic hormone; Alpha-MSH: alpha-melanocyte stimulating hormone; LH: luteinizing hormone; FSH: follicle stimulating hormone**

In addition to ACTH and corticosterone, α-MSH secretion from the intermediate lobe of the pituitary gland is also controlled by the dopaminergic system [134]. α-MSH levels were increased after $D_2$ receptor antagonist treatment [135,136] but changed not after $D_2$ receptor agonist treatment [135], suggesting that α-MSH release is under maximal inhibitory control of dopamine.

*Biomarkers of the HPA axis*

Although the basal mechanisms of the HPA axis are very well understood, it remains unclear at which levels dopamine drugs interfere. The dopamine system is active in the hypothalamus, the pituitary gland, as well as the adrenal gland. While α-MSH and ACTH reflect the response in the pituitary gland upon hypothalamic stimuli, the corticosterone response is secondary to ACTH, or elicited at the adrenal gland directly. Therefore, the interpretation of biomarker responses should rely simultaneous evaluation of α-MSH, ACTH and corticosterone in a longitudinal manner to enable the evaluation of dopamine drug effects at the different levels of the HPA axis.

## *Effects on the reproductive system*

*Signaling in the reproductive system*

The reproductive system also involves communication between the brain and the periphery. It is controlled by the neuroendocrine system through the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, which stimulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary gland. These hormones subsequently modulate the release of progesterone and estrogens (estrone, estradiol and estriol) in females, as well as testosterone in males from the reproductive glands, which act as a negative feedback on GnRH release.

*Effects of dopaminergic agents on the reproductive system*

The role of the dopaminergic system in the reproductive system is supported by a well-known side effect of $D_2$ receptor antagonists, i.e., sexual dysfunction [137,138]. Furthermore, dopamine release in the nigrostriatal, mesolimbic and medial preoptic area plays a crucial role in mating behavior and copulation [139,140], providing a mechanistic basis for the involvement of dopamine in sexual function. Other studies have investigated the dopaminergic drug effects on the sex hormones testosterone, progesterone and estrogen in plasma (Table II, Figure 2). prolactin was excluded from our analysis because of its well-known relation with dopaminergic agents; however, it is an important mediator of sexual function, supported by the higher frequencies of sexual disorders observed with strong inducers of prolactin (classical antipsychotics and risperidone) compared to weak inducers (e.g., clozapine and olanzapine) [138]. The antipsychotic drug-induced disorders are at least partially mediated via peripheral mechanisms, since the peripherally acting $D_2$ recep-

tor antagonist domperidone also caused significant changes in reproductive hormones [141].

The results observed for testosterone plasma concentrations were conflicting and mainly associated with high dose levels [142–144]. Furthermore, while the $D_2$ receptor antagonists chlorpromazine and metoclopramide caused a reduction in progesterone and estrogen levels [145–147], sulpiride, clozapine, risperidone, and haloperidol led to enhanced concentrations [148–151]. Similarly, LH and FSH were reduced after long-term chlorpromazine and fluphenazine treatment [146,152], while there was no effect observed after long-term sulpiride, risperidone and haloperidol treatment [143,149]. After short-term haloperidol treatment, however, increased levels of LH and FSH were observed [153]. Interestingly, the effect of short-term $D_2$ receptor antagonist treatment was observed in female but not in male rats [24,153].

The non-selective characteristics of the abovementioned $D_2$ receptor antagonists may explain these conflicting results, particularly since the effects were associated with large dose levels [16,20]. Moreover, sex hormones show a high degree of intra-individual variability and impact of treatment duration, the latter being illustrated by the increased testosterone levels observed after 5 days of domperidone treatment, while it was reduced after 30 days [141]. This dual effect highlights the importance of longitudinal sampling upon dopaminergic treatment.

Finally, in addition to the effects of dopaminergic drugs on prolactin and the sex hormones, $D_2$ receptor agonists enhanced oxytocin secretion, likely in a D3R-specific manner [154].

*Biomarkers of the reproductive system*

The reproductive system has multiple levels, i.e., the hypothalamus, the pituitary and the endocrine glands, where further understanding is required to develop an effective biomarker strategy. The prolactin response is already difficult to interpret. Although some studies indicated that it correlates to drug exposure in the brain [23,155], another study found plasma exposure a better predictor [156]. A prolactin response has been also observed with domperidone, which does not cross the BBB [141]. These observations suggest that the prolactin response is a composite of central and peripheral effects. Similarly, it is not known to which extent LH and FSH represent a central or a peripheral effect. oxytocin, however, represents a biomarker for central effects only, given that the release is solely controlled by the hypothalamus. The testosterone and progesterone responses are secondary to LH and FSH responses, although they may also have been elicited through a peripheral mechanism. Overall, similar to the HPA axis, the longitudinal evaluation of such

possible biomarkers is essential to understand the interaction between dopamine drugs and the reproductive system.

## *Effects on the insulin system*

### *Signaling in the insulin system*

It is well known that many antipsychotics, especially atypical, increase the risks for complicated disorders such as metabolic syndrome and type 2 diabetes mellitus [157]. Blood glucose levels are controlled by mainly two hormones; insulin and glucagon. Upon a rise in glucose levels, insulin is secreted from pancreatic β-cells, leading to the glucose uptake in the muscles and storage as glycogen in the liver. As a consequence, the insulin secretion is reduced. When blood glucose levels fall, glucagon is released from the pancreatic α-cells, causing glucose release from the liver.

### *Effects of dopaminergic agents on the insulin system*

Although insulin signaling is under PNS control [158], the role of dopamine is mainly at the periphery. It is argued that dopamine and insulin are co-secreted from the pancreatic beta cells, with dopamine providing a negative feedback on insulin secretion in a $D_2$-like receptor dependent manner [159]. However, both insulin and glucagon levels were not influence by short-term $D_2$ receptor agonist treatment (Table II, Figure 2) [160], highlighting that this mechanism does not play a major role. In contrast, glucose concentrations were increased after treatment with the $D_3$ agonist 7-OH-DPAT, which was antagonized by raclopride. Interestingly, this effect was confirmed for quinpirole, but not for bromocriptine [160]. Possibly, off-target mechanisms of bromocriptine normalize the $D_3$ receptor mediated effect on glucose. Both glucose and insulin levels were increased with $D_2$ receptor antagonists (Table II, Figure 2). Typically, the dose required to elicit a short-term glucose response was higher than the one needed for a corticosterone response [161], indicating that an off-target effect explains this response.

The results of long-term treatment are conflicting, with in general no effect on basal fasting glucose or insulin levels [36,144,148,162], although for some D2 receptor antagonists a stimulation of the insulin system has been observed [36,144,163,164]. Given the large variation in experimental design (sex, strain, fasting protocol, dose levels), it is difficult to identify the source of this discrepancy. Moreover, many $D_2$ receptor antagonists were found to share the off-target affinity for other receptors, such as serotonine, muscarinic and the histamine receptor, all involved in weight gain which is associated with insulin resistance and hyperglycemia [16,157,165]. Interestingly, the $M_3$ muscarinic receptor was found to be crucial in the control of insulin release [166]. It is thus likely that the short- and the long-term effects of $D_2$ receptor antagonists on the insulin system are mediated via other receptors than the $D_2$ receptor only.

*Biomarkers of the insulin system*

The insulin system has been well described in terms of biomarkers, including fasting plasma glucose, fasting serum insulin and glycated hemoglobin. Systematic and well-controlled studies that longitudinally evaluate these biomarkers in combination with dopamine treatment are needed to better understand their potential interaction.

## *Effects on the lipid metabolism*

*Metabolism and signaling in the lipid system*

Phospholipid and cholesterol pathways are the main pathways of lipid metabolism. Both pathways start with acetyl CoA, and depending on whether the enzyme SREB-1 or SREB-2 is present, the fatty acid or the cholesterol pathway is activated [148]. Fatty acids are subsequently converted to triglycerides or phospholipids, amongst others. Cholesterol and phospholipids are notably essential to maintain the cell membrane integrity [167]. A distorted lipid metabolism can lead to the loss of neural transmission and is involved in brain several disorders, including schizophrenia [168]. Moreover, misbalances in the lipid homeostasis may, for example, cause weight gain, atherosclerosis and cardiovascular problems. In this regard, the relation between dopaminergic drugs and the lipid metabolism is closely related to what is observed with the insulin system [157,169].

*Effects of dopaminergic agents on the lipid system*

The lipid metabolism has shown to be significantly altered after long-term treatment, while no studies were identified for short-term treatment (Table II, Figure 2). For instance, 2-3 week treatment with the $D_2$ receptor antagonists risperidone and olanzapine caused an increase in triacylglycerols and a decrease in free fatty acids plasma levels, which was not the case for the partial $D_2$ receptor agonist aripiprazole [18]. Another study showed that 4 weeks of treatment with clozapine and risperidone, but not haloperidol, raised the serum levels of total cholesterol, free fatty acids and triglycerides via modulation of the pathway that is responsible for their biosynthesis [148]. The fact that the $D_2$ receptor agonist ergocryptine, although relatively unselective for this receptor, has been reported to decrease total cholesterol and triglycerides concentrations [170], may indicate that these effects are mediated via $D_2$ receptors. However, given that not all $D_2$ receptor antagonists affect the lipid metabolism, other receptors than the $D_2$ receptor may be involved. Further investigations are needed to investigate through which mechanism dopaminergic agents affect the lipid metabolism.

*Biomarkers of the lipid metabolism*

Cholesterol, free fatty acids, triacylglycerols and triglycerides can be used as biomarker to evaluate dopamine treatment effect on the lipid metabolism. Additionally, a lipidomics-

based approach also revealed an increase of phosphatidylethanolamine as biomarker for antipsychotic efficacy [18].

## *Recommendations for biochemical biomarker strategies in CNS drug development*

This review provides an extensive overview into the effects of dopaminergic agents on multiple biological pathways in the CNS and the periphery, as well as the potential of easily accessible biomarkers to reflect these effects. Overall, there is a strong need for systematic searches for biomarkers that together can represent the system-wide effects of dopaminergic agents. Here, we provide the following recommendations to account for system-wide effects in early CNS drug development.

*1. Use proteomics and metabolomics-based biomarkers discovery for CNS drug effects*

We envision a crucial role for proteomics and metabolomics approach to further elucidate known and unknown pathways and to identify drug effect-related biomarkers [171]. Considering the potential lack of insights into the system-wide effects of a new compound in early drug development, these methodologies enable preclinical anticipation of wanted and unwanted effects [172]. This information can then be used to optimize the future dosing strategies. Also, using a targeted metabolomics approach with monoamines in the brain, it was shown that risperidone and clozapine are biochemically closer to the 5-$HT_{2A}$ antagonist M100907 than to haloperidol [71,173]. Interestingly, this pattern highly corresponded with behavioral outcome [71]. Indeed, many of the dopaminergic agents described in this review are non-selective. Pharmacological effects should be seen as a balance between multiple components of a network of affected biochemical pathways (Fig 4) [103]. CNS drug discovery should thus aim for rational development of non-selective drugs to attack the polygenic CNS disorders [16]. Proteomics and metabolomics will certainly provide additional and valuable tools for the investigation of the *in vivo* pharmacology [171].

*2. Use longitudinal data and mathematical modeling*

Mathematical modeling to understand CNS drug effects are further needed. A pharmacological interaction at one or more receptors will pass on to the neurotransmitter network, causing the net result on the individual neurotransmitters, as well as the balance between them, being not so intuitive. A mathematical evaluation is therefore needed to understand CNS drug effects [103,174,175]. In this regard, longitudinal data on biomarker levels is essential to calibrate these models. Indeed, the pattern of the response reveals information that cannot be obtained from single time point measures [4,176]. For example, it was observed that not only basal levels of dopamine and norepinephrine were decreased after long-term treatment, but also the effect size after pharmacological stimulation [37,46].

Moreover, it is also difficult to quantify the effect by a single time point in short-term treatments.

*3. Evaluate CNS drug effects in combination with pharmacokinetics*

This temporal pattern not only depends on the dynamic interactions within the biological system, but also on the exposure pattern of the drug and its possible active drug metabolites at the site of action. It is therefore important to take into account the pharmacokinetics when evaluating the pharmacodynamics. Only one study considered the steady state plasma concentrations of clozapine and its active metabolite N-desmethylclozapine in combination with a response measure [74]. The levels of the drug and the metabolite showed high variability between the animals. Moreover, the ratio between clozapine and its metabolite was dependent on the sex of the animal and the dose. Given the fact that the exposure of the drug and its metabolite drives the response, such variability can have a significant impact on the biomarker plasma levels. This is particularly true for CNS drugs, for which the exposure pattern in the brain is determined by a complex interaction of pharmacokinetics, BBB transport and distribution through the brain [175]. Moreover, the drug exposure is likely to be brain region-specific, which will lead to quantitative differences in drug-receptor interactions, depending on the brain region [177]. Thus, when pharmacokinetics is taken into account, pharmacodynamics can be compared between drugs of the same pharmacological class, excluding the interference of pharmacokinetic differences.

*4. Analyze brain, plasma and CSF biomarkers simultaneously*

Plasma (or urine) samples are typically used for biomarker identification, while CSF samples are getting more and more interest in CNS-related diseases. Interestingly, our literature search did not reveal pharmacological biomarker evaluations in CSF, even though it has been used for other drug classes [41] and discovery of pathological biomarkers [88]. Although plasma and CSF have the advantage to be accessible in humans, biomarker responses in these biofluids may give a biased view with regard to the actual effects in the brain. Many biomarkers, for example dopamine, do not cross the BBB. Even in the case they do (e.g., HVA) or if the biomarker is measured in CSF, it is difficult to know how is quantitatively relates to the effects in the brain. The current overview shows hardly any studies that simultaneously studied biomarker responses in $brain_{ECF}$ and plasma. One study measured plasma and brain cholesterol levels after long-term treatment with clozapine or haloperidol, but no significant correlation was found [178]. Another study could positively associate serum progesterone levels with brain allopregnanolone as a reflection of $GABA_A$ potentiation and anxiolytic effect after short-term treatment with olanzapine and clozapine [150]. Systematic and simultaneous biomarker evaluations in plasma and

brain are recommended to provide a quantitative relation between the central effect and the accessible biomarker response.

*5. Investigate the condition-dependency of pharmacological effects*

Dopaminergic effects are highly condition dependent. As an illustration, dopamine receptors are present on immune cells to reduce their activation level [179,180], but no effect of dopaminergic agents was found on immune markers such as C-reactive protein, interleukin-6 or tumor-necrosis-factor alpha [144,181–183]. On the other hand, haloperidol was found to have immune-modulatory and anti-inflammatory effects in an animal disease model of rheumatoid arthritis [184]. Indeed, $D_2$ receptor antagonists have been shown to normalize lipopolysaccharide-induced inflammation [185], indicating that only in an activated immune system, $D_2$ receptor antagonists have an effect on immune markers. Thus, while some markers may not respond under healthy conditions, these observations cannot directly be extrapolated to a diseased condition. Patients or diseased animals need to be evaluated as a population on its own.

## Conclusions

This review highlights that dopaminergic agents, even selective ones, have a wide array of biochemical effects. Indeed, dopaminergic drugs may interfere with at least 8 different systems in the brain, including dopamine signaling, norepinephrine signaling, ACh signaling, GABA-glutamate circuits, serotonin signaling, kynurenine metabolism, nitric oxide pathway, endocannabinoid system, and 4 systems in the periphery, i.e., HPA axis, reproductive system, insulin signaling, and lipid metabolism. All these systems need to be taken into account during drug development. Moreover, in line with earlier reviews, many dopaminergic drugs are non-selective [16,20,21]. Therefore, although we refer to 'dopaminergic drugs', the biochemical actions of these drugs may be elicited via non-dopamine receptors. A systems pharmacology approach is expected to provide deeper insight into the actions of dopaminergic drugs. With such approach it will become possible to anticipate unwanted effects, such as weight gain or sexual disorders. It is stressed that CNS drug development lacks accessible biomarkers that represent central effect. Hardly any studies were found that relate the central effect to an accessible (i.e. CSF, plasma, urine) biomarker response. Moreover, plasma samples were mostly obtained at a single time-point, thereby missing the insight into the longitudinal pattern of the effect. Overall, given that other neurotransmitter systems are similarly interconnected as the dopamine system and also widely expressed, we highlight the need for longitudinal system-wide biomarker evaluations to create greater understanding of CNS and to improve early CNS drug development.

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# CHAPTER 4

## REVEALING THE NEUROENDOCRINE RESPONSE AFTER REMOXIPRIDE TREATMENT USING MULTI-BIOMARKER DISCOVERY AND QUANTIFYING IT BY PK/PD MODELING

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## Abstract

To reveal unknown and potentially important mechanisms of drug action, multi-biomarker discovery approaches are increasingly used. Time-course relationships between drug action and multi-biomarker profiles, however, are typically missing, while such relationship will provide increased insight in the underlying body processes. The aim of this study was to investigate the effect of the dopamine D2 antagonist remoxipride on the neuroendocrine system.

Different doses of remoxipride (0, 0.7, 5.2 or 14 mg/kg) were administered to rats by intravenous infusion. Serial brain extracellular fluid (brainECF) and plasma samples were collected and analyzed for remoxipride PK. Plasma samples were analyzed for concentrations of the eight pituitary related hormones as a function of time. A Mann-Whitney test was used to identify the responding hormones, which were further analysed by PK/PD modeling.

A three-compartment PK model adequately described remoxipride PK in plasma and brainECF. Not only plasma PRL, but also adrenocorticotrophic hormone (ACTH) concentrations were increased, the latter especially at higher concentrations of remoxipride. Brain derived neurotropic factor (BDNF), follicle stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH) and thyroid stimulating hormones (TSH) did not respond to remoxipride at the tested doses, while oxytocin (OXT) measurements were below limit of quantification. Precursor pool models were linked to brainECF remoxipride PK by $E_{max}$ drug effect models, which could accurately describe the PRL and ACTH responses. To conclude, this study shows how a multi-biomarker identification approach combined with PK/PD modeling can reveal and quantify a neuroendocrine multi-biomarker response for single drug action.


**Keywords:** blood-brain barrier; central nervous system; dose-response; hormones; pharmacokinetic/ pharmacodynamic models

### Abbreviations

ACTH: adrenocorticotropic hormone; AL: anterior lobe; BBB: blood-brain barrier; BDNF: brain-derived neurotropic factor; ECF: extracellular fluid; FSH: follicle stimulating hormone; GH: growth hormone; IL: intermediate lobe; LH: luteinizing hormone; ODE: ordinary differential equation; OFV: objective function value; OXT: oxytocin; PACAP: pituitary adenylate cyclase-activating polypeptide; PHDA: periventricular hypothalamic dopaminergic; PK/PD: pharmacokinetic/ pharmacodynamic; PL: posterior lobe; PRL: prolactin; RSE: relative standard error; THDA: tuberohypothalamic dopaminergic; TIDA: tuberoinfundibular dopaminergic; TSH: thyroid stimulating hormone; VPC: visual predictive check

## Introduction

To better understand pharmacological effects of central nervous system (CNS) drugs on the whole biological system, including the unknown mechanisms of action, a holistic approach is key [1,2]. Unfortunately, the focus of current *in vivo* pharmacology is often on the known mechanism of action only [3]. Therefore, to obtain insight in multiple system components, increasing efforts are made to show the utility of a multi-biomarker discovery approach, both in disease conditions and upon drug administration [3,4]. With that, the pathophysiological and pharmacological influences are reflected by a multi-biomarker response.

Thereby it is not enough to investigate dose versus multi-biomarker response, because such relationships are not unique, i.e. condition dependent. It is therefore important to have insight into processes that govern drug distribution to target sites, target binding kinetics, signal transduction and homeostatic feedback mechanisms. Such insight is obtained by multilevel studies, i.e. measurement of different biomarker types in a time-dependent manner, and advanced pharmacokinetic/ pharmacodynamic (PK/PD) modeling [5–7].

PK/PD modeling was successfully applied for the selective dopamine D2 antagonist remoxipride, both in human [8] and rat [9], to predict the pharmacological response beyond the tested conditions. The pharmacological response was represented by prolactin (PRL), which is a well-known biomarker for D2 antagonism [10,11]. More specifically, dopamine binding to the D2 receptor inhibits the prolactin release from the pituitary into plasma, and therefore D2 antagonism induces its release [10]. Movin-Osswald & Hammarlund-Udenaes (1995) developed a PK/PD model to describe the PK of remoxipride in plasma in conjunction to its effect on the PRL kinetics (synthesis, release and elimination) in human subjects, to successfully predict the PRL response after different and repeated doses [8]. Then, in our lab, Stevens et al. (2012) developed a translational PK/PD model for remoxipride effects on PRL plasma concentrations in rats, using data on brain extracellular fluid (brainECF) concentrations that could be identified as target site concentrations [9]. With that, they successfully predicted the human PK/PD data of remoxipride and PRL of Movin-Osswald & Hammarlund-Udenaes (1995). This indicated that plasma PRL is a translatable biomarker of D2 antagonism.

So far, D2 antagonism was only reflected by a single hormone, PRL. This hormone is part of the neuroendocrine system, which consists of the hypothalamus, the pituitary and peripheral hormone glands (e.g. adrenal gland), containing a variety of hormones. The neuroendocrine hormones are highly regulated through feedback mechanisms of single hormones on their own secretion or that of others, both in a direct or an indirect manner. PRL and oxytocin (OXT), for example, interact through a positive feedback loop in female rats [12]. Through the neuroendocrine system, the brain controls the plasma hormone

levels in response to neurological stimuli. Thus, from a pharmacological perspective, a change in plasma hormone concentration may reflect a central drug action. This provides the unique opportunity to study central pharmacology on plasma hormone concentrations, overcoming the ethical and technical hurdles of taking samples from the human brain. Plasma hormones are released from the pituitary, which consists of the anterior lobe (AL), the intermediate lobe (IL) and the posterior lobe (PL). Dopaminergic neurons from the hypothalamus are involved in regulating all these parts of the pituitary [13]. Release of hormones (e.g. PRL) that are stored in the AL may be regulated by dopamine that is secreted from tuberoinfundibular dopaminergic (TIDA) neurons into the portal vein. Hormones from the IL and the PL (e.g. OXT) are directly released, possibly from tuberohypothalamic dopaminergic (THDA) neurons that project into the pituitary. This tight connection between the dopaminergic and the neuroendocrine system inspired us to use the neuroendocrine system as a source for multi-biomarker discovery of dopaminergic agents.

Indeed, next to the dopamine-PRL connection, other interactions between the dopaminergic system and neuroendocrine hormones have also been reported. For example, the dopamine agonist bromocriptine is used to treat hypersecretion of adrenocorticotrophic hormone (ACTH) in Nelson's disease [14] as well as hypersecretion of growth hormone (GH) in acromegaly [15]. Moreover, dopamine was found to inhibit secretion of GH from human pituitary cells [16], and dopamine D2 receptors have been identified on thyroid stimulating hormone (TSH) adenoma's [17]. Furthermore, stimulation of the D2 receptor leads to suppression of the luteinizing hormone (LH) by induction of the second messenger pituitary adenylate cyclase-activating polypeptide (PACAP) in gonadotrophs [18]. Therefore, to understand whether these interactions between the dopaminergic and neuroendocrine system are relevant to dopamine D2 antagonists *in vivo*, it seems of interest to investigate their broader neuroendocrine responses in a well-controlled animal study.

In this study we applied a multi-biomarker discovery approach to investigate the neuroendocrine response to remoxipride in rats. Serial sampling of brainECF and blood was performed to determine the remoxipride PK in plasma and brain, following the procedures as earlier described [19]. Blood samples were also analyzed for ACTH, brain derived neurotropic factor (BDNF), follicle-stimulating hormone (FSH), GH, LH, OXT, PRL and TSH to obtain a neuroendocrine multi-biomarker. We combined this approach with PK/PD modeling to gain a comprehensive understanding of the PK/PD relation between remoxipride and the neuroendocrine system.

# Methods

## *Animals*

All animal experiments were performed in accordance with the Dutch Law of Animal Experimentation. The study protocols (DEC14051/DEC13186) were approved by the Animal Ethics Committee in Leiden. Male Wistar rats (n=106, 264 +/- 17 g), Charles River, The Netherlands) were housed in groups for 6-9 days until surgery (Animal Facilities Gorlaeus Laboratories, Leiden, The Netherlands), under standard environmental conditions with ad libitum access to food (Laboratory chow, Hope Farms, Woerden, The Netherlands) and acidified water. Artificial daylight was provided from 7:30AM to 7:30PM.

## *Surgery*

Surgery was performed following the procedures as described earlier [19], with slight adaptations. In short, animals were kept under 2% isoflurane anesthesia while they underwent surgery. They received cannulas in the femoral artery for serial blood sampling and femoral vein for drug administration. A microdialysis guide (CMA 12 Elite PAES, Schoonebeek, The Netherlands) was implanted in caudate-putamen (AP -1.0; L 3.0; V -3.4) for serial brainECF sampling. 24 hour before the experiment, the microdialysis guide was replaced by a probe (CMA 12 Elite PAES, 4 mm polycarbonate membrane, cut-off 20 kDA, Schoonebeek, The Netherlands). Between surgery and experiments, the animals were kept individually in Makrolon type 3 cages for 7 days to recover from surgery.

## *Experiments*

Table I provides an overview of the groups and number of rats used (study numbers EW01 and WB02). All experiments started between 8:00AM and 8:30AM, with rats randomly assigned to receive 0, 0.7, 5.2 or 14 mg/kg remoxipride by a 10-minute i.v. infusion at the start of experiment (t=0 min). Microdialysate perfusion buffer was prepared as described earlier [20], and 60 minutes before the experiment the perfusion was started using a flow rate of 1 ul/min until the end of experiment (see table I for the sampling times). Samples with a deviated flow rate of >10% were discarded. Microdialysate samples were stored at 4 degrees Celsius during the experiment and at -80 degrees Celsius after the experiment until analysis.

The extraction efficiency (*in vivo* recovery) of the microdialysis probe was determined following an *in vivo* loss experiment with 20, 100, 300 and 1000 ng/ml remoxipride. The microdialysate concentrations were corrected for an extraction efficiency of 11 +/- 0.5% (mean +/- SEM, n=208). Blood samples of 200 ul were taken at serial time points (Table I) through the arterial cannula and collected in heparin-coated eppendorf tubes. Animals received 200 ul saline after each sampling. The samples were centrifuged (1000 rpm, 10 min) for separation of plasma and were subsequently stored at 4 degrees Celsius during the experiment and at -20 degrees Celsius after the experiment until analysis.

**Table I. Overview of groups of rats, their numbers, experimental conditions, and analyses that were performed.**

| | | | Plasma samples | | | | | BrainECF samples | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Analysis | | | | | Analysis | | |
| Study | Nr. of animals | Dose | Sampling times (min) | No. | Volume | Platform/ assay | Output | Sampling times (min)[d] | No.[a,b,c] | Volume | Platform | Output |
| **EW01 (DEC14051)** | 21 | 0.7 mg/kg (10 min i.v.) | -15, 2, 7, 10, 15, 22, 40, 60, 150 | 189 | 20 ul | Bioplex/ RTPMAG-86K | PRL | -35 – 135 [20 minute interval] | 56 | 15 ul | LCMS | REM |
| | | | | | 15 ul | LCMS | REM | | | | | |
| **WB02 (DEC13186)** | 61 | 0, 5.2, 14 mg/kg (10 min i.v.) | -15, 2, 10, 22, 30, 40, 60, 100, 180, 240 | 450 | 80 ul | Bioplex/ RTPMAG-86K | OXT | -50 – 230 [20 minute interval] | 559 | 15 ul | LCMS | REM |
| | | | | | 20 ul | Bioplex/ RTPMAG-86K | ACTH, BDNF, FSH, GH, LH, PRL, TSH | | | | | |
| | | | | 160 | 15 ul | LCMS | REM | | | | | |
| **WB02 (*in vivo* loss) (DEC13186)** | 8 | 20, 100, 300, 1000 ng/ml, | | | | | | -50 – 310 [20 minute interval] | 208 | 15 ul | LCMS | REM |
| **WB06 (DEC13186)** | 21 | 0.7, 3.5, 7 mg/kg (2 min i.v.) | -15, 2, 10, 22, 30, 40, 60, 100, 180, 240 | 210 | 20 ul | LCMS | REM | -50 – 290 [40 minute interval] | 168 | 15 ul | LCMS | REM |
| **JS05[e] (DEC6132)** | 25 | 4, 8, 16 mg/kg (30 min i.v.) | -15, 2, 10, 22, 30, 40, 60, 100, 180, 240 | 250 | 20 ul | LCMS | REM | -25 – 55 [10 minute interval]<br>70 – 230 [20 minute interval] | 150 | 15 ul | LCMS | REM |

**ACTH: adrenocorticotropic hormone; BDNF: brain derived neurotropic factor; FSH: follicle stimulating hormone; GH: growth hormone; LCMS: liquid chromatography tandem mass spectrometry; LH: luteinizing hormone; OXT: oxytocin; PRL: prolactin; REM: remoxipride; TSH: thyroid stimulating hormone**

[a] **The first two or three microdialysis samples were used for equilibration purposes and therefore discarded**

[b] **For 6 animals the microdialysis failed as evaluated by a deviation in the flow > 10%, those samples were discarded**

[c] **The in vivo loss animals received two probes, one in the right CP and another one in the left, doubling the number of samples**

[d] **Sampling times are the midpoints of the collection intervals**

[e] **For details on the JS05 study, the reader is referred to reference [22]**

## *Analytical methods*

*Remoxipride in plasma and microdialysates* – Remoxipride concentrations in plasma and microdialysates were determined following a previously published liquid chromatography tandem mass spectrometry method [21] with small modifications to improve the column lifetime. In short, formic acid instead of trifluoro acetic acid was added to the solvents of on-line solid phase extraction and liquid chromatography, while maintaining the same performance in peak shape and resolution.

*Plasma hormones* – The plasma hormone analysis was performed with Bio-Plex® MAG-PIX™ technology (Biorad Laboratories, Veenendaal, The Netherlands). The hormones ACTH, BDNF, FSH, GH, LH, PRL and TSH were analyzed using the commercially available multiplex assay for analysis of pituitary hormones (RTPMAG-86K, Rat pituitary magnetic bead panel, Merck Millipore, Darmstadt, Germany). We followed the protocol provided by the manufacturer, analyzing 10 ul of each sample in duplo. OXT was analyzed using the commercially multiplex assay for analysis of neuropeptides (RMNPMAG-83K, Rat/mouse neuropeptide magnetic bead panel, Merck Millipore, Darmstadt, Germany). We followed the protocol provided by the manufacturer, analyzing 80 ul of each sample. Data were acquainted and concentrations were calculated using the Bio-Plex® Data Pro™ software (Biorad Laboratories, Veenendaal, The Netherlands). Data below lower or above upper limit of quantifications were excluded, which was below 9% for all hormones, except for OXT for which most measurements were below limit of quantification (< 3.84 pg/ml).

## *Identification of responding hormones for further PK/PD analysis*

To select responding hormones, the plasma hormone concentration-time data were first corrected for individual hormone baseline concentrations as obtained before administration of remoxipride (t=-15 min). These data were used to compare the experimental groups that received 5.2 or 14 mg/kg remoxipride with the placebo group (for baseline hormone values during the experimental period). A Mann-Whitney Wilcoxon non-parametric test was performed to compare the baseline corrected concentrations at each time-point for each hormone. Hormones that showed a significant difference ($p<0.05$) for at least one time-point were selected for further PK/PD analysis. All data analyses were performed using R version 3.1.1.

## *PK/PD modeling*

NONMEM® version 7.3.0 with subroutine ADVAN13 was used to perform the PK/PD modeling on the remoxipride concentrations in plasma and brainECF, and the hormone concentrations in plasma. In addition to data from the studies described above, plasma and brainECF remoxipride concentrations were taken from a previously performed study in which 4, 8 and 16 mg/kg remoxipride was administered by a 30-minute i.v. infusion

(table I) [22]. Criteria to develop the best model were i) significant drop in objective function value (OFV) calculated as -2loglikelihood ratio (> 3.84, $p < 0.05$, $df = 1$); ii) parameter precision; iii) goodness-of-fit; iv) correlations; v) condition number; vi) shrinkage; vii) bootstrap and viii) visual predictive check (VPC). Furthermore, the PK/PD model for PRL was externally validated on available data from Stevens et al. 2012 [9].

A sequential PK/PD modeling approach was applied, in which the posthoc parameter estimates of the PK model were used as input for the PK/PD model. For the PK model data were log-transformed and an exponential error model was found to best describe the residual variation of the data, whereas for the PD models a proportional error model was selected.

## Results

### *Pharmacokinetics*

A three-compartment model was identified to describe the free remoxipride concentrations in plasma and brainECF (figure 1, middle part). Inclusion of saturable remoxipride

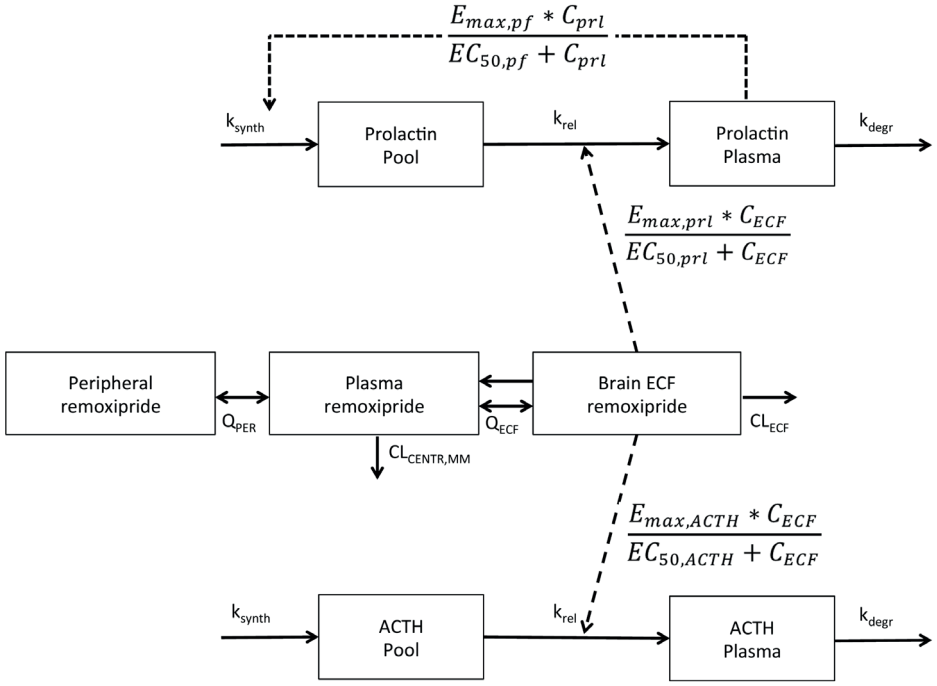



**Figure 1. Schematic overview of the final PK/PD model describing the pharmacokinetics of remoxipride in plasma and brain, as well as the pharmacodynamics for both PRL and ACTH. For explanation of the abbreviations the reader is referred to Table IV and Table V.**

clearance from plasma, described by Michaelis Menten kinetics, was found to improve the description of the data (dOFV -94, figure S1). This was particularly observed after low and high remoxipride doses (figure S1). Furthermore, in addition to passive blood-brain-barrier (BBB) transport, distribution from brainECF to plasma (dOFV -238) or elimination from brainECF (dOFV -239) was found to improve the model fit. However, the model with distribution from brainECF to plasma, showed imprecise parameter estimates (> 1000%) and therefore the model with elimination from brainECF was selected. Parameter estimates showed good precision (RSE < 30%), and bootstrap showed accurate estimates (table II).

**Table II. Remoxipride pharmacokinetics (PK). Parameter estimates and bootstrap results for the PK model following different doses of remoxipride. CL: clearance; CV: coefficient of variation; $k_m$: remoxipride concentration at half maximal clearance rate; Q: passive distribution between compartments; RSE: relative standard error of estimate; $V_{max}$: maximal clearance rate; V: volume of distribution.**

| Parameter | | | Bootstrap (n=50) | |
|---|---|---|---|---|
| | Parameter estimate | RSE (%) | Bootstrap mean | CV (%) |
| $V_{max,centr}$ (uM $h^{-1}$) | 5.9 | 25 | 5.9 | 32 |
| $k_{m,centr}$ (uM) | 2.9 | 27 | 3.0 | 31 |
| $V_{central}$ (L) | 0.14 | 8 | 0.13 | 10 |
| $Q_{PL\text{-}periph}$ (L $h^{-1}$) | 2.6 | 8 | 2.6 | 9 |
| $V_{periph}$ (L) | 0.52 | 15 | 0.52 | 14 |
| $Q_{PL\text{-}ECF,passive}$ (L $h^{-1}$) | 2.7 | 12 | 2.7 | 14 |
| $CL_{ECF,el}$ (L $h^{-1}$) | 3.1 | 14 | 3.1 | 18 |
| $V_{brainECF}$ (L) | 3.5 | 10 | 3.5 | 12 |
| **Residual error** | | | | |
| Plasma | 0.26 | 13 | 0.26 | 14 |
| ECF | 0.51 | 16 | 0.52 | 18 |

## *Pharmacodynamics*

### *Multi-biomarker discovery*

As shown in figure 2A, not only plasma PRL but also ACTH was found to respond to remoxipride treatment ($p < 0.05$). The other hormones (BDNF, FSH, GH, LH and TSH) did not show a significant response, while for oxytocin most measurements were below limit of quantification. Plasma PRL showed a response after both 5.2 mg/kg and 14 mg/kg, whereas ACTH only showed a response after 14 mg/kg remoxipride. The response time profiles of plasma PRL were similar for 5.2 mg/kg and 14 mg/kg (figure 2B). Apparently the response maximum was already reached after 5.2 mg/kg. Therefore, an additional study was performed in which plasma PRL was measured after 0.7 mg/kg remoxipride (EW01 study, table I). The plasma PRL concentrations after 0.7 mg/kg remoxipride were not only lower, but also exhibited a different longitudinal pattern. After 5.2 mg/kg and 14 mg/kg, but not after 0.7 mg/kg PRL showed a two-phasic decline (figure 2B). For plasma ACTH, a

quick response was observed after 14 mg/kg, with ACTH levels back to baseline within one hour. No response was observed after 5.2 mg/kg (figure 2C).

*PK/PD model for PRL*

For PRL, a pool model was identified linking brainECF remoxipride to the PRL release, including a positive feedback of PRL on its own synthesis (figure 1, upper part). First of all, although PRL in the placebo group initially decreased and subsequently increased (figure 2B), this trend was small relative to the pharmacological response (~3 ng/ml vs. ~45 ng/ml). Therefore, a steady baseline was assumed. Second, a turnover model outperformed a pool model as indicated by a significant difference in the OFV (table III, models A&B vs. C&D, dOFV > 3.84, *df* = 1). However, inclusion of a positive feedback component of plasma PRL (figure 1) on its own synthesis led to a significant improvement in comparison with both a pool model and a turnover model (table III, models A and C vs. E dOFV > 7.81, *df* = 3). Moreover, this model explained the two-phasic decline (figure 3A), which was not possible without positive feedback (fig-

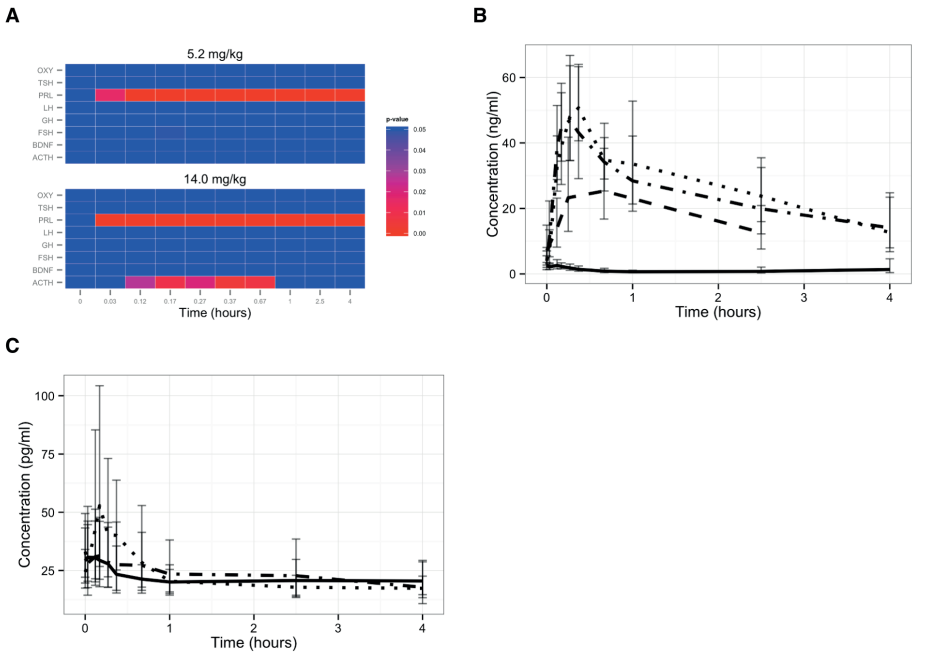



**Figure 2. Biomarker responses upon remoxipride treatment. A) Heatmap showing the differential response of the different hormones after 5.2 mg/kg and 14 mg/kg remoxipride. Red squares indicate a significant difference compared to the control group that received saline (*p < 0.05*). B) Different response time curves for PRL and C) ACTH after 0 (solid), 0.7 mg/kg (dashed) 5.2 (dot-dashed) or 14 mg/kg (dotted). Data are geometric mean +/- geometric SD.**

ure 3B). The positive feedback parameters ($E_{max,pf}$, $EC_{50,pf}$) were fixed to the values identified by Stevens et al. (2012), assuming that these system- specific parameters are not different between our earlier and more recent studies. Finally, remoxipride in brainECF could better explain the PRL response than remoxipride in plasma (table III, model E vs. F, dOFV > 3.84, *df* = 1). Parameter estimates were reasonably precise (RSE <30%), and bootstrap evaluation showed good accuracy (table IV). The VPC showed a good agreement of the model with the data (figure 3A). The model was found to well-describe the data from Stevens et al. 2012 [9], although the upper variation was slightly overestimated (figure S2).

**Table III. Steps in PK/PD model development for the models describing the PRL and ACTH responses.**

| Model | Description | OFV |
|---|---|---|
| | ***PRL*** | |
| A | Turnover model with drug effect from remoxipride in brainECF on PRL release | 1861 |
| B | Turnover model with drug effect from remoxipride in plasma on PRL release | 1897 |
| C | Pool model with drug effect from remoxipride in brainECF on PRL release | 1909 |
| D | Pool model with drug effect from remoxipride in plasma on PRL release | 1916 |
| **E** | **Pool model with drug effect from remoxipride in brainECF on PRL release + positive feedback of PRL in its own synthesis [best model]** | **1848** |
| | ***ACTH*** | |
| A | Turnover model with drug effect ($E_{max}$ model) from remoxipride in ECF on ACTH release | 1957 |
| B | Turnover model with drug effect ($E_{max}$ model) from remoxipride in plasma on ACTH release | 1869 |
| **C** | **Pool model with drug effect ($E_{max}$ model) from remoxipride in ECF on ACTH release [best model]** | **1862** |
| D | Pool model with drug effect ($E_{max}$ model) from remoxipride in plasma on ACTH release | 1861 |
| E | Pool model with drug effect (linear slope model) from remoxipride in ECF on ACTH release | 1860 |

*PK/PD model for ACTH*

Also for ACTH a pool model was identified, linking remoxipride in brainECF to the release of ACTH into plasma (figure 1, lower part). First of all, as indicated by a significant difference in the OFV, a pool model performed better than a turnover model (table II, model A&B vs. C&D, dOFV > 3.84, *df* = 1). Second, although it was not possible to discriminate between the model with brainECF remoxipride or plasma remoxipride explaining the ACTH response (table II, model C vs. D, dOFV < 3.84, *df* = 1), the brainECF model (model C) showed better parameter precision (28% vs. 89%). Moreover, the VPC showed better agreement, albeit a minor difference, with the data for this model (figure 3C) as compared to model D, with plasma remoxipride coupled to the response (figure 3D). Finally, because ACTH only showed a response after a high remoxipride dose, different drug effect models were compared. An $E_{max}$-model showed an equal model fit as a linear slope model (table III, model D vs. E, dOFV < 3.84, *df* = 1). However, to have a beginning of a clue about the potency of the ACTH response as compared to the PRL response, we sticked to the $E_{max}$-model.

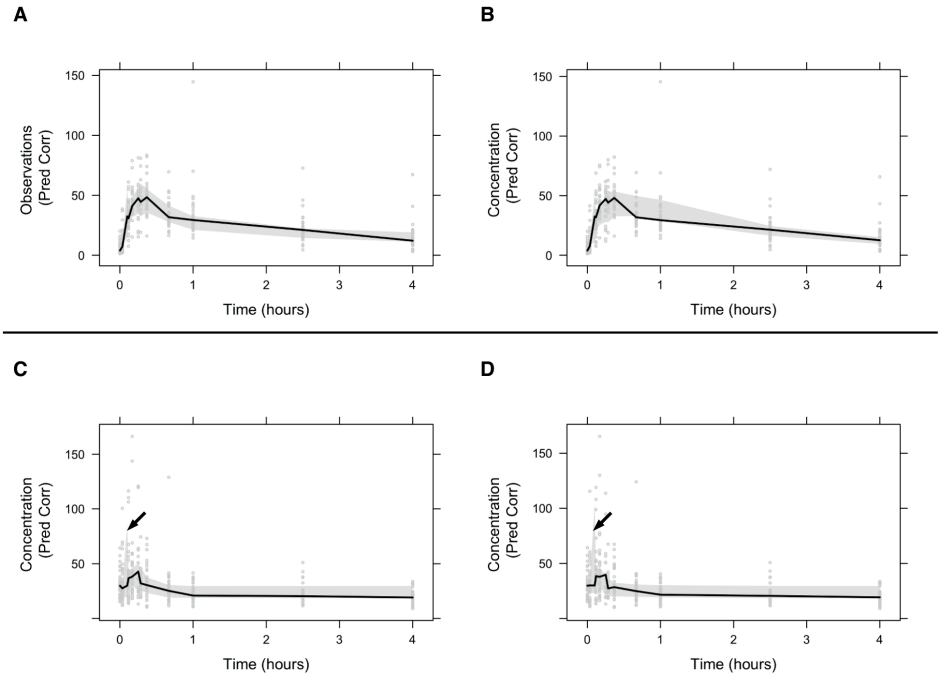



**Figure 3. Prediction corrected visual predictive checks (n=1000) for the remoxipride PK/PD model describing PRL after 0.7, 5.2 and 14 mg/kg (top) and ACTH after 0, 5.2 and 14 mg/kg (bottom). The pool models with (A) and without (B) positive feedback are shown for the PRL response. The models with ECF (C) and plasma (D) as target site of action are shown. Solid lines are the observed medians and the shaded area shows the 95% prediction interval around the simulated median. The extent to which this area captures the observed median is a measure of correct model specification. Ideally, at each point 50% of the prediction interval is above the observed median, and 50% below. The large prediction intervals in the third bin (see arrow) of figures (C) and (D) are explained by the fact that this bin contained only two observations.**

Since the degradation rate of ACTH ($k_{degr,acth}$) was not identifiable on basis of the current data, it was fixed to a value of 24.5 $h^{-1}$, as obtained in rats during the ACTH decline after a stress response [23].

Also, the maximal drug effect parameter ($E_{max,rem,ACTH}$) was not identifiable. However, because this parameter is a composite of unknown underlying parameters, a literature value was not available. Therefore, $E_{max,rem,ACTH}$ was chosen on basis of the sensitivity of the OFV to $E_{max,rem,ACTH}$ values of 1, 7.5, 10, 15, 20 and 100. Up till a value of 10 the OFV dropped significantly, but from a value of 10 or higher, it showed no significant difference (dOFV < 3.84). Therefore, $E_{max,rem,acth}$ was fixed to 10. Here it must be noted that, with changing $E_{max,rem,ACTH}$, the $EC_{50}$ changed almost linearly with the change in $E_{max}$, while other parameters remained the same. Parameter estimates of the best model (table III, model C) showed good precision (RSE < 30%) and accuracy (table V), and the VPC showed good agreement between the model and the data (figure 3C).

**Table IV. Parameter estimates and bootstrap results for the remoxipride PK/PD model describing the PRL response. Bsl: baseline;$E_{max}$: maximal effect; CV: coefficient of variation; $EC_{50}$: drug concentration at half maximal effect; IIV: interindividual variability; $k_{synth}$: PRL synthesis rate in the lactotroph; $k_{rel}$: PRL release rate from the lactotroph to plasma; $k_{degr}$: degradation rate of PRL in plasma; pf: positive feedback; prl: prolactin; rem: remoxipride; RSE: relative standard error of estimate.**

| Parameter | | | Bootstrap (n=100[c]) | |
|---|---|---|---|---|
| | Parameter estimate | RSE (%) | Bootstrap mean | CV (%) |
| $E_{max,rem,prl}$ | 13.3 | 12 | 13.8 | 16 |
| $EC_{50,rem,prl}$ (uM) | 0.064 | 28 | 0.074 | 63 |
| $Bsl_{prl}$ (ng $ml^{-1}$) | 7.3 | 7 | 7.2 | 6 |
| $k_{synth,prl}$ (ng $ml^{-1}$ $h^{-1}$) | 41.7[a] | | 41.2 | |
| $k_{rel,prl}$ ($h^{-1}$) | 0.35 | 12 | 0.36 | 18 |
| $k_{degr,prl}$ ($h^{-1}$) | 5.72[b] | | 5.72 | |
| $E_{max,pf,prl}$ | 3.47[b] | | 3.47 | |
| $EC_{50,pf,prl}$ (ng $ml^{-1}$) | 12.4[b] | | 12.4 | |
| **IIV** | | | | |
| $Bsl_{prl}$ | 0.075 | 19 | 0.071 | 43 |
| **Residual error** | | | | |
| Proportional | 0.13 | 8 | 0.13 | 13 |

[a] **$k_{synth}$ was calculated from $bsl_{prl}$*$k_{degr,prl}$.**
[b] **values were obtained from literature.**
[c] **100 out of 100 bootstrap runs minimized successfully.**

**Table V. Parameter estimates and bootstrap results for the remoxipride PK/PD model describing the ACTH response. Bsl: baseline; $E_{max}$: maximal effect; CV: coefficient of variation; $EC_{50}$: drug concentration at half maximal effect; IIV: interindividual variability; $k_{synth}$: ACTH synthesis rate in the lactotroph; $k_{rel}$: ACTH release rate from the lactotroph to plasma; $k_{degr}$: degradation rate of ACTH in plasma; pf: positive feedback; prl: ACTH; rem: remoxipride; RSE: relative standard error of estimate.**

| Parameter | | | Bootstrap (n=100[d]) | |
|---|---|---|---|---|
| | Parameter estimate | RSE (%) | Bootstrap mean | CV (%) |
| $E_{max,rem,ACTH}$ | 10[a] | | 10 | |
| $EC_{50,rem,ACTH}$ (uM) | 2.61 | 19 | 2.66 | 30 |
| $Bsl_{ACTH}$ (pg $ml^{-1}$) | 24.6 | 8 | 24.6 | 5 |
| $k_{synth,ACTH}$ (pg $ml^{-1}$ $h^{-1}$) | 603[b] | | 603 | |
| $k_{rel,ACTH}$ ($h^{-1}$) | 3.13 | 18 | 3.03 | 21 |
| $k_{degr,ACTH}$ ($h^{-1}$) | 24.5[c] | | 24.5 | |
| **IIV** | | | | |
| $Bsl_{prl}$ | 0.15 | 26 | 0.14 | 26 |
| **Residual error** | | | | |
| Proportional | 0.12 | 5 | 0.12 | 17 |

[a] **Parameter was not identifiable and the value was chosen on basis of a sensitivity analysis.**
[b] **$k_{synth}$ was calculated from $bsl_{prl}$*$k_{degr,prl}$.**
[c] **Values were obtained from literature.**
[d] **89 out of 100 bootstrap runs minimized successfully.**

## Discussion

Our goal was to apply the multi-biomarker discovery approach in a quantitative manner, with remoxipride as a paradigm compound. To that end, hormones from the neuroendocrine system were analyzed as a source for a multi-biomarker to represent remoxipride effect. We showed that not only PRL, but also ACTH acts as biomarker for remoxipride pharmacology. Both these hormones are synthesized in the so-called 'troph' cells in the anterior pituitary, from which they are continuously released. In our study, we quantified the effect of remoxipride on the PRL and ACTH release, as described by pool models, thereby gaining a comprehensive understanding of remoxipride effect on the neuroendocrine system. With that, we revealed the *in vivo* concentration effect relation of brainECF remoxipride with the hormones PRL and ACTH enabling the prediction of a neuroendocrine response for other doses of remoxipride.

### *Pharmacokinetics*

The brainECF concentrations were described by a three-compartment PK model, which was developed first to be subsequently linked to the PK/PD model. This model included drug elimination from the brain, confirming a previously developed PK model for remoxipride in rats [22]. Whether this represented remoxipride active transport from brainECF to plasma, metabolism of remoxipride in the brain, or efflux of remoxipride from brainECF to cerebrospinal fluid (CSF) remains to be answered. No studies on remoxipride being a substrate for efflux transporters have been reported, to the knowledge of the authors. Interestingly, O-demethylase activity has been observed in the rat brain [24], suggesting that remoxipride metabolism in the brain may have occurred. Furthermore, whereas Stevens et al. (2011) found linear elimination from plasma, we identified saturable elimination, described by Michaelis Menten kinetics. They applied a 30-minute infusion time, but we used a 10-minute infusion time with similar doses. This led to a 1.5 times higher maximal plasma remoxipride concentrations in the current study, making it more likely to observe saturation of metabolic clearance. This is not surprising for remoxipride since in rats it is eliminated mainly through liver enzymatic processes of demethylation and aromatic hydroxylation [25]. However, in humans remoxipride is metabolized mainly via oxidation [25], which may not be saturated at these concentrations. Indeed, linear elimination was identified for remoxipride in humans [26].

### *Pharmacodynamics*

Dopaminergic control over the neuroendocrine system is established via the tuberoinfundibular system, which consists of TIDA, THDA and periventricular hypothalamic dopaminergic (PHDA) neurons that connect the hypothalamus to the pituitary [13]. Therefore, we were interested in plasma hormones that are released from the AL or the PL of the pituitary as a reflection of central pharmacology of dopaminergic agents. Whereas PRL

has been used as a biomarker for central dopaminergic activity, exerted via the tuberoinfundibular system, other neuroendocrine hormones have not been used for this purpose. Surprisingly, despite the potential to respond to dopaminergic perturbation as described in the introduction, FSH, LH, GH, TSH and BDNF did not show a response to a single dose of remoxipride. Thus, a D2 agonistic effect on these hormones, or presence of the D2 receptor on the secretory cells does not necessarily imply a response to dopamine D2 antagonism. Also, within four hours we could not identify feedback regulation on these five hormones by the two responding hormones. However, using the multi-biomarker discovery approach on eight hormones, we identified not only PRL, but also ACTH as a biomarker for the D2 antagonist remoxipride. Unfortunately, we were not able to measure OXT since its levels were lower than the limit of quantification, although it is likely that there is an interaction between PRL and OXT [12].

### *PK/PD model for PRL*

To use prolactin as a biomarker for prediction of the response after a second remoxipride administration, it is important to take into account underlying tolerance mechanisms, either being the depletion of the lactotroph (pool model) [8,9] or the stimulation of dopamine production by PRL (agonist-antagonist interaction model) [27]. Although both models were able to account for the tolerance, only the pool model could explicitly separate drug-specific and system-specific parameters, enabling a proper translational step from animal to human [9]. The models seem to have similar flexibility to fit the prolactin response after a D2 antagonist challenge [27–29], and we also found a similar goodness-of-fit between the pool and the AAI models (AIC 1860 versus AIC 1844). In fact, to be able to discriminate between these models, a continuous infusion with a D2 antagonist should be applied. According to the pool model, the plasma PRL concentrations will drop to baseline after the pool is empty. Following the AAI model, the plasma PRL concentrations will reach a higher steady state during the continuous exposure. In this study, a pool model was found to describe the prolactin response with brainECF remoxipride related to the release of PRL. Furthermore, in agreement with Stevens et al. (2012), the inclusion of a positive feedback of PRL on its own synthesis significantly improved the model with particularly the two-phasic decline being better described (figure 3B). This feedback mechanism works by increasing the ‘refilling’ of the lactotrophs after release of PRL, possibly mediated through stimulation of the PRL receptor on the lactotrophs [9,10]. Although the inclusion of the positive feedback improved the data fitting, caution must be taken with this type of non-linear models. Bakshi et al. (2016) published a tutorial on mathematical analysis of ordinary differential equation (ODE) model behavior, showing that the pool model with positive feedback has two steady states and interesting stability behavior. This means that the model may converge to one or the other steady state, depending on the specific simulated trajectory, which could be affected by small changes

in dose or parameter values. Such behavior is not physiological, and caution should be taken when extrapolating to other parameter regions or dosages. Still, the model proved powerful in translating the PRL response from animal to human, provided inclusion of an if-condition that forced the prolactin concentrations to remain above baseline prolactin concentrations [9,30]. Simulations with the current model including the if-condition up to ten hours, showed a convergence to the correct baseline (Suppl fig 1).

Furthermore, dopamine D2 receptors influence PRL release both at the level of the hypothalamus (TIDA neurons) and the pituitary (lactotrophs) [10]. Stimulation of PRL release via D2 receptor antagonism at the TIDA neurons would be mediated through reduced dopamine release into the pituitary, whereas stimulation via the lactotrophs would be mediated through antagonism directly at the level of the pituitary. Reduced dopamine release via D2 antagonism at the TIDA neurons is driven by remoxipride in brainECF, whereas D2 antagonism at the lactotrophs is driven by remoxipride in plasma, since the pituitary is exposed to blood. With data on remoxipride both in plasma and brainECF, we could compare these hypotheses to find that PRL release was driven by remoxipride in brainECF (table III). However, domperidone, another D2 antagonist which hardly penetrates the brain, also stimulates prolactin release [31]. Indeed, drugs with limited brain penetration exhibit a low $ED_{50}$ of D2 receptor occupancy in the pituitary as compared to that in the striatum, which correlated with the $ED_{50}$ of prolactin release (peripheral effect) as compared to $ED_{50}$ of apomorphine induced stereotype behavior (central effect) [32]. This suggests that direct antagonism of pituitary D2 receptors is responsible for the prolactin release. Unfortunately, no such data exists on D2 antagonists with high brain penetration ($k_{p,uu}$ > 1) to investigate whether the reduced dopamine release in the pituitary will become dominant in stimulating the prolactin release. On basis of temporal PK/PD analysis we found brainECF remoxipride driving the PRL response. Therefore, because remoxipride highly penetrates the brain ($k_{p,uu}$ ~ 1), it is suggested that for D2 antagonists with high brain penetration, the reduced dopamine release into the pituitary becomes dominant in driving the PRL response.

Finally, the estimated $EC_{50,rem,prl}$ of 64 nM was 2-4 fold different from *in vitro* binding potencies of 113 nM [33] and 240 nM [34] for remoxipride on striatal D2 receptors measured in rat brain homogenate, but comparable to the $EC_{50}$ of 80 nM found for the previously developed pool model on basis of in vivo data [9]. The discrepancy between *in vivo* and *in vitro* estimates might be explained by a role of active metabolites [25]. Although their concentrations in plasma are 10-1000 times lower compared to remoxipride [25], four metabolites (FLA797, FLA908, NCQ436, NCQ469) showed 2-200 times higher *in vitro* affinity for the D2 receptor than the parent remoxipride [33]. Moreover, these metabolites showed *in vivo* activity on DOPA accumulation in rat striatum [35]. Interestingly, this

could be an alternative explanation of the two-phasic decline of prolactin (figure 2B). In that case, the PRL response would be the consequence of D2 receptor binding of both remoxipride and its active metabolites. Since the PK profile of the active metabolite lags behind that of remoxipride [25], the first part of the PRL response would be explained by remoxipride effect, whereas the second part would be driven by the active metabolites. Nevertheless, the contribution of active metabolites to the *in vivo* remoxipride potency is tentative and in further research remoxipride should be analyzed in conjunction with its active metabolites to obtain a complete picture of its PK/PD characteristics.

*ACTH as a biomarker of adrenergic receptor antagonism*

To the knowledge of the authors, no studies have been performed for the ACTH response upon remoxipride, but other dopaminergic agents have been investigated. The relation between dopaminergic agents and ACTH is rather intriguing. Although the dopamine D2 agonist bromocriptine is used to treat ACTH hypersecretion [14], suggesting an inhibiting effect, this appears to be specific for tumor cells [36]. On the contrary, ACTH release in rats was stimulated by the dopamine D2 agonists quinpirole and apomorphine, as well as by the dopamine reuptake inhibitor GBR12909. These effects were blocked by the dopamine D2 antagonists haloperidol and sulpiride suggesting a D2 specific response [37,38]. However, counterintuitively, haloperidol could also stimulate ACTH release [39,40], which was also observed for the D2 antagonists thioproperazine [41] and eticlopride [42]. Yet, sulpiride did not stimulate ACTH release [37].

Since the stimulatory effect of D2 agonists on ACTH release has been confirmed to be D2 specific, it is likely that the effect of the D2 antagonists is an off-target effect that these drugs have in common.

Although 5-$HT_{1A}$ agonists can induce ACTH release [43], remoxipride and haloperidol have no affinity for the 5-$HT_1$ receptors [34]. Moreover, 5-$HT_{1A}$ agonists are known to stimulate GH release [43], which was not observed for remoxipride in the present study. On the other hand, the *in vitro* affinity to adrenergic receptors of both remoxipride (to α2 receptor) and haloperidol (to α1 receptor) was only 10 - 50 times lower than to dopamine $D_2$ receptors [34,44]. Maximal ECF remoxipride concentrations that were observed in rats receiving 14.0 mg/kg (1.3 uM – 3.1 uM) exceed the $EC_{50,prl}$ (0.064 uM) by 20 – 50 times. In contrast, sulpiride has negligible affinity to adrenergic receptors (at least 100 times lower than its affinity to $D_2$ receptor) [34,44]. In addition, haloperidol showed considerable *in vivo* receptor occupancy and functional activity at central adrenergic receptors [45,46]. The endogenous ligands for adrenergic receptors are epinephrine and norepinephrine, which are known to inhibit the release of corticotropin releasing hormone (CRH) from the hypothalamus to the pituitary [47]. In the pituitary, CRH stimulates the release of

ACTH from the corticotrophs, which was found to be the pathway for thioproperazine to stimulate ACTH release [41]. This is in line with our observation that ACTH release is likely to be linked to remoxipride in brainECF, suggesting a drug-receptor interaction at the hypothalamus level. Following these observations, it is thus well possible that the effect of remoxipride on ACTH is elicited via antagonism of the centrally located adrenergic receptor, leading to an increased release of CRH and ACTH.

*PK/PD model for ACTH*

The mechanism of ACTH being released upon remoxipride administration thus seems similar to that of PRL, albeit via another pathway. Whereas PRL is released from lactotrophs, stimulated by reduction of dopamine, ACTH is secreted from corticotrophs, stimulated by induction of CRH. This provides further evidence for our observation that a pool model described the ACTH response (table III). Furthermore, as discussed above, ACTH is suggested as a biomarker for an adrenergic response. Although it was not possible to determine the exact $EC_{50,ACTH}$ (2.61 uM), since it was dependent on the $E_{max,ACTH}$ that was fixed to an arbitrary value of at least 10, we can conclude that it is at least 40 times higher than $EC_{50,prl}$ (0.064 uM). This confirms the earlier suggestion that ACTH represents an off-target effect of remoxipride, possibly via the adrenergic receptor.

Thus, by simultaneous analysis of eight hormones in plasma for a multi-biomarker approach, we could identify ACTH as additional biomarker for remoxipride in rats. Together with PRL, this biomarker provides insights into the effects of remoxipride on the neuroendocrine system through different pathways. However, whereas animals in preclinical experiments are very similar, having the same genetic background and environmental conditions, humans are highly heterogeneous in terms of genetic background, lifestyle, disease, age, and other factors that may influence drug efficacy [48]. Such variation may impact the response of the neuroendocrine system to D2 antagonists. For example, corticotrophs in pituitary tumors have higher D2 receptor expression and become sensitive to dopamine D2 agents [36], which would impact the ACTH response, but not the PRL response. Thus taking into account a multi-biomarker would give more precise insights in the PK/PD processes on an individual level, providing opportunities for personalized medicine.

Furthermore, as discussed above, the D2 antagonists haloperidol [40], thioproperazine [41], eticlopride [42] and remoxipride all stimulate ACTH release, whereas sulpiride [37] does not show such response. With PRL only, it is shown that some D2 antagonists cause hyperprolactinemia, but others not, providing a sub-classification of D2 antagonists. A multi-biomarker provides further sub-classification, for example distinguishing sulpiride from haloperidol, thioproperazine, eticlopride and remoxipride. Thus, our approach en-

ables detailed sub-classification and provides comprehensive insights in differential effects among drugs. As such, the approach should ultimately be applied to a series of dopamine $D_2$ antagonists and agonists.

Finally, since now we could reveal the neuroendocrine response by combining a multi-biomarker discovery approach with PK/PD modeling, we have provided a conceptual basis to use for example metabolism for a multi-biomarker paradigm. This is expected to provide a much more extensive multi-biomarker than the biomarkers used in the current study. With the metabolomics technique, more than one hundred endogenous metabolites can be measured, providing a promising next step with an untargeted biomarker discovery approach rather than the relatively targeted approach exploited in our study. These metabolite responses are then analyzed by multivariate statistics [49], which would identify the underlying shared responses among the metabolites and provide a multi-biomarker. Metabolomics has been successfully applied for identification of new biomarkers of drug effects [2–4], although no studies have been performed that combined it with PK/PD modeling. Therefore, further studies should expand on the current study by using metabolomics for untargeted biomarker discovery.

In conclusion, often multi-biomarker discovery and PK/PD modeling are separated fields, limiting the insights that can be obtained in *in vivo* pharmacological studies. In our study, we overcame this limitation by combining these fields, revealing remoxipride effects not only on PRL, but also ACTH. Moreover, using PK/PD modeling we revealed that both hormone responses were i) likely to be driven by remoxipride in brainECF and ii) described by a pool model according to the underlying physiology of hormone release from 'troph' cells. With that, we quantified the dose response over a large dose range, enabling the prediction of neuroendocrine responses after different doses of remoxipride. More generally speaking, our study shows how multi-biomarker discovery can reveal and PK/PD modeling can quantify the multiple neuroendocrine responses for single drug action.

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## Supplementary Materials

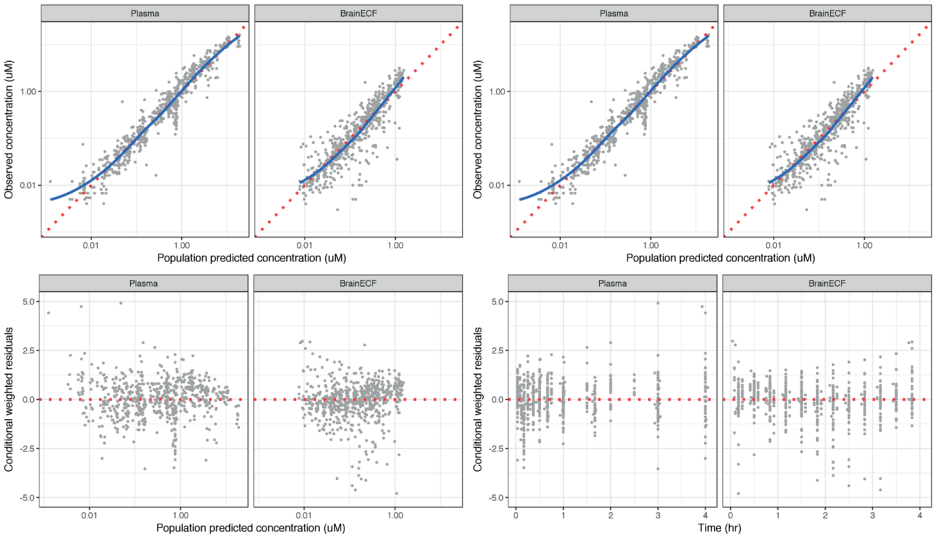



**Figure S1A. Goodness-of-fit of remoxipride PK model in plasma and brain$_{ECF}$ A) Observed versus individual predicted remoxipride plasma concentrations. B) Observed versus population predicted remoxipride plasma concentrations. C) Conditionally weighted residuals versus individual predictions. D) Conditionally weighted residuals versus time.**

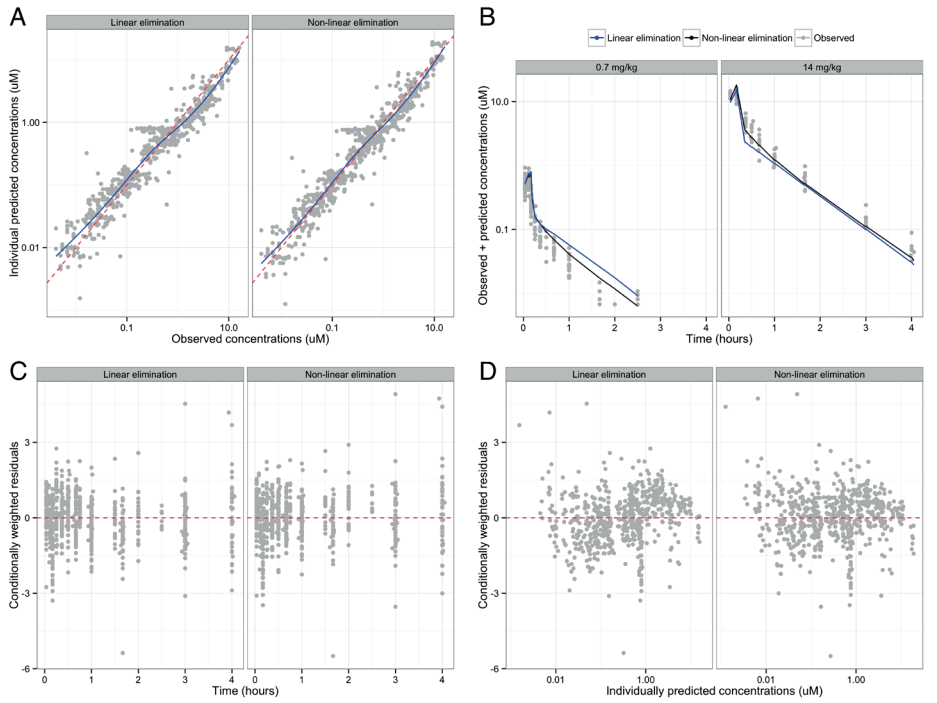


**Figure S1B. Goodness-of-fit of remoxipride PK model comparing linear plasma elimination and non-linear plasma elimination. A) Observed versus individual predicted remoxipride plasma concentrations. B) Concentration time graphs of observed and individual predicted remoxipride concentrations in plasma for a low dose (0.7 mg/kg) and a high dose (14 mg/kg) as examples. C) Conditionally weighted residuals versus time. D) Conditionally weighted residuals versus individual predictions.**

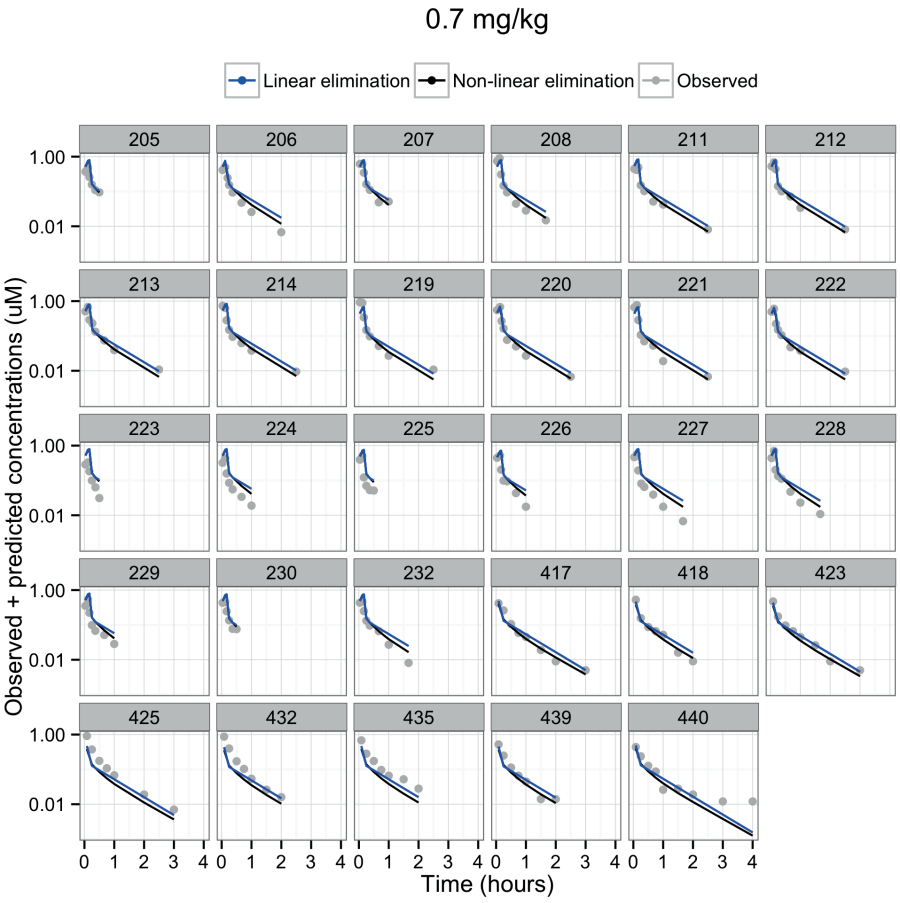



**Figure S1C. Individual remoxipride concentration profiles in plasma comparing observed concentrations with individual predicted concentrations with linear and non-linear elimination from plasma**

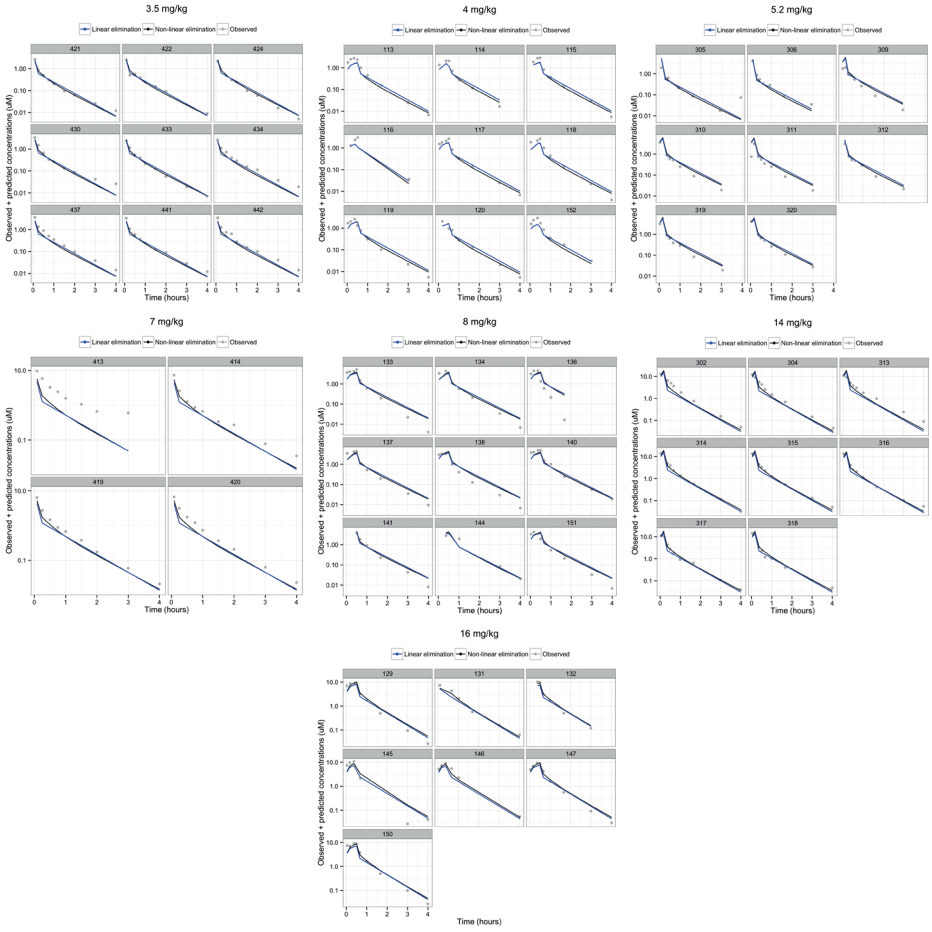



**Figure S1D-J. Individual remoxipride concentration profiles in plasma comparing observed concentrations with individual predicted concentrations with linear and non-linear elimination from plasma**

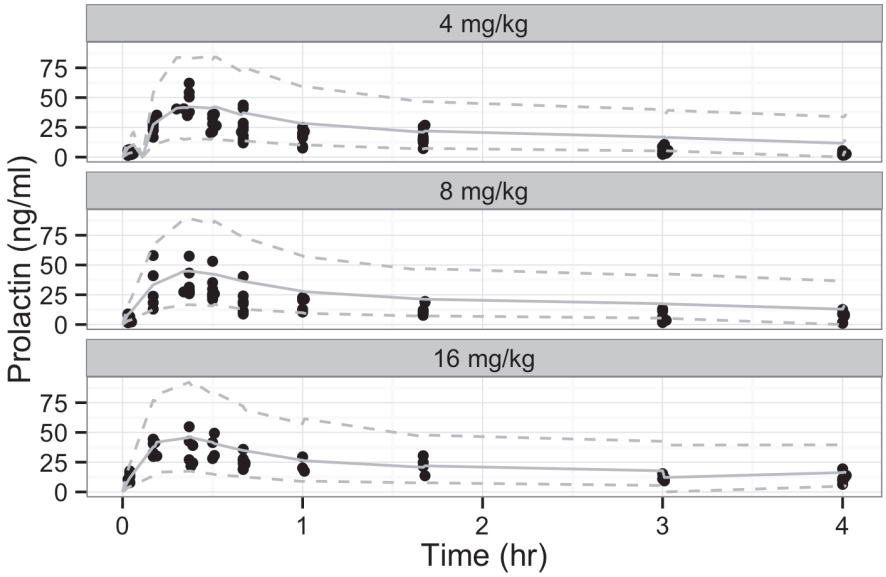



**Figure S2. External validation of the PRL PK/PD model on data from Stevens et al. 2012. 4, 8 and 16 mg/kg remoxipride was administered to rats by a 30 min. i.v. infusion. Observed data (black dots) are compared to simulated median (solid grey line) and 5% and 95% percentiles (dashed grey lines).**

# CHAPTER 5

## FINGERPRINTS OF CNS DRUG EFFECTS: A PLASMA NEUROENDOCRINE REFLECTION OF $D_2$ RECEPTOR ACTIVATION USING MULTI-BIOMARKER PK/PD MODELING

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## Abstract

Because biological systems behave as networks multi-biomarker approaches increasingly replace single-biomarker approaches in drug development. To improve the mechanistic insights into CNS drug effects, a plasma neuroendocrine fingerprint was identified using multi-biomarker pharmacokinetic/pharmacodynamic (PK/PD) modeling. Short- and longer-term $D_2$ receptor activation was evaluated using quinpirole as paradigm compound.

Rats (n=44) received 0, 0.17 or 0.86 mg/kg of the $D_2$ agonist quinpirole intravenously. Quinpirole concentrations in plasma and brain extracellular fluid ($brain_{ECF}$), as well as plasma concentrations of 13 hormones and neuropeptides, were measured. Experiments were performed at day 1 and repeated after seven-day subcutaneous drug administration. PK/PD modeling was applied to identify the *in vivo* concentration-effect relations and neuroendocrine dynamics.

The quinpirole pharmacokinetics were adequately described by a two-compartment model with an unbound $brain_{ECF}$-to-plasma concentration ratio of 5. The release of adenocorticotrophic hormone (ACTH), growth hormone (GH), prolactin (PRL), and thyroid stimulating hormone (TSH) from the pituitary was influenced. Except for ACTH, $D_2$ receptor expression levels on the pituitary hormone-releasing cells predicted the concentration-effect relationship differences. Baseline levels (ACTH, PRL, TSH), hormone release (ACTH), and potency (TSH) changed with treatment duration.

The integrated multi-biomarker PK/PD approach revealed a fingerprint reflecting $D_2$ receptor activation. This forms the conceptual basis for *in vivo* evaluation of on- and off-target CNS drug effects. The effect of treatment duration is highly relevant given the long-term use of $D_2$ agonists in clinical practice. Further development towards quantitative systems pharmacology models will eventually facilitate mechanistic drug development.


**Keywords:** CNS drugs, neuroendocrine system, biomarkers, quantitative systems pharmacology, dopamine agonists, PK/PD modeling

### Abbreviations

ACTH: adenocorticotropic hormone; BBB: blood-brain-barrier; BDNF: brain-derived neurotropic factor; CNS: central nervous system; CRH: corticotropic releasing hormone; FSH: follicle stimulating hormone; GH: growth hormone; LH: luteinizing hormone; OFV: objective function value; PK/PD: pharmacokinetic/pharmacodynamic; PRL: prolactin; RSE: relative standard error; TIDA: tuberoinfundibular dopaminergic; TSH: thyroid stimulating hormone

# 1. Introduction

Besides insufficient information on drug distribution into and within the brain, a main cause of attrition in central nervous system (CNS) drug development is the lack of translational pharmacodynamic biomarkers, i.e. preclinical biomarkers that are predictive for clinical effect [1–3]. This enables the mechanistic extrapolation of drug effects from animals to humans [3,4].

It is important that these biomarkers are accessible in humans. This poses a challenge for CNS drug development, given that sampling from the human brain is highly limited. However, the pituitary hormones and peptides of the neuroendocrine system are released upon signals from the CNS, in particular the hypothalamus, providing an opportunity to study central drug effects in plasma. Dopamine, for example, is released from the tuberoinfundibular dopamine (TIDA) neurons into the median eminence of the pituitary to control the release of prolactin (PRL) from the lactotrophs into plasma [5]. It has been shown that dopamine $D_2$ agonists stimulate the release of dopamine into the median eminence [6,7]. This principle has been used to evaluate the dopaminergic drug efficacy with PRL [8–13], including the translation of these effects from rats to humans [10,12].

Realizing that biological systems behave as networks, single biomarker approaches are increasingly replaced by multi-biomarker approaches [14,15]. Although PRL is a sensitive biomarker for dopamine $D_2$ receptor activation, it is also sensitive to serotonin and thyroid releasing hormone [16]. A multi-biomarker approach is envisioned to provide a more specific reflection of $D_2$ receptor activation. Indeed, the dopaminergic system has multiple connections to the neuroendocrine system, including the release of PRL, growth hormone (GH), and thyroid stimulating hormone (TSH) [17,18]. With that, it is important to identify the pharmacokinetic/pharmacodynamic (PK/PD) parameters that can be scaled from animals to humans [4]. Moreover, dopaminergic drug effects may change with increasing duration of treatment following sensitization and tolerance, as was shown for $D_2$ agonists [19].

The aim of the current study was, therefore, to characterize both the short-term and longer-term interaction of the dopaminergic system with the neuroendocrine system, in order to obtain a fingerprint biomarker of $D_2$ receptor activation. The selective $D_{2/3}$ agonist quinpirole will be used as a paradigm compound. Here we present a PK/PD fingerprint of quinpirole with adenocorticotrophic hormone (ACTH), GH, PRL and TSH as neuroendocrine biomarkers.

# 2. Methods

## *2.1. Animals, surgery, and experiment*

*Animals.* Animal studies were performed in agreement with the Dutch Law of Animal Experimentation and approved by the Animal Ethics Committee in Leiden, the Netherlands (study protocol DEC12247). Male Wistar rats (n = 44) were housed in groups for 6-9 days until surgery (Animal Facilities Gorlaeus Laboratories, Leiden, The Netherlands). Animals were held under standard environmental conditions while artificial daylight was provided from 7:30 AM to 7:30 PM. They had ad libitum access to food (Laboratory chow, Special Diets Services, Tecnilab BMI, Someren, The Netherlands) and acidified water.

*Surgery.* The surgery was performed following previously reported procedures [20]. The rats received 2% isoflurane anesthesia while undergoing surgery. After induction of the isoflurane, 0.09 ml Buprecare® (AST Farma B.V., Oudewater, The Netherlands) was administered intramuscular. Cannulas were placed in the femoral artery for serial blood sampling and the femoral vein for drug administration. Probe guides (CMA/12) with dummy probes were implanted in caudate putamen in both hemispheres (1.0 mm anterior, 3.0 mm lateral, 3.4 mm ventral, relative to bregma) and replaced by the probes (CMA/12 Elite – 4 mm) 24 hours before the experiment. After the surgery, the animals received 0.15 ml Ampicillan® (Dechra Veterinary Products B.V., Bladel, The Netherlands) and 3 ml 0.9% NaCl subcutaneously. The rats were individually held in Makrolon type 3 cages for 7 days to recover and weighed on a daily basis to evaluate the recovery.

*Experiments.* The rats were randomly assigned to receive 0 (n=12), 0.17 mg/kg (n=16), or 0.86 mg/kg (n=16) intravenous quinpirole between 10:45 AM and 11:15 AM on the first experiment day. The smaller group size for the control group was chosen, because less variation was expected in the data; i.e. there is no inter-individual variation from PK and the resultant PD processes. The statistical non-linear mixed effect analysis (see section *2.3. Data analysis*) is able to handle unbalanced study designs. The microdialysate samples were collected from -200 to 180 minutes (20-minute interval, 1.5 ul/min, 120 min equilibration time) in polypropylene microvolume inserts (250 ul, Waters) containing an antioxidant mix of 10 ul 0.02M formic acid/0.04% ascorbic acid in water. Blood samples of 200 ul were collected in heparin-coated Eppendorf tubes at -5, 5, 7.5, 10, 15, 25, 45, 90, 120 and 180 min and centrifuged (1000 x g, 10 min, 4°C) to separate the plasma. All samples were stored at -80°C until analysis. After the experiment, the cannulas were filled with a saline-heparin solution (venous) or a PVP-heparin solution (arterial), while a dummy replaced the probes. The rats received their quinpirole dose subcutaneously, until the second experiment on day 8, which was executed as on day 1. After the experiment, the rats were sacrificed following an overdose of Nembutal®.

## *2.2. Chemical analysis of the samples*

### *2.2.1. Quinpirole analysis in plasma and microdialysate*

Quinpirole (Bio-Connect, Huissen, The Netherlands) was analyzed using liquid chromatography-tandem mass spectrometry (LCMS/MS). Calibration standards were prepared in plasma with 0, 5, 10, 20, 50, 100, 200, 400 and 500 ng/ml and in buffered perfusion fluid (bPF) with 0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 ng/ml quinpirole. Quality controls (QC's) were prepared in plasma with 5, 10, 50 and 500 ng/ml and in bPF with 1, 6, 30 and 150 ng/ml quinpirole. Of the microdialysate samples, 20 µL was transferred to microvolume inserts (BGB Analytik, Harderwijk, the Netherlands and spiked with 20 µL of 40 ng/ml internal standard ropinirole-D4 (Bio-Connect, Huissen, the Netherlands). Of the plasma samples, 20 µL was spiked with 20 µL of the same internal standard and 20 µL water before deproteination with 1 mL acetonitrile (AcN). After centrifuging (20,000 x g, 10 min), the supernatant was transferred to an Eppendorf vial and dried by CentriVap vacuum centrifugation (Labconco, Kansas City, Missouri). The residue was dissolved in 40 µL 5 % AcN. After centrifuging (20,000 x g, 10 min) the supernatant was transferred to microvolume inserts and inserted into 1.5 ml screw cap vials.

The vials were placed into the Nexera X2 UHPLC-MS/MS system (Shimadzu, 's Hertogenbosch, the Netherlands) at 10°C. 5 µl of the sample was injected into the system, operated by LCQuan software (version 2.7, Thermo Fisher Scientific, Breda, the Netherlands)) and the MS Finnigan TSQ quantum ultra-mass spectrometer (Thermo Fisher Scientific, Breda, The Netherlands), operated by XCalibur software (version 2.5, Thermo Fisher Scientific, Breda, The Netherlands). An Acquity UPLC BEH C18 column (130Å, 1.7 µm, 2.1 mm X 50 mm; Waters, Etten-Leur, The Netherlands) was used with a flow rate of 0.4 ml/min and a column temperature of 40°C. The mobile phases were prepared in 10 mM ammonium acetate in water (adjusted to pH 7 with formic acid). The aqueous mobile phase ($MP_{AQ}$) contained 5% and the organic mobile phase ($MP_{ORG}$) 95% AcN. A gradient was applied with 10% $MP_{ORG}$ (0 – 0.5 min) to 100% $MP_{ORG}$ (0.5 – 2.0 min) and kept at 100% $MP_{ORG}$ (2.0 – 2.8 min), after which the column was re-equilibrated with 10% $MP_{ORG}$ (2.8 – 3.0 min). The retention time of quinpirole and ropinirole-D4 was 1.8 and 2.24 minutes, respectively. The MS was used in positive electrospray ionization mode and all compounds were monitored by Selective Reaction Monitoring (SRM). The ionization voltage, capillary energy, capillary temperature and desolvation temperature were set to 3.50 kV, 3 V, 150°C, and 400°C, respectively. The transition ion pair was 220.18 m/z → 161.00 m/z, 16 V for quinpirole and 265.22 m/z → 132.07 m/z, 32 V for ropinirole-D4. The quality of the method was assured following the guidelines for bioanalysis [21]. The unbound fraction of quinpirole in plasma was determined to be 71 ± 3% (concentration-independent) by filtrating plasma samples using high-speed filtration (Centrifree®, Merck Millipore, Amsterdam, The Netherlands, 2000 x g, 10 min) and calculating the ratio of unbound to total plasma concentrations.

The measured total plasma concentrations of quinpirole were corrected accordingly, to obtain unbound plasma concentrations. The recovery of quinpirole over the microdialysis probe was determined to be 5.4 ± 1.7% (n = 191) using the retrodialysis method [22]. The measured microdialysate quinpirole concentrations were corrected for probe recovery to report the $brain_{ECF}$ concentrations.

*2.2.2. Pituitary hormones and neuropeptides in plasma*

The pituitary hormones (ACTH, brain-derived neurotropic factor (BDNF), follicle stimulating hormone (FSH), GH, luteinizing hormone (LH), PRL and TSH) and neuropeptides (α-melanocyte stimulating hormone, β-endorphin, neurotensin, Orexin A, oxytocin, Substance P) were analysed by multiplex assays (RTPMAG-86K and RMNPMAG-83k, Merck Millipore, Darmstadt, Germany) on a Bio-Plex® MAGPIX™ system (BioRad Laboratories, Veenendaal, The Netherlands). With the RTPMAG-86k, 10 uL and with the RMNPMAG-83k, 50 uL plasma was used for analysis according to the protocol provided by the manufacturer.

## *2.3. Data analysis*

*2.3.1. PK/PD modeling software and criteria*

The PK/PD models to describe the quinpirole and the hormone concentrations in $brain_{ECF}$ and plasma were developed by a two-stage approach (the PK parameters were fixed before developing the PD models), using a non-linear mixed effect population approach in NONMEM® version 7.3.0 with subroutine ADVAN13. The inter-individual variability around the parameters and the residual error were described by an exponential distribution (suppl. Equation 1, 2). Model selection was based on successful convergence, objective function value (OFV), parameter precision and visual evaluation of the model predictions as compared to the observations.

*2.3.2. Pharmacokinetic model development*

Two- and three-compartment models were compared, both with linear or non-linear clearance from plasma for their description of unbound quinpirole concentrations in plasma and $brain_{ECF}$. Here it should be noted that a two-compartment model refers to one compartment describing plasma and another compartment describing $brain_{ECF}$ quinpirole concentrations. The transport into and out of the brain across the blood-brain-barrier (BBB) in these models was estimated with two separate distribution clearances. The experiment day was evaluated as covariate on one of the model parameters. The selected model was evaluated on additional data to guarantee external validity, and the details of which are described in the supplementary information.

*2.3.3. Pharmacodynamic model development*

For each hormone, baseline, PK/PD and covariate models were developed in a step-wise manner. Baseline patterns were evaluated on placebo data following supplementary equations Part I, 4 – 7. The selected baseline models were, together with the pharmacokinetic model, integrated into the PK/PD models. The PK/PD models were defined as a combination of the following characteristics: i) baseline model for each hormone; ii) plasma or $brain_{ECF}$ as target site; iii) the slope, the $E_{MAX}$, the alternative $E_{MAX}$ [23], the on-off, or no drug effect model; iv) and the direct response, the turnover or the pool model as link model (suppl. Equations Part I, 9-16). The best model was automatically selected on basis of model convergence and OFV. Finally, the selected PK/PD models were evaluated for an effect of experiment day using step-wise covariate model building [24] (suppl. Equations Part I, 17-19).

*2.3.4 Estimation of signal transduction efficiency*

The quantitative relation between receptor binding and pharmacological effect depends on the signal transduction efficiency [25,26], which is made explicit in the operational model of agonism [27]. Therefore, the selected PK/PD models were simulated and fitted by the operational model (Equation 1) [25,27]:

$$E = \frac{E_m * \tau * C}{k_A + (1 + \tau) * C} \quad (1)$$

In which $E_m$ is the systems maximum, $\tau$ is the transduction efficiency, and $k_A$ is the affinity for the $D_2$ receptor. It was assumed that the target site of action is in the $brain_{ECF}$. Furthermore, the assumption was made that quinpirole is selective for the dopamine $D_2$ receptor, and the GH, PRL and TSH responses were modulated via the TIDA neurons (Figure 2). Therefore, the affinity of quinpirole to the $D_2$ receptor was estimated equal among all hormones, while the signal transduction efficiency of GH, PRL and TSH was assumed dependent on pituitary $D_2$ receptor expression obtained from literature [28]. The $D_2$ receptor expression for somatotrophs, lactotrophs and thyrotrophs was calculated as the number of 'troph' cells expression the $D_2$ receptor relative to the total number of 'troph' cells. This relation to the signal transduction efficiency was made explicit following equation 2:

$$\tau = \tau_0 * e^{slp * receptor\ expression} \quad (2)$$

Where $\tau$ is estimated for GH, PRL and TSH on basis of the pituitary $D_2$ receptor expression.

# 3. Results

## *3.1. Pharmacokinetics of quinpirole in plasma and brain$_{ECF}$*

A two-compartment model best described the pharmacokinetics of quinpirole in plasma and brain$_{ECF}$ with linear first-order elimination from plasma and a net active influx from plasma to brain$_{ECF}$ (Suppl. Equation 3). The parameter estimates were precise and accurate (Table I), and the model could well describe the quinpirole concentrations in plasma and brain$_{ECF}$ over a large dose range (Figure 1A). Although there is a slight over-prediction of quinpirole concentrations in brain$_{ECF}$, external validation showed good extrapolative ability of the model (Figure 1B).

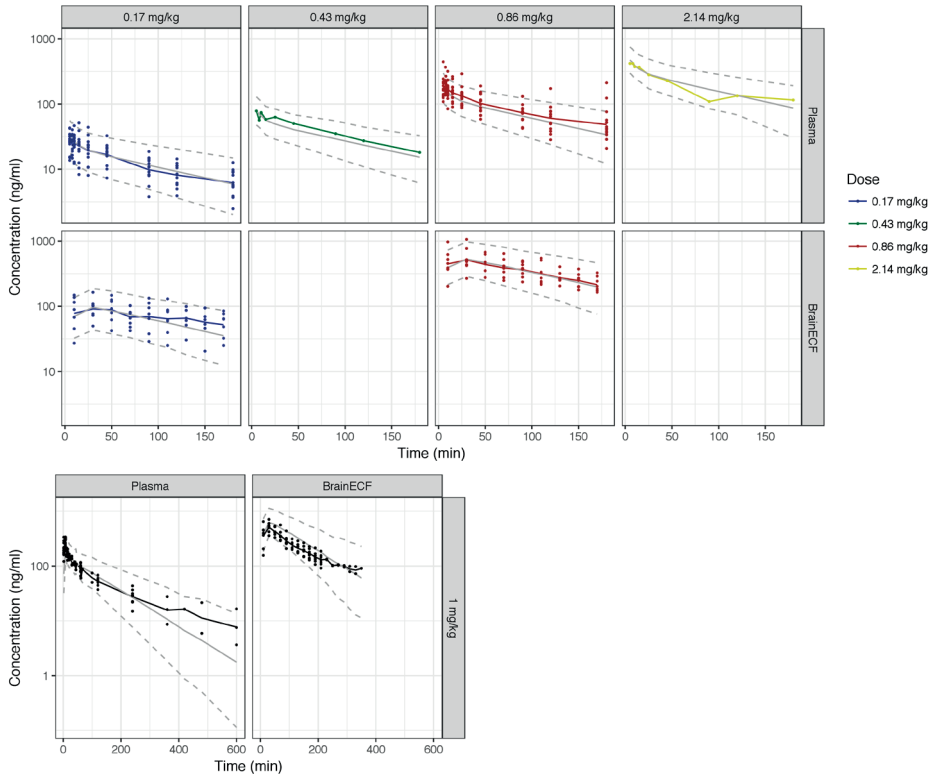



**Figure 1. Visual predictive check (A) and external validation (B) for the quinpirole pharmacokinetic model in plasma and brain$_{ECF}$. The colored dots represent the observed data, with the solid colored lines showing the mean of the observations. The solid grey line shows the mean, and the dashed grey lines the 90% confidence interval of 500 simulations. *The experiments in which the animals received 0.43 mg/kg and 2.14 mg/kg represented experimental protocol deviations (higher dose), and were included in PK model development only.**

**Table I. Parameter estimates of the quinpirole pharmacokinetic model**

| Parameter | Model evaluation | | Bootstrap ($n_{btstr}$ = 168) | |
|---|---|---|---|---|
| | Estimate | (RSE)[shr] | Estimate | (CV) |
| $CL_{PL,o}$ (L $h^{-1}$) | 0.71 | (9%) | 0.70 | (9%) |
| IIV $CL_{PL,o}$ | 0.12 | (32%) [7%] | 0.12 | (35%) |
| $CL_{PL,ECF}$ (L $h^{-1}$) | 2.5 | (20%) | 2.5 | (19%) |
| $CL_{ECF,PLASMA}$ (L $h^{-1}$) | 0.52 | (24%) | 0.55 | (24%) |
| $k_{p,uu}$ ($CL_{PL,ECF}$/$CL_{ECF,PL}$) | 5 | | | |
| $V_{CENTRAL}$ (L) | 1.0 | (6%) | 1.0 | (7%) |
| $V_{ECF}$ (L) | 0.12 | (13%) | 0.013 | (17%) |
| RUV $C_{QP,PL}$ | 0.08 | (24%) [3%] | 0.08 | (24%) |
| RUV $C_{QP,ECF}$ | 0.12 | (28%) [2%] | 0.12 | (30%) |

**C: concentration; CL: clearance; CV: coefficient of variation; ECF: brain extracellular fluid; h: hour; IIV: inter-individual variability; $k_{p,uu}$: ratio of unbound $brain_{ECF}$ and plasma drug concentration; L: liter; $n_{btstr}$: number of successful bootstrap model runs out of a total of 200 runs; PL: plasma; RSE: relative standard error; RUV: residual unexplained variability; shr: shrinkage; V: volume of distribution**

### *3.2.1. Responding pituitary hormones and neuropeptides in plasma*

On basis of automated model selection, the hormones luteinizing hormone, PRL and TSH showed a placebo response described by circadian rhythm with a period of 120 minutes, the Bateman equation, or exponential decay, respectively (Suppl. Figure 1A). A model with no baseline pattern best described the other hormone baselines. ACTH, GH, PRL and TSH responded to quinpirole treatment with diverse PK/PD relations, while no effect was observed on the neuropeptides, BDNF, FSH and LH, following automated model selection (Suppl. Figure 1B, Table II). Except for $k_{deg,ACTH}$ (relative standard error (RSE) = 282%) and $EC_{50,Prl}$ (RSE = 99%), the parameters were identified with reasonable precision (Table III) and the models could describe the data well (suppl Equations Part II, suppl. Figure 2).

**Table II. The PK/PD effects of quinpirole on ACTH, GH, PRL and TSH, including the PK/PD model type and target site of drug action that was identified**

| Hormone | Effect | PK/PD model | Target site |
|---|---|---|---|
| ACTH | + | Slope model & Pool model with stimulation of $k_{REL}$ | Plasma |
| GH | - | $E_{MAX}$ model & Turnover model with inhibition of $k_{REL}$ | $Brain_{ECF}$ |
| PRL | - | $E_{MAX}$ model & Turnover model with inhibition of $k_{REL}$ | $Brain_{ECF}$ |
| TSH | - | $E_{MAX}$ model & Turnover model with inhibition of $k_{REL}$ | $Brain_{ECF}$ |

**ACTH: adenocorticotropic hormone; ECF: extracellular fluid; Effect: + increased release, - reduced release; GH: growth hormone; $k_{REL}$: hormone release rate; PRL: prolactin; TSH: thyroid stimulating hormone**

### *3.2.2. Target site of effect*

No statistically significant difference was identified comparing the best models for ACTH, GH, PRL and TSH with either plasma or $brain_{ECF}$ as target site (Table III).

**Table III. Parameter estimates of the PK/PD models for quinpirole effect on ACTH, GH, PRL and TSH with plasma and $brain_{ECF}$ as target site. In bold the parameters of the selected models.**

| | **Plasma** | **$Brain_{ECF}$** |
|---|---|---|
| **ACTH** | **Estimate (RSE)** | **Estimate (RSE)** |
| **OFV** | -31.3 | -32.9 |
| **Baseline (pg/ml)** | 3.74 (17%) | 3.71 (17%) |
| **$IIV_{Baseline}$** | 0.68 (71%) [0%] | 0.68 (72%) [0%] |
| **Slope ($[ng/ml]^{-1}$)** | 0.873 (43%) | - |
| **$E_{MAX}$** | - | 2.35 (11%) |
| **$EC_{50}$ (ng/ml)** | - | 54.1 (40%) |
| **$K_{DEG}$ ($min^{-1}$)** | 0.0146 (24%) | 308 (282%) |
| **$K_{REL}$ ($min^{-1}$)** | 0.00760 (29%) | 0.00421 (31%) |
| **RUV** | 0.27 (21%) [3%] | 0.27 (21%) [3%] |
| **GH** | | |
| **OFV** | 667.6 | 664.1 |
| **Baseline (pg/ml)** | 1002 (n.a.) | 992 (25%) |
| **$E_{MAX}$** | -1 (n.a.) | -1 (39%) |
| **$S_0$ ($[ng/ml]^{-1}$)** | 0.0545 (n.a.) | 0.00985 (53%) |
| **$EC_{50}$ (ng/ml)** | 18.4 (calc.) | 101 (calc.) |
| **$K_{DEG}$ ($min^{-1}$)** | 0.0228 (n.a.) | 0.0282 (22%) |
| **RUV** | 2.48 (13%) | 2.45 (13%) |
| **PRL** | | |
| **OFV** | 377.0 | 373.5 |
| **Baseline (pg/ml)** | 284 (25%) | 262 (25%) |
| **$IIV_{Baseline}$** | 0.70 (28%) [4%] | 0.67 (28%) [4%] |
| **$D_{Plac}$ (pg/ml)** | 8.72 (fix) | 8.72 (fix) |
| **$K_{IN,\ Plac}$ ($min^{-1}$)** | 1.65 (fix) | 1.65 (fix) |
| **$K_{DEC,\ Plac}$ ($min^{-1}$)** | 1.55 (fix) | 1.55 (fix) |
| **$E_{MAX}$** | -0.961 (21%) | -0.959 (13%) |
| **$EC_{50}$ (ng/ml)** | 0.0983 (275%) | 0.933 (99%) |
| **$K_{DEG}$ ($min^{-1}$)** | 0.584 (22%) | 0.0652 (22%) |
| **RUV** | 0.79 (18%) [3%] | 0.79 (18%) [3%] |
| **TSH** | | |
| **OFV** | -272.2 | -270.0 |
| **Baseline (pg/ml)** | 305 (5.3%) | 293 (4.7%) |
| **$IIV_{Baseline}$** | 0.047 (30%) [8%] | 0.045 (30%) [9%] |

**Table III. Parameter estimates of the PK/PD models for quinpirole effect on ACTH, GH, PRL and TSH with plasma and brain$_{ECF}$ as target site. In bold the parameters of the selected models. (continued)**

| | Plasma | Brain$_{ECF}$ |
|---|---|---|
| **ACTH** | **Estimate (RSE)** | **Estimate (RSE)** |
| **$K_{DEC, Plac}$ (min$^{-1}$)** | 0.00489 (fix) | 0.00489 (fix) |
| **$E_{MAX}$** | -0.819 (36%) | -0.794 (32%) |
| **$EC_{50}$ (ng/ml)** | 31.2 (30%) | 178 (34%) |
| **$K_{DEG}$ (min$^{-1}$)** | 0.0781 (13%) | 0.126 (20%) |
| **RUV** | 0.17 (19%) [2%] | 0.18 (19%) [2%] |

**ACTH: adenocorticotropic hormone; $D_{Plac}$: the extent of the placebo effect; ECF: extracellular fluid; $EC_{50}$: concentration at half maximal drug effect; $E_{MAX}$: maximal drug effect; GH: growth hormone; $k_{DEC}$: dose-independent hormone decay; $k_{DEG}$: hormone elimination rate; $k_{REL}$: hormone release reate; OFV: objective function value; PRL: prolactin; RSE: relative standard error; RUV: residual unexplained variability; $S_0$: $E_{MAX}$/ $EC_{50}$; TSH: thyroid stimulating hormone**

*3.2.3. Mechanistic evaluation of quinpirole effect on ACTH, GH, PRL, and TSH*

The concentration-effect relations between quinpirole and every single hormone are depicted in Figure 3, assuming brain$_{ECF}$ as target site (table III). Prolactin was most sensitive to quinpirole with a potency of 0.93 ng/ml, while ACTH, GH and TSH responded with a potency of 54 ng/ml, 101 ng/ml and 178 ng/ml, respectively (Table III). The operational model could fit the simulated concentration-effect relationships well (Figure 3, Table IV). Within this model, the signal transduction efficiency values (τ) of GH, PRL and TSH could be related to the pituitary receptor expression on the somatotrophs, lactotrophs and the thyrotrophs, respectively. In contrast, the ACTH concentration-response relationship could not be fitted under the assumption of signal transduction efficiency being dependent on pituitary $D_2$ receptor expression (suppl. Figure 3).

*3.2.4. One-day versus eight-day treatment responses*

The pharmacokinetics of quinpirole were not significantly influenced by eight-day drug treatment. In contrast, the pharmacodynamics showed a significant change for ACTH, PRL and TSH (suppl. Table I). The differences between the responses after short- and long-term treatment are graphically presented in Figure 4. The basal levels of ACTH were increased independent of dose, while the hormone release rate was increased in a dose-dependent manner. This resulted in a lower maximal ACTH response after 8-day treatment with a high dose as compared to a low dose of quinpirole. The basal PRL concentrations after eight days were increased with dose, while the extent of the placebo effect was decreased, independent of dose. The basal levels of TSH have decreased with eight-day treatment regardless the dose, while the sensitivity to quinpirole ($EC_{50}$) was decreased in a dose-dependent manner.

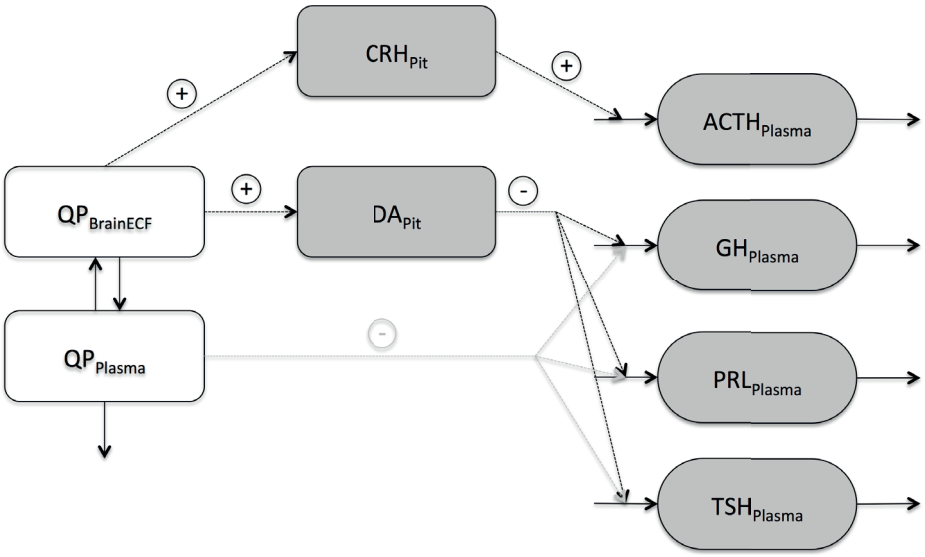



**Figure 2. The interaction between quinpirole and the neuroendocrine system with the pharmacokinetics as white compartments and the pharmacodynamics as grey compartments. Quinpirole stimulates TIDA neurons in the hypothalamus to increase the release of dopamine into the pituitary. Dopamine inhibits the release of GH, PRL and TSH into plasma. ACTH was stimulated by quinpirole, suggesting a pathway other than TIDA neuron stimulation. The main effect site is assumed to be the brain, given the high quinpirole in brain$_{ECF}$ as compared to plasma. QP: quinpirole; DA: dopamine; CRH: corticotropic releasing hormone; ACTH: adenocorticotropic hormone; GH: growth hormone; PRL: prolactin; TSH: thyroid stimulating hormone.**

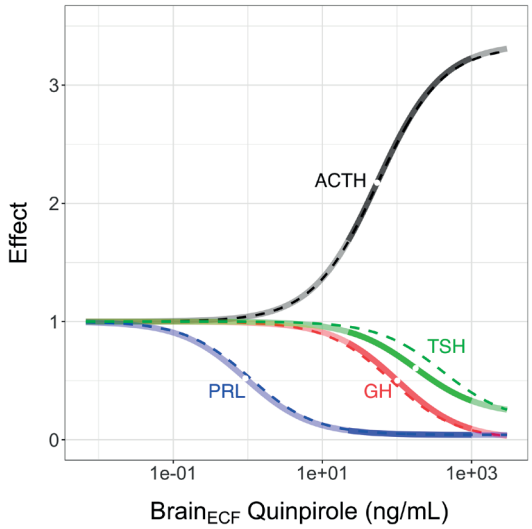



**Figure 3. A) Simulated concentration-effect relations for ACTH (black), TSH (green), GH (red) and PRL (blue) on basis of the parameter estimates in table III. The dark segments represent the quinpirole concentration range measured in brain$_{ECF}$. The dotted lines represent the fit with the operational model, in which the signal transduction efficiency τ for GH, PRL and TSH is dependent on $D_2$ receptor expression following equation 2.**

**Table IV. Relative $D_2$ receptor expression on the troph cells in the rat anterior pituitary, the signal transduction efficiency $\tau$, and the systems maximal effect $E_m$ estimated from the operational model in equation 1. The $k_A$ was estimated 805 ng/ml (see equation 1), while the $\tau_{GH}$, $\tau_{PRL}$, $\tau_{TSH}$ was described by $\tau=0.24*e^{0.106*receptor\ expression}$**

| | $D_2$ receptor expression [28] | $\tau$ | $E_M$ |
|---|---|---|---|
| Corticotrophs (ACTH) | 20% | 13.8 | 2.51 |
| Somatotrophs (GH) | 34% | 8.7 | -1.11 |
| Lactotrophs (PRL) | 76% | 743 | -0.96 |
| Thyrotrophs (TSH) | 13% | 0.94 | -1.76 |

**ACTH: adenocorticotropic hormone; GH: growth hormone; PRL: prolactin; TSH: thyroid stimulating hormone**

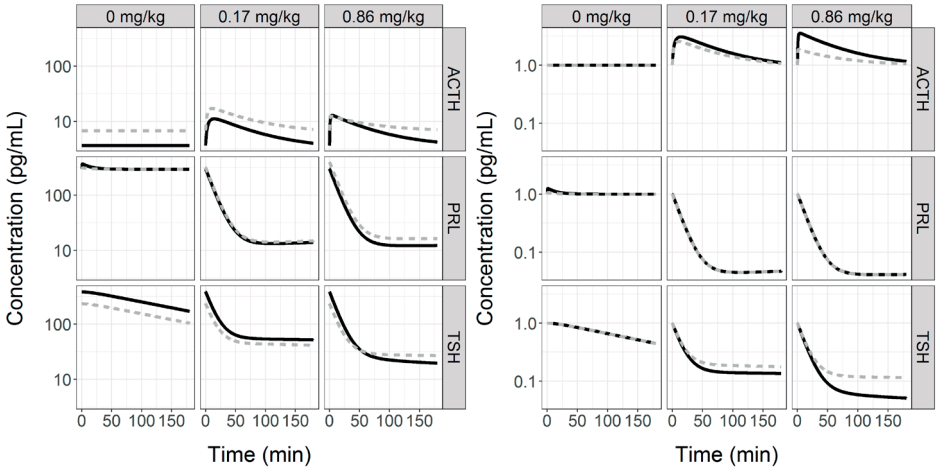



**Figure 4. Simulated hormone actual (A) and baseline normalized (B) concentration-time profiles of ACTH, PRL and TSH after one administration (solid black line) and 8 administrations (dashed grey line).**

## 4. Discussion and conclusion

This study systematically evaluated the effects of quinpirole on the neuroendocrine system following a PK/PD based multi-biomarker approach. Quinpirole showed a high rate of transport over the blood-brain-barrier with an unbound partition coefficient ($k_{p,uu}$) of 5. ACTH, GH, PRL, and TSH responded to quinpirole, each with a unique target site concentration-effect relationship, providing a fingerprint of $D_2$ receptor activation. Additionally, while no changes were found in PK, the pharmacodynamics changed with eight-day administration both dependent and independent of the quinpirole dose. This study underlines the need for integrative multi-biomarker evaluations of drug effects to comprehend the system-wide pharmacological profile.

### *4.1. Can the target site of effect be determined?*

Since the ultimate purpose is to identify peripheral biomarkers of the central drug effect, an important question is whether we can consider $brain_{ECF}$ concentrations as the target site concentrations of the effect of quinpirole. We have shown that, on basis of statistical significance, it was not possible to discriminate between $brain_{ECF}$ or plasma as target site of effect. Given that the $D_2$ receptors on the 'troph' cells are accessible from plasma, and the release of these hormones have been modified by systemic dopamine infusion [29,30], it is suggested that these hormones are released upon peripheral drug action. On the other hand, the release of these hormones is tightly controlled by signals from the hypothalamus that are highly connected to dopamine and other neurotransmitter systems. Considering this, the rate and extent of drug distribution into the brain may determine the dominant target site of effect. For the $D_2$ antagonist remoxipride ($k_{p,uu}$ = 1) [11]) , $brain_{ECF}$ could be considered as target site to release PRL into plasma, while for the $D_2$ antagonist risperidone ($k_{p,uu}$ = 0.45 [31]), plasma could be considered as target site [10,11,32]. Quinpirole is found to be subjected to active influx: although no information on the transporter is available in literature, it is observed that, under steady state conditions, the free drug concentration in $brain_{ECF}$ is as much as five times higher than in plasma ($k_{p,uu}$ = 5, Table I). Therefore, although we could not provide a statistical determination, it is presumed that the main effect of quinpirole on the neuroendocrine system is mediated via the brain rather than via the periphery.

### *4.2. Interpretation of the unique concentration-effect relationships*

Dopamine activity in the brain is reflected in the tuberoinfundibular dopamine pathway that consists of three types of neurons that project from the hypothalamus to the pituitary: 1) the TIDA neurons; 2) the periventricular hypophyseal dopamine neurons, and 3) the tuberohypophyseal dopamine neurons [5]. TIDA neurons release dopamine into the long portal veins of the pituitary to which the 'troph' cells are exposed. While quinpirole has affinity for both the $D_2$ and the $D_3$ receptor [33], the effects on the neuroendocrine system are putatively mediated via the $D_2$ receptor because of the following findings. First, , the enhancing effect of quinpirole on ACTH release was reversed with administration of the $D_2$ receptor antagonist sulpiride [34,35]. Second, the effect of quinelorane, which is similarly specific for the $D_{2/3}$ receptor, on the neuroendocrine TIDA neurons was antagonized by the selective $D_2$ receptor antagonist raclopride [6]. Third, while the selective $D_2$ agonist PNU-95,666 activated the TIDA neurons and inhibited PRL release, this was not the case for the selective $D_3$ agonist PD128907 [7]. In contrast, studies with selective $D_2$ and $D_3$ agonists in ovariectomized estrogen-primed female rats showed a decrease of TIDA neuron activity and an increase of subsequent PRL release [36,37]. However, the estrogen-priming in these studies prevents a direct comparison between these results and our results, since estrogen interferes with TIDA neuron activity as well as the sensitivity of

the pituitary to dopamine [38,39]. Indeed, our study design is more similar to that of the studies observing activation of TIDA neuron activity and suppression of PRL release, i.e. they studied male rats, or diestrous female rats with low estrogen levels [6,7]. Therefore, we assume that the stimulation of TIDA neuron activity by of quinpirole in our study is $D_2$ specific. Dopamine $D_2$ receptors were identified not only on lactotrophs (PRL), but also on corticotrophs (ACTH), somatotrophs (GH), gonadotrophs (FSH, LH), and thyrotrophs (TSH) [17,28]. Also, dopamine agonists inhibited the release of ACTH, GH, PRL and TSH *in vitro*, likely mediated via the $D_2$ receptors [40–42]. Overall, it is thus expected that ACTH, GH, PRL, and TSH concentrations decrease with quinpirole treatment upon the stimulation of TIDA neuron activity that enhances dopamine release into the pituitary to bind to the pituitary dopamine receptors on the 'troph' cells. Interestingly, the secretion of ACTH was increased, indicating a different mechanism of action not via the TIDA neurons. The hypothalamic mediator of ACTH release is corticotrophin-releasing hormone (CRH). CRH is under control of several neurotransmitters, for example, norepinephrine and gamma-aminobutyric acid. Since the effect of quinpirole on ACTH was found to be $D_2$ receptor specific [34], it is likely that CRH or the controlling neurotransmitters are influenced in a $D_2$ specific manner (Figure 2).

According to these mechanisms the assumptions of $D_2$ receptor selectivity for all hormones and the pituitary $D_2$ receptor expression dependent signal transduction for GH, PRL and TSH were made (Figure 2, Table IV). Conceptually, the differences in signal transduction efficiency may also be explained by differences in fractional receptor occupancy needed to elicit a certain level of pituitary hormone release, i.e. the release of some hormones may be more sensitive dopamine receptor activation than that of other hormones. However, our assumptions are confirmed by a good fit of the operational model on the simulated concentration effect relationships as depicted in Figure 3. Physiologically, this suggests a receptor expression dependent sensitivity of the hormones to the increase of pituitary dopamine following the central quinpirole effect. In fact, it indicates that $\tau$ indeed is a system-specific parameter. The opposed direction of the ACTH response, and the deviation of $\tau_{ACTH}$ from the relation between receptor expression and $\tau$ indicates a different mechanism of action of $D_2$ receptor activation on ACTH release (Figure 3, Table IV, suppl. Figure 3). Altogether, the systems response expressed in terms of signal transduction efficiency provides a fingerprint that is specific for $D_2$ receptor stimulation in the brain.

### *4.3. Habituation, tolerance and homeostatic feedback mechanisms*

There are three mechanisms through which the differences between day 1 and day 8 are explained (Figure 4). First of all, the dose-independent changes are likely the consequence of habituation; the animals' response to the daily injection procedure returns to basal levels with longer-term administration. Indeed, the ACTH and TSH basal levels and the

PRL placebo response changed over the period of quinpirole administration (Figure 4). Second of all, pharmacodynamic tolerance may occur as a consequence of long-term drug administration [43]. Tolerance is the mechanism of physiological adaptation to continuous external stimuli, for example, the change in receptor expression. Pharmacodynamic tolerance was identified for the TSH response, as indicated by the dose-dependent change of $EC_{50}$ (Figure 4, suppl. Table I). Assuming a $D_2$ dependent mechanism, this cannot be explained by reduced hypothalamic $D_2$ receptor expression, since this was not observed for the other hormones. Moreover, $D_2$ receptor expression was found not to change with long-term $D_2$ agonist exposure [44]. Possibly, the balance between other mediators of TSH release and dopamine has changed, thereby influencing the transduction efficiency. Third of all, the differences between the experiment days can be explained by homeostatic feedback mechanisms. The release rates of ACTH and PRL were increased in a dose-dependent manner, suggesting a positive and negative feedback, respectively (Figure 4, Suppl. Table I). These hormones are components of highly complex networks that include multiple negative and positive feedback mechanisms that are affected by eight-day administration of quinpirole. The net effect is reflected in the current analysis, showing that these networks have changed to another equilibrium.

### *4.5. Strengths, limitations and future research*

Our integrated PK/PD approach included multiple hormones and neuropeptides that provide comprehensive insight into the interaction between quinpirole and the neuroendocrine system to reveal a fingerprint reflecting $D_2$ receptor activation. Nevertheless, it has a few limitations that will be discussed in this section. First of all, the 3-hour duration of the experiments limited the evaluation of the full pharmacodynamic response. While ACTH levels were back to baseline at the end of the experiment, GH, PRL and TSH levels were still decreased. This may have limited the precise identification of the PK/PD model, although, in general, the parameter estimates showed good precision. Second, a wider dose range may have enabled better identification of the $EC_{50}$ parameter in case of, for example, ACTH that was best described by a slope model. However, since a relatively untargeted approach was applied, it was not possible to anticipate the dose range beforehand. Moreover, the current choice of doses was based on an experimental regimen, reflecting the therapeutic range [45,46], in order to gain pharmacologically relevant insights. Third, the choice of hormones and neuropeptides, although guided by pharmacological knowledge, was based on the available platforms rather than based on the physiology. While this provides a non-biased evaluation of neuroendocrine effects of quinpirole, there is a series of hormones that will be of interest for further research, for example, the downstream signals of the pituitary hormones, such as will be discussed below.

Suggestions for further investigation include the validation of the $D_2$ receptor activation fingerprint with other selective $D_2$ agonists, for example, quinelorane and ropinirole [33]. Furthermore, we suggest efforts towards unraveling the mechanisms underlying the quinpirole-hormone relationships that were identified in the current study. Such investigation should include: i) the measurement of quinpirole and dopamine in the hypothalamus using microdialysis [47]; ii) the measurement of quinpirole and CRH, GHRH, dopamine, and TRH in the pituitary using microdialysis [48]; iii) the measurement of ACTH, GH, PRL, and TSH in plasma; iv) the measurement of corticosterone, IGF-1, triiodithyronine, thyroxine as downstream signals of ACTH, GH and TSH, respectively; v) a study duration of at least 6 hours of experiment. This takes into account the duration of quinpirole exposure (~4 hours) as well as the delay of the hormone responses.

Such data will form the basis of a quantitative systems pharmacology model describing the interaction between quinpirole and the neuroendocrine system in terms of purely drug- and system-specific parameters. This will also allow the separation of central and peripheral quinpirole effect since the drug concentration will be evaluated in both the hypothalamus and the pituitary. Moreover, the upstream hormones that are released from the hypothalamus will exclusively reflect the hypothalamic interaction with the drug. Eventually, such model can be evaluated with different lengths of chronic administration periods to mechanistically understand the tolerance and homeostatic feedback mechanisms.

### *4.6. Conclusion*

The current study has made the case for an integrated and system-wide approach to understand the interaction between dopaminergic pharmacology and the neuroendocrine system. It was shown that, under standard experimental conditions, quinpirole interferes with the hypothalamus-pituitary-adrenal axis (ACTH), the growth hormone system (GH), parts of the reproductive system (PRL), and the thyroid function (TSH). With this multi-biomarker approach, a fingerprint of transduction efficiency values was obtained that is specific for $D_2$ receptor activation. In contrast to PRL alone, as classical biomarker, this multi-biomarker fingerprint provides a specific reflection of $D_2$ receptor activation. Our study also indicated a clear change of the PK/PD relationship with comparing short-term and longer-term administration. This is highly relevant, considering the long-term use of $D_2$ receptor agonists in clinical practice. Further understanding of the underlying tolerance and homeostatic feedback mechanisms will increase the proper application of these drugs in clinical practice.

In conclusion, this study provided further insights into the interaction between dopaminergic pharmacology and the neuroendocrine system. Using a multi-biomarker approach,

a fingerprint of $D_2$ receptor activation was obtained. This forms the conceptual basis for the in vivo evaluation of the on- and off-target effects of drug effects in the CNS. Further efforts towards quantitative systems pharmacology model development will eventually lead to mechanistic translational dopaminergic drug development.

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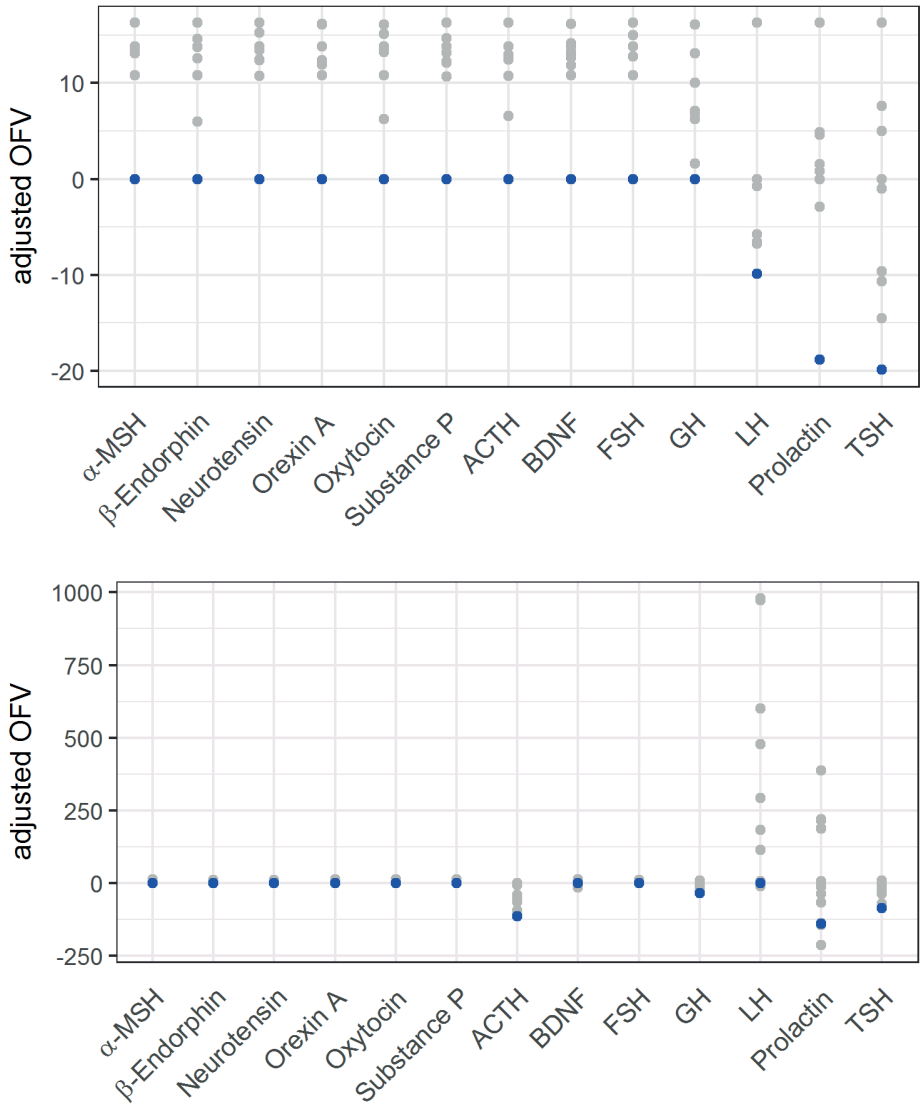
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**Supplementary Table I. Parameter estimates for ACTH, PRL and TSH with and without covariate effect**

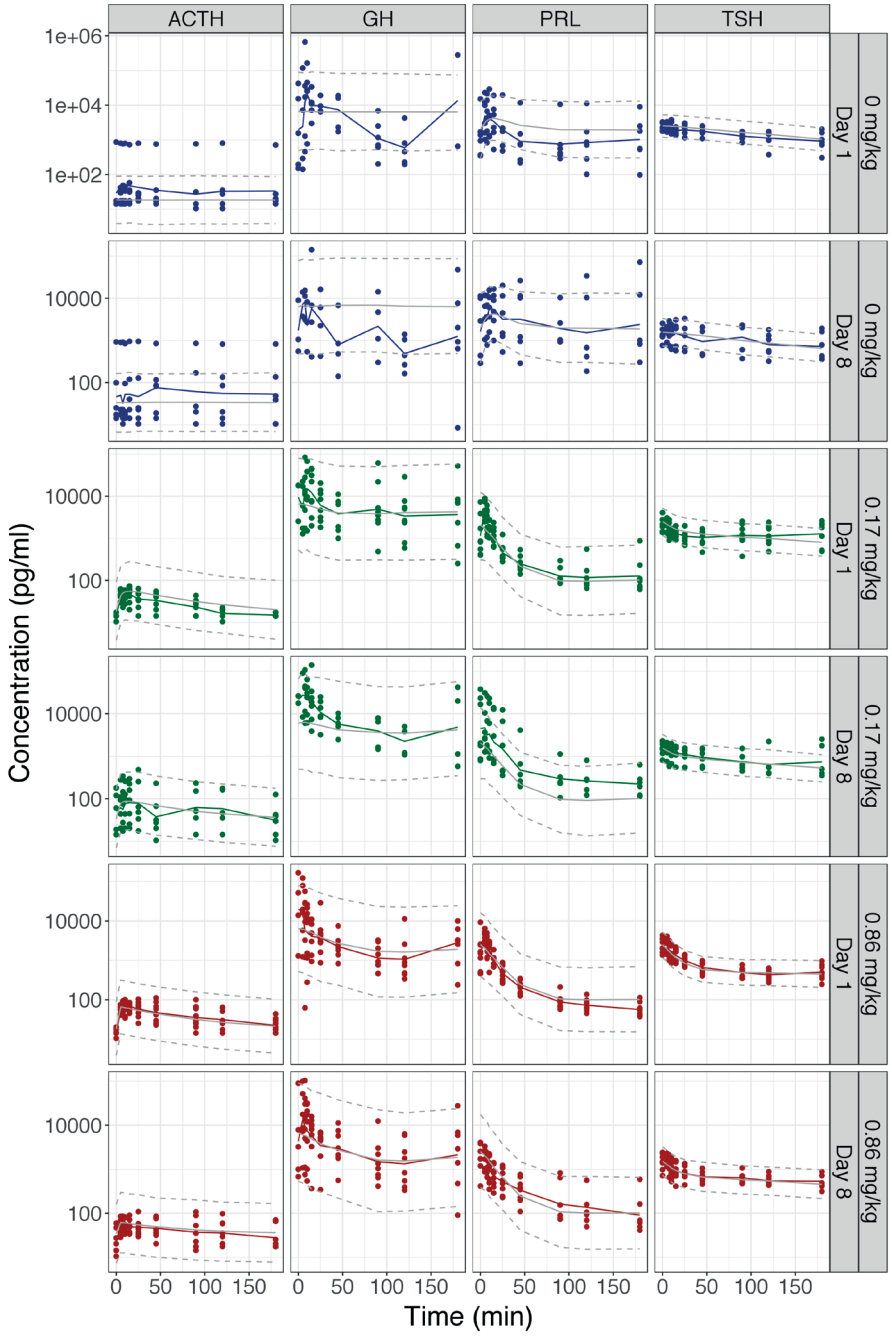
| | | Without covariate effect | With covariate effect |
|---|---|---|---|
| **ACTH** | | | |
| **OFV** | | -31.3 | -117.3 |
| **$Baseline_{DAY1}$ (pg/ml)** | | 3.74 (17%) | 2.80 (19%) |
| **$Baseline_{DAY8}$ (pg/ml)** | | 3.74 (17%) | 2.80 (19%) * 1.85 (37%) |
| | **$IIV_{Baseline}$** | 0.68 (71%) [0%] | 0.71 (67%) [0%] |
| **Slope ($[ng/ml]^{-1}$)** | | 0.87 (43%) | 0.79 (45%) |
| **$K_{DEG}$ ($min^{-1}$)** | | 0.0146 (24%) | 0.0150 (22%) |
| **$K_{REL,DAY1}$ ($min^{-1}$)** | | 0.00760 (29%) | 0.0055 (30%) |
| **$K_{REL,DAY8}$ ($min^{-1}$)** | | 0.00760 (29%) | 0.0055 (30%) * (1 + 2.1 (41%) * dose)[a] |
| | **RUV** | 0.27 (21%) [3%] | 0.22 (16%) [3%] |
| **PRL** | | | |
| **OFV** | | 373.5 | 290.9 |
| **$Baseline_{DAY1}$ (pg/ml)** | | 262 (25%) | 296 (38%) |
| **$Baseline_{DAY8}$ (pg/ml)** | | 262 (25%) | 296 (38%) * (1 + 0.34 * dose)[a] |
| | **$IIV_{Baseline}$** | 0.67 (28%) [4%] | 0.69 (28%) [2%] |
| **$Extent_{Plac,DAY1}$ (pg/ml)** | | 8.72 (fix) | 8.72 (fix) |
| **$Extent_{Plac,DAY8}$ (pg/ml)** | | 8.72 (fix) | 8.72 (fix) * 0.34 (11%) |
| **$K_{IN,\ Plac}$ ($min^{-1}$)** | | 1.65 (fix) | 1.65 (fix) |
| **$K_{DEC,\ Plac}$ ($min^{-1}$)** | | 1.55 (fix) | 1.55 (fix) |
| **$E_{MAX}$** | | -0.959 (13%) | -0.963 (11%) |
| **$EC_{50}$ (ng/ml)** | | 0.933 (99%) | 0.556 (174%) |
| **$K_{DEG}$ ($min^{-1}$)** | | 0.0652 (22%) | 0.068 (5.9%) |
| | **RUV** | 0.79 (18%) [3%] | 0.63 (18%) [3%] |
| **TSH** | | | |
| **OFV** | | -270.0 | -362.1 |
| **$Baseline_{DAY1}$ (pg/ml)** | | 293 (4.7%) | 384 (7.0%) |
| **$Baseline_{DAY8}$ (pg/ml)** | | 293 (4.7%) | 384 (7.0%) * 0.61 (21%) |
| | **$IIV_{Baseline}$** | 0.045 (30%) [9%] | 0.071 (27%) [4%] |
| **$K_{DEC,\ Plac}$ ($min^{-1}$)** | | 0.00489 (fix) | 0.00489 (fix) |
| **$E_{MAX}$** | | -0.794 (32%) | -0.948 (149%) |
| **$EC_{50,DAY1}$ (ng/ml)** | | 178 (34%) | 27.1 (26%) |
| **$EC_{50,DAY8}$ (ng/ml)** | | 178 (34%) | 27.1 (26%) * (1 + 2.84 (48%) * dose)[a] |
| **$K_{DEG}$ ($min^{-1}$)** | | 0.126 (20%) | 0.070 (13.2%) |
| | **RUV** | 0.18 (19%) [2%] | 0.14 (17%) [3%] |

[a] **dose in mg/kg**

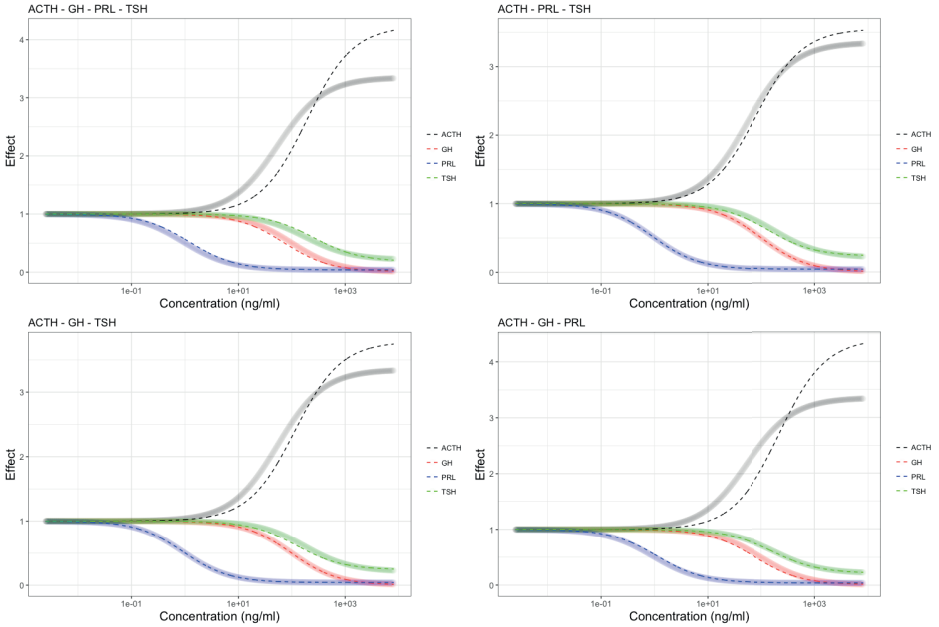
**ACTH: adenocorticotropic hormone; ECF: extracellular fluid; $EC_{50}$: concentration at half maximal drug effect; $E_{MAX}$: maximal drug effect; GH: growth hormone; $k_{DEC}$: dose-independent hormone decay; $k_{DEG}$: hormone elimination rate; $k_{REL}$: hormone release reate; OFV: objective function value; PRL: prolactin; RSE: relative standard error; RUV: residual unexplained variability; $S_0$: $E_{MAX}/EC_{50}$; TSH: thyroid stimulating hormone**




**Supplementary Figure 1. Visualization of the automated model selections for the baseline model (A) and the PK/PD model (B) on basis of adjusted objective function value (suppl. Equations part I, 8). Grey dots represent the adjusted objective function value for each evaluated model, while blue dots represent the selected models.**




**Supplementary Figure 2. Visual predictive check of the quinpirole PK/PD models for ACTH, GH, PRL and TSH at experiment day 1 and 8. The colored dots represent the observed data, with the solid colored lines showing the mean of the observations. The solid grey line shows the mean, and the dashed grey lines the 90% confidence interval of 500 simulations.**




**Supplementary Figure 3. A) Simulated concentration-effect relations for ACTH (black), TSH (green), GH (red) and PRL (blue) on basis of the parameter estimates in table III as compared to the fits by the operational model (equation 1). Each figure represents a different scenario with regard to the hormones included in the relation between tau and $D_2$ receptor expression (equation 2). The signal transduction efficiency τ is assumed dependent on pituitary $D_2$ receptor expression for the hormones indicated above each figure. For the hormone not included, the signal transduction efficiency τ is estimated separately.**

# Supplementary Equations

*Inter-individual and residual variability*

$$\vartheta_i = \vartheta_{pop} * e^{\eta i} \quad \text{(Eq. 1)}$$

$$Log(C_{obs\,,\,i\,,\,j}) = Log(C_{pred}\,,\,i\,,\,j) + \varepsilon_{i\,,\,j} \quad \text{(Eq. 2)}$$

$\boldsymbol{\vartheta_i}$ is the estimated parameter for individual i; $\boldsymbol{\vartheta_{pop}}$ is the estimated parameter for the population; $\boldsymbol{\eta_i}$ follows a normal distribution with mean 0 and variance $\omega^2$; $\boldsymbol{C_{obs,i,j}}$ is the observed concentration data point for individual i at timepoint j; $\boldsymbol{C_{pred,i,j}}$ is the predicted concentration for data point for individual i at timepoint j; $\boldsymbol{\varepsilon_{i,j}}$ follows a normal distribution with mean 0 and variance $\sigma^2$.

*Pharmacokinetic model*

$$\frac{dA_{QP\,,\,PL}}{dt} = -\frac{CL_{PL\,,\,o}}{V_{PL}} * A_{QP\,,\,PL} - \frac{CL_{PL\,,\,ECF}}{V_{PL}} * A_{QP\,,\,PL} + \frac{CL_{ECF\,,\,PL}}{V_{ECF}} * A_{QP\,,\,ECF} \quad \text{(Eq. 3a)}$$

$$\frac{dA_{QP\,,\,ECF}}{dt} = \frac{CL_{PL\,,\,ECF}}{V_{PL}} * A_{QP\,,\,PL} - \frac{CL_{ECF\,,\,PL}}{V_{ECF}} * A_{QP\,,\,ECF} \quad \text{(Eq. 3b)}$$

*With* $A_{QP\,,\,PL\,,\,0} = Dose\,,\quad A_{QP\,,\,ECF\,,\,0} = 0$

*Where* $C_{QP\,,\,PL} = \frac{A_{QP\,,\,PL}}{V_{PL}}\,,\quad C_{QP\,,\,ECF} = \frac{A_{QP\,,\,ECF}}{V_{ECF}}$

$\boldsymbol{A_{QP,PL}}$ is the amount of quinpirole in plasma; $\boldsymbol{A_{QP,ECF}}$ is the amount of quinpirole in brain$_{ECF}$; $\boldsymbol{CL_{PL,o}}$ is the elimination clearance of quinpirole from plasma; $\boldsymbol{V_{PL}}$ is the volume of distribution of quinpirole in plasma; $\boldsymbol{CL_{PL,ECF}}$ is the clearance of quinpirole from plasma to brain$_{ECF}$; $\boldsymbol{CL_{ECF,PL}}$ is the clearance of quinpirole from brain$_{ECF}$ to plasma; $\boldsymbol{V_{ECF}}$ is the volume of distribution of quinpirole in brain$_{ECF}$; $\boldsymbol{C_{QP,PL}}$ is the concentration of quinpirole in plasma; $\boldsymbol{C_{QP,ECF}}$ is the concentration of quinpirole in plasma.

*Baseline models*

*No pattern*

$$C_{HORM\,,\,BSL} = BSL_{HORM} \quad \text{(Eq. 4)}$$

*Circadian rhythm function*

$$C_{HORM\,,\,BSL} = BSL_{HORM} + A * cos\left(\frac{2\pi}{p} * (time - \varphi)\right) \quad \text{(Eq. 5)}$$

*Placebo Bateman function*

$$C_{HORM\,,\,BSL} = BSL_{HORM} + \frac{D * k_e}{k_{DEC} - k_{IN}} * \left(e^{-kINtime} - e^{-kDECtime}\right) \quad \text{(Eq. 6)}$$

*Placebo exponential decay function*

$$C_{HORM\,,\,BSL} = BSL_{HORM} * e^{-\,kDECtime} \quad \text{(Eq. 7)}$$

$\boldsymbol{C_{HORM,BSL}}$ is the hormone concentration given no drug effect; $\boldsymbol{BSL_{HORM}}$ is the hormone concentration at baseline at time = 0; $\boldsymbol{A}$ is the amplitude; $\boldsymbol{p}$ is the period; $\boldsymbol{\varphi}$ is the phase shift; $\boldsymbol{D}$ determines the extent of the placebo response; $\boldsymbol{k_a}$ the rate at which the placebo response occurs; $\boldsymbol{k_e}$ the rate at which the placebo response disappears.

*Objective function value*

$$adjOFV = OFV_{test} - OFV_{ref} + inv.\chi^2\,(\,1 - p.value\,,\,df\,) \quad \text{(Eq. 8)}$$

***adjOFV*** is the adjusted objective function value to compare two models. An adjOFV below 0 indicates a significant improvement of the test model over the reference model. The **inv. $\boldsymbol{\chi^2}$** is a statistical test to compare two models. For example, a significant improvement with a p-value of 0.05 and 1 degree of freedom is equivalent to a decrease of 3.84 points in OFV according to the $\chi^2$-test.

## *Drug effect models*

*Slope model*

$$E = 1 + slope * C_{QP} \quad \text{(Eq. 9)}$$

*$E_{MAX}$ model*

$$E = 1 + \frac{E_{MAX} * C_{QP}}{EC_{50} + C_{QP}} \quad \text{(Eq. 10)}$$

*Alternative $E_{MAX}$ model*

$$E = 1 + \frac{S_0 * E_{MAX} * C_{QP}}{E_{MAX} + S_0 * C_{QP}} \quad \text{(Eq. 11)}$$

*On-off model*

$$E = \begin{cases} 0\,, & \& \; C_{QP} = 0 \\ E_{MAX}\,, & \& \; C_{QP} > 0 \end{cases} \quad \text{(Eq. 12)}$$

$\boldsymbol{E}$ is the magnitude of drug effect; ***Slope*** is the parameter that determines the strength of the drug effect; $\boldsymbol{C_{QP}}$ is the drug concentration at the target site, either plasma or $brain_{ECF}$; $\boldsymbol{E_{MAX}}$ is the maximal effect; $\boldsymbol{EC_{50}}$ is the drug concentration at half maximal effect; $\boldsymbol{S_0}$ is defined as $\boldsymbol{E_{MAX}/EC_{50}}$.

## *Link models*

*No effect*

$$C_{HORM\,,\,PL} = C_{HORM\,,\,BSL} \quad \text{(Eq. 13)}$$

*Direct response model*

$$C_{HORM,PL} = C_{HORM,BSL} * E \quad \text{(Eq. 14)}$$

*Turnover model (effect on hormone release)*

$$\frac{dC_{HORM,PL}}{dt} = k_{DEG} * C_{HORM,BSL} * E - k_{DEG} * C_{HORM,PL} \quad \text{(Eq. 15)}$$

*Pool model (effect on hormone release)*

$$\frac{dC_{HORM,Pool}}{dt} = k_{DEG} * C_{HORM,BSL} - k_{REL} * E * C_{HORM,Pool} \quad \text{(Eq. 16a)}$$

$$\frac{dC_{HORM,PL}}{dt} = k_{REL} * E * C_{HORM,Pool} - k_{DEG} * C_{HORM,PL} \quad \text{(Eq. 16b)}$$

$\boldsymbol{C_{HORM,PL}}$ is the hormone concentration in plasma; $\boldsymbol{k_{DEG}}$ is the hormone turnover rate; $\boldsymbol{k_{DEG} * C_{HORM,BSL}}$ is equivalent to $\boldsymbol{k_{IN}}$, the hormone production rate within the pool compartment; $\boldsymbol{C_{HORM,POOL}}$ is the hormone concentration in the pool; $\boldsymbol{k_{REL}}$ is the hormone release rate from the pool into plasma.

*Covariate models*

$$\vartheta_{DAY} = \vartheta_{pop} * ( 1 + COV ) \quad \text{(Eq. 17)}$$

$$\vartheta_{DAY} = \vartheta_{pop} * ( 1 + COV_{SLP} * Dose ) \quad \text{(Eq. 18)}$$

$$\vartheta_{DAY} = \vartheta_{pop} * ( 1 + \frac{COV_{MAX} * Dose}{COV_{50} + Dose} ) \quad \text{(Eq. 19)}$$

$\boldsymbol{\vartheta_{DAY}}$ is the estimated parameter for the specific day; $\boldsymbol{\vartheta_{pop}}$ the population parameter; $\boldsymbol{COV}$ the dose independent covariate parameter; $\boldsymbol{COV_{SLP}}$ the dose dependent covariate parameter following a linear relation; $\boldsymbol{COV_{MAX}}$ and $\boldsymbol{COV_{50}}$ the dose dependent covariate parameters following a non-linear relation. The covariate effect is set to zero for day 1.

## Supplementary Methods

### *Additional PK experiment as external validation of the quinpirole PK model*

The experimental procedures applied in this additional experiment have been previously described (Wong et al. Eur J Pharm Sci. 2018:111;514-525), which involved similar procedures as those in the current manuscript with some modifications. In brief, 15 male Wistar rats were used, and 7 of which received microdialysis surgery in addition to the femoral artery and vein cannulations. Two microdialysis guides (CMA 12 Guide Cannula, Aurora Borealis Control BV, Schoonebeek, the Netherlands) were embedded in the brain striatum (AP − 1.0; L 3.2; V − 3.5 mm relative to bregma) and cerebellum (AP − 2.51; L 2.04; V

− 3.34 mm, at an angle of 25° from the dorsoventral axis (toward anterior) and 11° lateral from the anteroposterior axis relative to lambda). The rats were given 7 days to recover from surgery. One day before the experiment, the microdialysis guides were substituted by the microdialysis probes (CMA 12 Elite Polyarylethersulfone, 4 mm membrane, cut-off 20 kDA, Aurora Borealis Control BV, Schoonebeek, the Netherlands).

On the day of experiment, rats received an IV infusion of quinpirole 1 mg/kg at the start of experiment (t = 0 min). The duration of the infusion was either 10 min (for 12 rats) or 0.5 min (for 3 rats). Plasma and brain microdialysate were collected and analyzed in the same manner as in the current manuscript. The plasma and striatum ECF data were used to validate the PK model.

# CHAPTER 6

## MULTIVARIATE PHARMACOKINETIC/ PHARMACODYNAMIC (PKPD) ANALYSIS WITH METABOLOMICS SHOWS MULTIPLE EFFECTS OF REMOXIPRIDE IN RATS

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## Abstract

The study of central nervous system (CNS) pharmacology is limited by a lack of drug effect biomarkers. Pharmacometabolomics is a promising new tool to identify multiple molecular responses upon drug treatment. However, the pharmacodynamics are typically not evaluated in metabolomics studies, although being important properties of biomarkers.

In this study we integrated pharmacometabolomics with pharmacokinetic/pharmacodynamic (PKPD) modeling to identify and quantify the multiple endogenous metabolite dose-response relations for the dopamine D2 antagonist remoxipride.

Remoxipride (vehicle, 0.7 or 3.5 mg/kg) was administered to rats. Endogenous metabolites were analyzed in plasma using a biogenic amine platform and PKPD models were derived for each single metabolite. These models were clustered on basis of proximity between their PKPD parameter estimates, and PKPD models were subsequently fitted for the individual clusters. Finally, the metabolites were evaluated for being significantly affected by remoxipride.

In total 44 metabolites were detected in plasma, many of them showing a dose dependent decrease from baseline. We identified 6 different clusters with different time and dose dependent responses and 18 metabolites were revealed as potential biomarker. The glycine, serine and threonine pathway was associated with remoxipride pharmacology, as well as the brain uptake of the dopamine and serotonin precursors.

This is the first time that pharmacometabolomics and PKPD modeling were integrated. The resulting PKPD cluster model described diverse pharmacometabolomics responses and provided a further understanding of remoxipride pharmacodynamics. Future research should focus on the simultaneous pharmacometabolomics analysis in brain and plasma to increase the interpretability of these responses.


**Keywords:** Systems pharmacology; PK/PD modeling; pharmacometabolomics; biomarkers; CNS drugs; D2R antagonists

### *Abbreviations*

AAAD: Aromatic Amino Acid Decarboxylase; AQC: 6-AminoQuinolyl-N-hydroxysuccinimidyl Carbamate; ASCA: Anova Simultaneous Component Analysis; BCAA: Branched Chain Amino Acids; brainECF: Brain extracellular fluid; CNS: Central Nervous System; D2R: dopamine D2 Receptor; DOPAC: 3,4-dihydroxyphenylacetic acid ; FWER: Family Wise Error Rate; HVA: Homovanillic acid; L-DOPA: L-3,4-dihydroxyphenylalanine; MeOH: Methanol; MS: Mass Spectometry; NMDA: N-Methyl-D-Aspartate; OFV: Objective Function Value; PCA: Principal

Component Analysis; PD: Pharmacodynamics; PK: Pharmacokinetics; PLS-DA: Partial Least Squares Discriminant Analysis; QC: Quality Control; RSD: Relative Standard Deviation; RSE: Relative Standard Error; RV: Residual Variability; SRM: Single Reaction Monitoring; TCEP: Tris(2-CarboxyEthyl)Phosphine; UPLC: Ultra high Performance Liquid Chromatography; VIP: Variable Importance Projection; WCSS: Weighted Cumulative Sum of Squares

## Introduction

Central nervous system (CNS) drug development is difficult and attrition rates are high [1]. While important progress has been made in the insight into human brain pharmacokinetics (PK) in response to plasma PK, insights into the relation to the time dependent CNS drug effects are limited [2–4]. It is therefore essential to utilize biomarkers that provide proof of pharmacology and dosing guidance for early clinical drug development [2,4–9]. Preferably, these biomarkers are measured in the blood, since blood can be easily obtained from humans.

Biomarker discovery is increasingly driven by (pharmaco)metabolomics [10–15]. It measures the end-products of cellular biochemical reactions under a drug-perturbed, disease or control condition, and is as such a phenotypic measure, sometimes referred to as the "metabotype" [16]. As an example, a pharmacometabolomics approach has been successfully applied in CNS drug research for identification of serum biomarkers of antipsychotic drug efficacy [17] or toxicity [18].

An important limitation so far has been that pharmacometabolomic studies are often performed at single time points while many biological processes change with time. A single time point evaluation thus limits the ability to accurately quantify the extent and duration of drug effects. Whereas for single time point studies multivariate data analysis mostly is performed using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) [19], more advanced methods are needed, and have been developed, to evaluate time-dependent effects in metabolomics data. For example, an extension of PCA was developed called ANOVA Simultaneous Component Analysis (ASCA), allowing for multivariate evaluation in multiple dimensions (e.g. dose, time and response) [20]. Still, a remaining limitation with this method is that the variables are treated as categorical data, while factors as dose and time typically are continuous variables. Furthermore, longitudinal clustering approaches are promising for the evaluation of multivariate longitudinal data, although its application until now has been mainly on gene expression data [21–24].

Not only the time course of the effect biomarker is important for the understanding of drug effects, but also the causal relation between drug dose and biomarker response

(Danhof et al. 2005). This relation is governed by processes of drug distribution to the target site [2,25], receptor binding [26] and activation [27], signal transduction [23,27] and homeostatic feedback [28]. These processes are typically non-linear, which increases the complexity from a data analysis perspective. Quantitative insights in drug effects are obtained by a combination of studies that measure biomarkers at different causal levels in a time-dependent manner and pharmacokinetic/pharmacodynamic (PKPD) modeling [2,5,29–31].

In this study we integrated pharmacometabolomics with PKPD modeling to identify and quantify multiple endogenous metabolite dose-response relations for the paradigm compound remoxipride. Rats received remoxipride in different dose levels and we obtained serial plasma samples for analysis of multiple endogenous metabolites. PKPD models were subsequently developed to fit the longitudinal dose-response data of each single metabolite. Biomarker clusters were identified on basis of the PKPD parameters to derive a PKPD model that fitted the cluster responses. Potential biomarkers and putative pharmacological pathways of remoxipride effect were identified using this approach; we obtained comprehensive insight in its differential effects on the endogenous metabolism.

## Methods

### *Animal studies*

Animal studies were performed in agreement with the Dutch Law of Animal Experimentation and approved by the Animal Ethics Committee in Leiden, the Netherlands (study protocol DEC13186). Male Wistar rats (n= 28, 278 +/- 15 g, Charles River, The Netherlands) were housed in groups for 6-9 days until surgery (Animal Facilities Gorlaeus Laboratories, Leiden, The Netherlands). Animals were held under standard environmental conditions while artificial daylight was provided from 7:30AM to 7:30PM. They had ad libitum access to food (Laboratory chow, Hope Farms, Woerden, The Netherlands) and acidified (to prevent infection) water.

### *Surgery and experiment*

Surgery was done according to previously reported procedures [25]. In brief, animals received 2% isoflurane anesthesia while undergoing surgery. Cannulas were placed in the femoral artery for serial blood sampling and the femoral vein for drug administration. Microdialysis guides (CMA 12 Elite PAES, Schoonebeek, The Netherlands) were placed in caudate putamen (AP −1.0; L 3.0; V −3.4, relative to bregma) and replaced by microdialysis probes (CMA 12 Elite PAES, 4 mm polycarbonate membrane, cut-off 20 kDA, Schoonebeek, The Netherlands) before the experiment. For 7 days, animals were individually held in Makrolon type 3 cages to recover from surgery. The start of the experiments was between

8:00AM and 8:30AM and rats were randomly assigned receiving 0 mg/kg (n=5), 0.7 mg/kg (n=8), or 3.5 mg/kg (n=9) remoxipride by i.v. bolus (2 min infusion) at the start of experiment (t=0 min). Microdialysis was performed using buffered perfusion fluid and a flow rate of 1µl/min. Blood samples of 200 µl were collected in heparin-coated eppendorf tubes at -15, 2, 10, 22, 30, 40, 60, 100, 180 and 240 min, after which animals received 200 ul saline to compensate for the lost blood volume. Plasma was separated by centrifuging (1000 g, 10 min) and was stored at 4°C during the experiment and at -20°C after the experiment until analysis.

### *Metabolomics analysis*

Metabolomics analysis in the plasma samples was performed using an amine platform, according to a previously described method [32]. The amine platform covers amino acids and biogenic amines employing an Accq-tag derivatization strategy adapted from the protocol supplied by Waters (Etten-Leur, The Netherlands). 5 µL plasma was spiked with an internal standard solution and reduced with TCEP (tris(2-carboxyethyl)phosphine) followed by deproteination by addition of MeOH. After centrifuging (9400xg, 10 min, 10°C), the supernatant was transferred to a deactivated autosampler vial (Waters) and dried under N2. The residue was reconstituted in borate buffer (pH 8.5) with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatisation reagent (Waters). Microdialysate samples underwent the same procedure, but without deproteination. After reaction, the vials were transferred to an autosampler tray and cooled to 10°C until the injection (1.0 µL) of the reaction mixture into the UPLC-MS/MS system. This consisted of an ACQUITY UPLC system with autosampler (Waters) coupled online with a Xevo Tandem Quadrupole mass spectrometer (Waters), and operated using Masslynx data acquisition software (version 4.1; Waters). The samples were analyzed by UPLC-MS/MS using an Accq-Tag Ultra column (Waters). The Xevo TQ was used in the positive-ion electrospray mode and all metabolites were monitored in Selective Reaction Monitoring (SRM) using nominal mass resolution. Acquired data were evaluated using Quanlynx software (Waters), by integration of assigned SRM peaks and normalization using proper internal standards. For analysis of amino acids their 13C15N-labeled analogs were used. For other amines, the closest-eluting internal standard was employed. Blank samples were used to correct for background, and in-house developed algorithms were applied using the pooled QC samples to compensate for drift in the sensitivity of the mass spectrometer with and over different batches [33]. Quality assurance of metabolite measurements was performed only reporting compounds with a QC relative standard deviation ($RSD_{QC}$) under 15%.

### *Data exploration, PKPD modeling and clustering*

Outliers were detected for each metabolite using Tukeys' Test (equation 1) [34], by comparing concentrations to the range:

$[Q_1 - 3 * (Q_3 - Q_1) , Q_3 + 3 * (Q_3 - Q_1)]$ , (Equation 1)

in which $Q_1$ and $Q_3$ are the lower and upper quartiles per metabolite, respectively.

1.3% of the data points were designated as outlier, and replaced by the median of the metabolite concentration of the dose group in which the data point existed. Most of the outliers came from one specific sample in the vehicle group (see figure S1). Sequential PKPD modeling approach was applied on the non-scaled metabolite concentrations, using NONMEM® version 7.3.0 with subroutine ADVAN13. Posthoc parameter estimates of a previously developed PK model were used as input for the PKPD model [35]. This model provided remoxipride concentrations both in plasma and brain extracellular fluid ($brain_{ECF}$).

A proportional error model was used in which the residual variability (RV, $\varepsilon_{ijk}$) follows a normal distribution with zero mean and an estimated variance (equation 2).

$$R_{obs\,,\,ij} = R_{pred\,,\,ij} * (1 + \varepsilon_{ijk}) \quad \text{(Equation 2)}$$

Criteria for model evaluation were the drop in objective function value (OFV) calculated as -2loglikelihood ratio (> 3.84, $p < 0.05$, $df = 1$), the precision of the parameter estimates (relative standard error (RSE) < 30%) and the visual evaluation of the goodness-of-fit. 44 models were developed linking the remoxipride $brain_{ECF}$ concentrations to the metabolite responses. The drug effect was described by an $E_{MAX}$ equation (equation 3), which was coupled to the metabolite production rate ($k_{IN}$) in a turnover model (equation 4) as follows:

$$Drug\ effect(DE) = \frac{E_{MAX} * [C_{REM}]}{EC_{50} + [C_{REM}]}, \quad \text{(Equation 3)}$$

$$\frac{\partial R}{\partial t} = k_{IN} * (1 - DE) - k_{OUT} * R , \quad \text{(Equation 4)}$$

in which $C_{REM}$ is the remoxipride concentration in $brain_{ECF}$, $E_{MAX}$ is the maximal inhibition, $EC_{50}$ is the concentration at half maximal effect, $k_{IN}$ is the metabolite production rate (which is derived from the metabolite baseline * $k_{OUT}$), $k_{OUT}$ is the metabolite elimination rate, and $R$ is the metabolite concentration in plasma.

We identified clusters in the scaled parameters $E_{MAX}$, $EC_{50}$ and $k_{OUT}$ using the k-means method, with scaling performed according to equation 5. K-means clustering aims to minimize the within-cluster sum of squares (WCSS), which may reach a local minimum, depending on the chosen initial cluster means. Therefore, the algorithm was repeated 5000 times, and the model with the lowest WCSS was selected.

$$\tilde{P}_{ij} = \frac{log\,(P_{ij}) - \overline{log\,(P_i)}}{sd_{log\,(Pi)}}, \qquad \text{(Equation 5)}$$

in which $P_{ij}$ is parameter value *i* for metabolite j.

A range of 4 – 10 clusters of metabolites was obtained on basis of an elbow plot (figure S2). For each candidate clustering, a PKPD model was developed estimating a single $E_{MAX}$, $EC_{50}$ and $k_{OUT}$ per cluster and a separate baseline and RV per metabolite. The best model was selected on basis of ΔOFV < 16.27 ($p < 0.001$, $df = 3$), as compared with the next candidate cluster model.

Parameter estimates for $E_{MAX}$, $EC_{50}$ and $k_{OUT}$ appeared similar between some clusters and allowed model simplification by sharing parameters among different clusters. The initial sharing was based on similarity of parameter estimates. The reduction was performed in a stepwise approach. The first step consisted of reducing three separate models each sharing only $E_{MAX}$, $EC_{50}$ or $k_{OUT}$. In a second step, dual combinations of these models were evaluated. The third and last step consisted of testing a shared value for all three parameters. In all three steps, the reduced models were rejected if they were significantly different from the non-reduced model ($p < 0.05$).

Finally, the best model was compared to a baseline model that did not include a drug effect component (i.e. $DE = 0$ in equation 5). A ΔOFV significance threshold was calculated to be 16.00 for each single metabolite, taking into account the family wise error rate (FWER) using Bonferroni correction. The results were compared to a partial least squares discriminant analysis (PLS-DA) on the data pooled per dose group, using the R-package mixOmics [36] after log-transformation and autoscaling of the data (excluding t=0). A Variable Importance in Projection (VIP) on the first principal component was calculated for each metabolite. Metabolites with a VIP score > 1 were reported as contributing significantly to a dose response relation for remoxipride and compared to those selected from the PKPD clustering approach. The methods were compared by a weighted Cohen's kappa-analysis.

# Results

## *PKPD models of remoxipride effect on individual metabolites*

The biogenic amine analysis detected 44 metabolites in plasma with good reproducibility ($RSD_{QC} \leq 15\%$). Unfortunately, due to metabolite degradation and detection limits, the biogenic amines could not reliably be measured in microdialysate samples. The plasma metabolites showed a general dose dependent decrease from baseline (t = 0h) in the treatment groups (figure 1) with different longitudinal patterns, some of them showing a slow and others a more rapid return to baseline (figure 1). The placebo group showed

an increase from baseline for many metabolites, which we initially attempted to describe by the mathematical bateman function that previously has been used to describe such placebo response [37]. This, however, did not result in an improved description of the data as compared to a model without a placebo effect included (Bonferroni corrected $p > 0.05$).

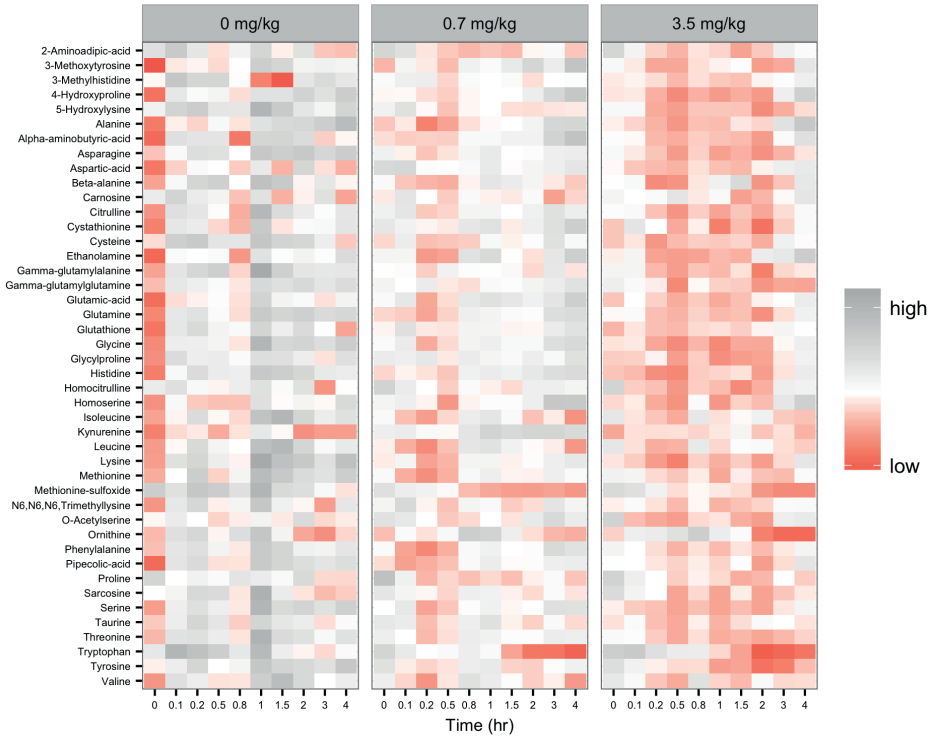



**Figure 1 Heatmap showing the longitudinal response for each metabolite in the different remoxipride dose groups. Data are log-transformed and autoscaled, and mean responses are shown.**

Since the metabolite responses were decreasing after treatment, the effect of remoxipride was mathematically described as an inhibition of the metabolite production rates (equations 4 & 5) for each individual metabolite, in a turnover model. The $E_{MAX}$ for the metabolite kynurenine approached zero, indicating that remoxipride had no effect on this metabolite (figure 2). Furthermore, some metabolites showed a similar parameter pattern (e.g. glycine versus lysine), whereas others exhibited different characteristics (e.g. threonine versus tryptophan) (figure 2, indicated in pink). Particularly, the $EC_{50}$ and $k_{out}$ estimates were different for some metabolites (figure 2, figure S3)).

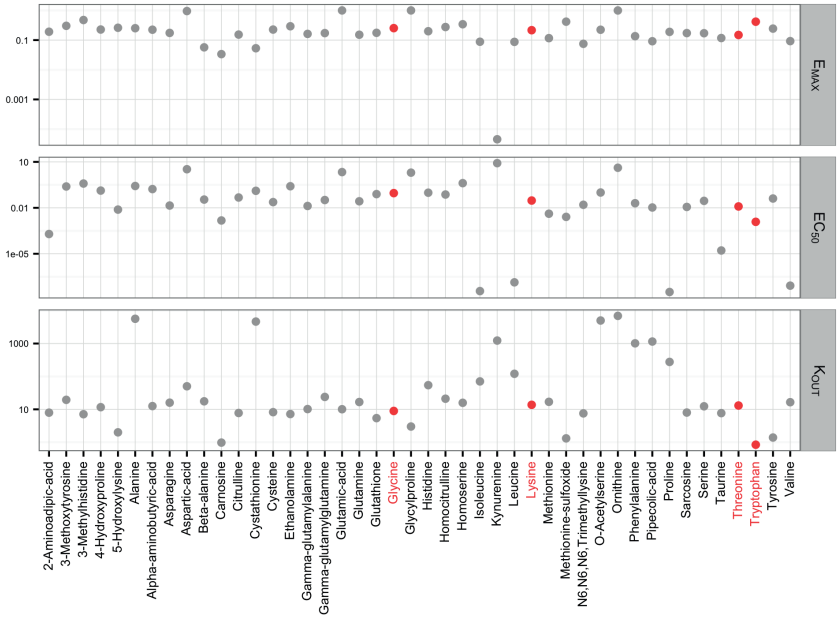



**Figure 2. Parameter estimates of the 44 PKPD models describing the individual metabolite responses. The red colors are indicating examples of metabolite with similar (glycine and lysine) or distinct (threonine and tryptophan) parameter estimates.**

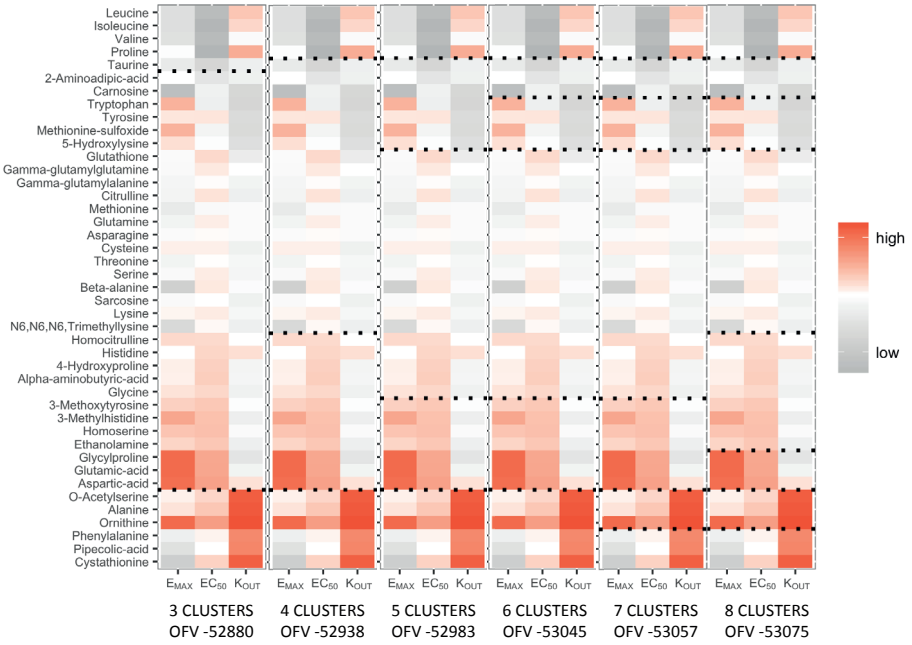



**Figure 3. K-means clustering results for 3 – 8 candidate clusters. Black dotted lines indicate the cluster separation. OFV values are shown for each candidate PKPD cluster model.**

*PKPD models of remoxipride effect on clusters of endogenous metabolites*

Metabolite clusters were identified on basis of the parameter estimates for $E_{MAX}$, $EC_{50}$ and $K_{OUT}$. Using the multi-model k-means clustering approach and subsequent cluster-based turnover model development (see methods section), the model with 6 clusters was found to best fit the data (figure 3). This model was significantly different from the 5-cluster model (*ΔOFV > 16.27*, *p < 0.001, df = 3*), but not from the 7-cluster model (*ΔOFV < 16.27*, *p > 0.001, df = 3*).

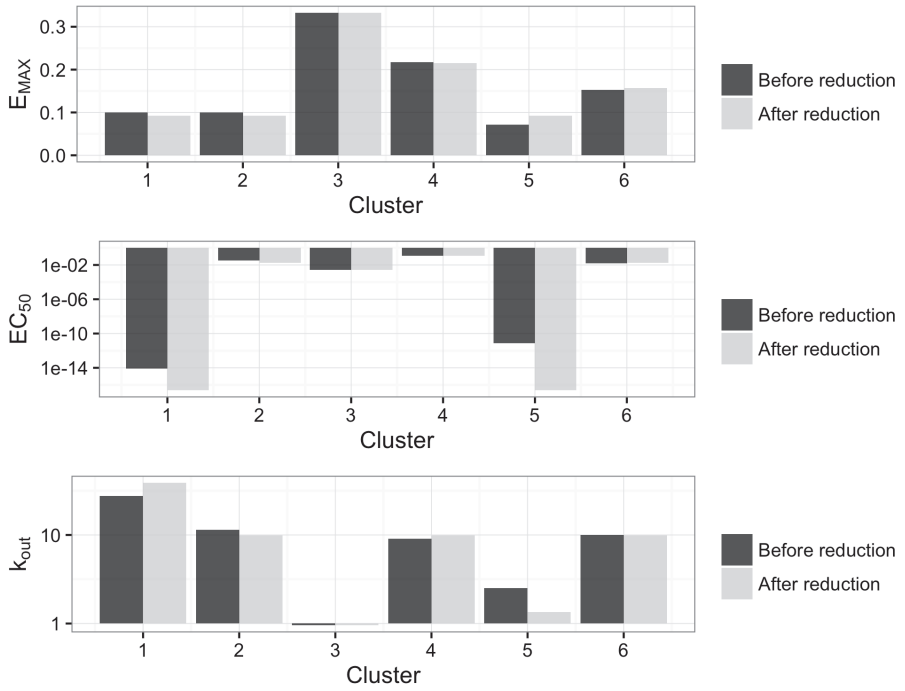



**Figure 4. The effect of parameter reduction on the parameter estimates of $E_{MAX}$, $EC_{50}$ and $k_{OUT}$ were evaluated for each cluster, comparing the estimates before (black bars) and after (grey bars) reduction.**

Parameter sharing led to a further simplification of the model with 6 less parameters. The more complex model was not significantly different from the simplified model (*ΔOFV < 12.59, p > 0.05, df = 6*) and the parameter estimates were highly similar (figure 4). We identified four different $E_{MAX}$, four different $EC_{50}$, and four different $k_{OUT}$ parameters (figure 4, Table 1). The parameter estimates in cluster 1 and 5 were imprecise (Table 1) and did not show a significant effect when compared with the model not including drug effect (figure 6). Moreover, the $EC_{50}$ approached 0 for these clusters and was therefore fixed at a value close to 0. It is concluded that a remoxipride effect could not be reliably identified. Although the kynurenine response (cluster 0) showed a possible trend in the placebo

**Table 1. Parameter estimates for the PKPD cluster model describing the multiple metabolite responses in 6 different response clusters.**

| | Parameter | Estimate (RSE%) |
|---|---|---|
| *Cluster 1* | $E_{MAX}$ | 0.093 (7) |
| (4 metabolites) | $EC_{50}$ (uM) | ~ 0 (fix) |
| | $k_{out}$ ($h^{-1}$) | 39 (182) |
| *Cluster 2* | $E_{MAX}$ | 0.093 (7) |
| (6 metabolites) | $EC_{50}$ (uM) | 0.019 (19) |
| | $k_{out}$ ($h^{-1}$) | 9.9 (15) |
| *Cluster 3* | $E_{MAX}$ | 0.33 (19) |
| (4 metabolites) | $EC_{50}$ (uM) | 0.0027 (72) |
| | $k_{out}$ ($h^{-1}$) | 0.96 (23) |
| *Cluster 4* | $E_{MAX}$ | 0.22 (23) |
| (7 metabolites) | $EC_{50}$ (uM) | 0.12 (43) |
| | $k_{out}$ ($h^{-1}$) | 9.9 (15) |
| *Cluster 5* | $E_{MAX}$ | 0.093 (7) |
| (3 metabolites) | $EC_{50}$ (uM) | ~ 0 (fix) |
| | $k_{out}$ ($h^{-1}$) | 1.3 (51) |
| *Cluster 6* | $E_{MAX}$ | 0.16 (6) |
| (19 metabolites) | $EC_{50}$ (uM) | 0.019 (19) |
| | $k_{out}$ ($h^{-1}$) | 9.9 (15) |

**Note: cluster 0 is not included since it represented the metabolite (kynurenine) that was not affected by remoxipride.**

group (0 mg/kg), this was not consistent in the other dose groups (figure 5). Parameter estimates in cluster 2, 3, 4 and 6 could be precisely determined (RSE < 30%), except for the $EC_{50}$ in cluster 3 (RSE > 50%). The predicted centroids (i.e. the time and dose dependent average cluster response) showed good agreement with the observed centroids (figure 5). Ornithine (cluster 2) was excluded from this graph, since the effect on ornithine was in the positive direction. The single metabolite responses for ornithine and the other metabolites were reasonably to well predicted (figure S5).

## *Identification of potential plasma biomarkers for remoxipride effect*

As indicated in figure 6 and table SI, the model including the drug effect significantly outperformed the model without drug effect for 18 metabolites ($\Delta OFV > 16.00$, *adjusted* $p < 0.05$, $df = 3$).

3 metabolites (cluster 3) showed a high impact of remoxipride ($E_{MAX}/EC_{50}$ = 122), 13 metabolites (cluster 2 and 6) a medium impact ($E_{MAX}/EC_{50}$ = 5 – 8), whereas 2 metabolites

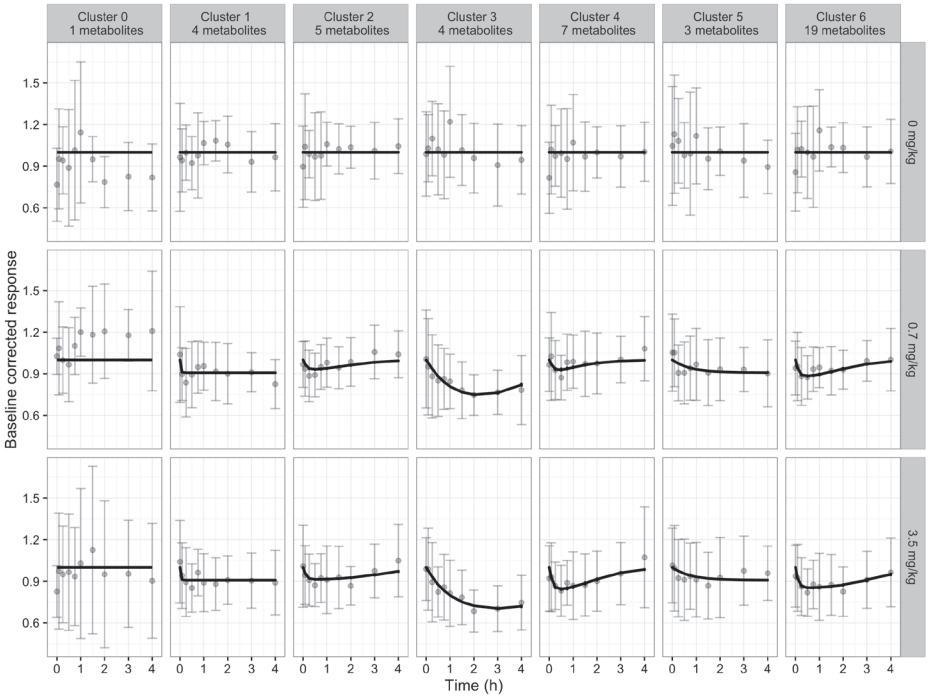



**Figure 5. Goodness of fit on basis of the cluster centroids (or means) for each cluster and remoxipride dose. Predicted response is indicated by the black solid line, whereas the observed data is indicated by the grey dots (mean) and error bars (+/- standard deviation).**

(cluster 4) showed a low impact ($E_{MAX}/EC_{50}$ = 2). The turnover rate was high (9.9 /h) for cluster 2, 4 and 6, and low (0.96 /h) for cluster 3.

The PLS-DA revealed 18 metabolites with a VIP score > 1 with 13 metabolites overlapping and a Cohen's kappa of 0.38, suggesting a fair agreement between the two methods (Table 2).

## Discussion

This study showed how the integration of pharmacometabolomics and PKPD modeling led to identification and significant description of 4 clusters of pharmacodynamic patterns. The model predicts the diverse longitudinal effects of remoxipride on endogenous metabolites in plasma using a clustering approach. We propose 18 metabolites as potential biomarkers of remoxipride pharmacology.

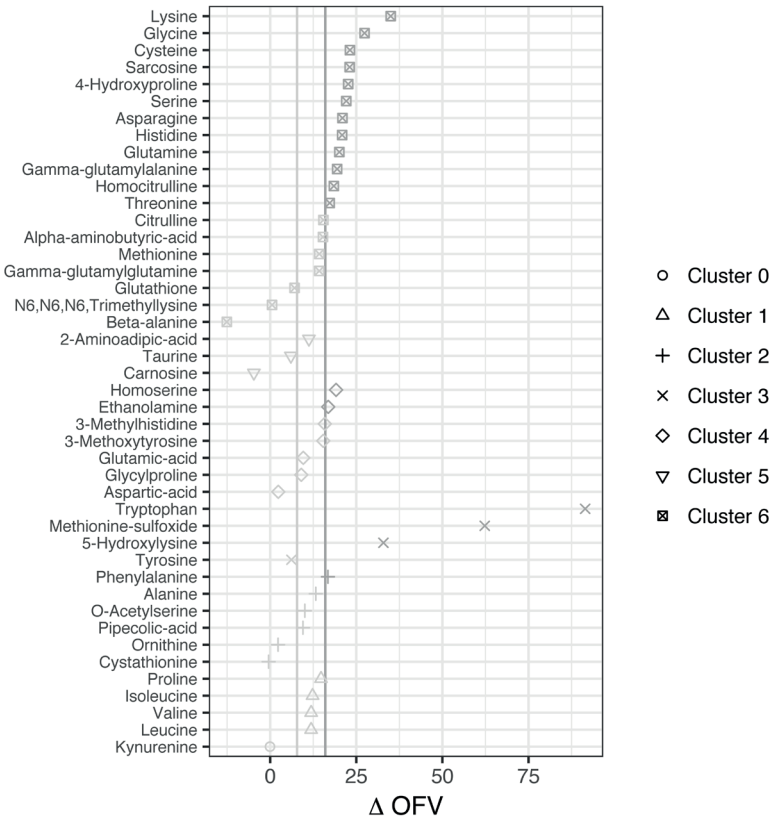



**Figure 6. ΔOFV for each metabolite between the baseline model with no drug effect component and the best model with drug effect component. The light grey line indicates the significance threshold with no bonferroni correction (α = 0.05), whereas the dark grey line indicates the significance threshold with bonferroni correction (α = 0.05/44). Clusters are indicated by the different symbols.**

Earlier clustering approaches have been dedicated to cluster time dependent multivariate responses. As a next step, the current method deals with the complex non-linear (concentration-effect relations are typically sigmoidal), time dependent (biological processes differ in their rates of change upon pharmacological treatment) and multivariate dose response data by step-wise integration of PKPD modeling and clustering. The model is therefore suited for predicting the multivariate dose-response relation for remoxipride with time and dose. Moreover, the model provides pharmacological meaning with the parameters that determine the concentration-effect relation ($E_{MAX}$, $EC_{50}$) and the longitudinal behavior of the response ($k_{OUT}$).

Many metabolites identified by the PKPD clustering method were also obtained by PLS-DA (Table 2), although PLS-DA assumes linear dose-response relations, and does not account

**Table 6. Metabolites identified to show a significant dose response with PLS-DA and PKPD based clustering**

| Metabolite | PLS-DA[a] | PKPD based clustering[b] |
|---|---|---|
| 3-Methylhistidine | X | |
| 4-Hydroxyproline | | X |
| 5-Hydroxylysine | X | X |
| Asparagine | X | X |
| Beta-alanine | X | |
| Citrulline | X | |
| Cysteine | X | X |
| Ethanolamine | | X |
| Gamma-glutamylalanine | X | X |
| Glutamine | X | X |
| Glycylproline | X | |
| Glycine | X | X |
| Histidine | X | X |
| Homocitrulline | | X |
| Homoserine | | X |
| Lysine | X | X |
| Methionine | X | |
| Methionine-sulfoxide | X | X |
| Phenylalanine | | X |
| Sarcosine | | X |
| Serine | X | X |
| Threonine | X | X |
| Tryptophan | | X |
| Tyrosine | X | |
| **Total** | 17 | 18 |

**[a] Metabolites with a VIP score > 1; [b] Metabolites with a ΔOFV > 15.99; Cohen's kappa = 0.38**

for the time dependent response behavior. Other metabolites were only identified by one of the methods. This raises the question under which conditions the methods are in agreement and when they contradict each other. As an illustrative example, homoserine shows a longitudinal dose-dependent response, which was captured by the PKPD clustering, despite the high variability. The dose-dependency was not visible if the serial data is pooled per dose group for PLS-DA analysis (figure S4, A1 vs. A2). On the other hand, glycylproline was only identified by PLS-DA. This is explained by a decrease with 3.5 mg/kg remoxipride relative to the other dose levels when pooling the data per dose group, which does not appear as a dose dependent decrease from baseline (figure S4, B1 vs. B2). Random variation in the data interfered thus both for homoserine and glycylproline with the pooled dose

response analysis (figure S4 - A2, B2, C2), and thus with PLS-DA. This suggests that the PKPD clustering method outperforms PLS-DA if random variation dominates the response. In contrast to homoserine and glycylproline, tyrosine showed a clear dose response, also as a longitudinal decrease from baseline, and was only identified by PLS-DA (figure S4, C1 vs. C2). Whereas the PKPD clustering method failed to identify tyrosine, it showed a significant response in the single metabolite model (*ΔOFV = 31.19*). The clustering thus negatively affected the fitting of the tyrosine response, while overall the 6-cluster model was identified as the best model. It is concluded that the clustering could not identify the cluster for the unique tyrosine response pattern. Further investigation did not show other cases in which the single metabolite model outperformed the cluster model.

There are clusters associated with metabolic pathways, providing a biological context of the clustering results. The branched chain amino acids (BCAA) are clustered into cluster 1, although this cluster showed no significant effect of remoxipride (figure 6). Cluster 6 is associated with the glycine, serine and threonine metabolism. Others have also found an association of D2R antagonism with this pathway, for example a decrease of glycine in plasma [17], a decrease of glycine and serine [38] as well as an increase of homoserine in brain tissue [39]. Serine is actively transported into the brain, where it is converted to glycine and phosphatidylcholine, both implicated in memory function [40]. Serine and glycine both modulate NMDA receptors, which play a main role in the glutamate pathway in the brain. Although plasma glutamate itself was not changed by remoxipride, such interaction may exist in the brain. This would not be surprising, since dopamine and glutamate systems in the brain are highly interrelated [41]. Furthermore, cluster 3 included tyrosine and tryptophan, the precursors of dopamine and serotonin, respectively. Dopamine levels are increased in different brain regions after treatment with D2R antagonists [42]. Furthermore, both tyrosine and tryptophan are converted to their neurotransmitters by the aromatic amino acid decarboxylase (AAAD) enzyme, of which the activity was increased after remoxipride and other D2R antagonists treatment [43]. The decreased tyrosine and tryptophan levels in plasma may therefore be explained by the increased uptake into the brain to refill their brain stores after increased conversion to dopamine and serotonin. These connections to pathways show how remoxipride has a potential interaction with multiple biological pathways. Further studies to these interactions should confirm the hypotheses that are generated by this study.

The different time and concentration dependent patterns in our data suggest a multilevel interaction between remoxipride and the metabolic system. It is not deducible what the exact origin of these differences is, but there are possible explanations. It might be partly caused by on-target versus off-target effects, considering the large differences in $E_{MAX}/EC_{50}$ ratio between the clusters (table SI). Although remoxipride is very selective compared to

other dopamine D2R antagonists, it also has affinity for other receptors, for example the σ-receptors [44]. The differential patterns might also be explained by remoxipride having a potential effect in multiple tissues. The dopamine D2 receptor is not only expressed in the brain, but also in many other tissues [45]. Different tissues may have different receptor concentrations affecting $E_{MAX}$ and $EC_{50}$, and different drug distribution characteristics influencing $k_{OUT}$. Indeed, it is likely that the $k_{OUT}$ is determined by distribution rather than by enzymatic conversion. Typically, enzymatic conversion rates of biogenic amines are >1000 /h (BRENDA Enzyme Database, 2017), while their BBB transport rates are in the range of 0.1 – 10 /h [47], similar to the $k_{OUT}$ values that we identified. Finally, even when bound to the same receptor in the same tissue, multiple downstream pathways might have been affected with differential time and concentration dependent patterns. This idea is clearly illustrated by the differential gene expression patterns in the liver after antagonism of the glucocorticoid receptor [23].

We are aware of limitations that are to be addressed in future studies. Unfortunately, the information on the dopaminergic pathway was limited because the analytical reproducibility was not sufficient for dopamine and its metabolite 3-methoxytyrosine. Moreover, dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) were not measurable by the current analytical platform. Activity of the dopamine pathway in the current experiment is nevertheless illustrated by significant response of tyrosine in the single metabolite model.

Furthermore, data that we obtained on metabolite concentrations in brainECF could not be used because of assay limitations. The relation between metabolite concentrations in plasma and the brain (or CSF) is not straightforward; they do not always correlate [48–51]. Good insight into this relation is crucial for the application of blood-based biomarkers in CNS pharmacology. Simultaneous analysis of biomarker-data in brain and blood would be highly valuable in translational CNS drug development because the brain provides information on drug effects at the site of action, while blood is better accessible in humans. Moreover, such analysis would enable the separation of effects in the brain from those in the periphery. Further work should improve the application of metabolomics on microdialysate samples to enable the identification of the longitudinal biomarker response in brain and plasma simultaneously.

Taking into consideration these discussions, our analysis framework that we developed on preclinical data is also promising in a clinical context. Pharmacometabolomics is increasingly used to provide insights into between-subject variability in drug response [14]. It is similarly important, or perhaps even more so, to identify the particular causes of variable

drug responses when analyzing larger and typically more variable clinical datasets. Application of PKPD based multivariate data analysis is envisioned to increase understanding of inter-individual variability of pharmacometabolomics responses. Additionally, the current framework provides the basis for interspecies translation of pharmacometabolomics responses. Applying the principles of allometric scaling can be used to scale the clearances and rate constants, while physiological information with regard to receptor functionality can be implemented to scale the $E_{MAX}$ and the $EC_{50}$ parameters [52,53]. Interestingly, the metabotype is highly conserved among mammalian species [11]. It is therefore anticipated that the combination of PKPD based multivariate data analysis and interspecies scaling will improve the dose selection in early clinical development.

In conclusion, we have laid out the basis for the integration of pharmacometabolomics and PKPD modeling. The developed PKPD cluster model predicts the different biochemical responses in plasma for a range of remoxipride doses and provided comprehensive insights in its drug effects. The study design with multiple dose levels and time serial sampling, together with an analytical method that measured a large number of metabolites enabled this model-based approach that mathematically linked the PK and the multiple PD responses. Remoxipride showed 6 differential response patterns, indicating a multilevel interaction between the drug and the biochemical system. In particular, the glycine, serine and threonine pathway, as well as the precursors of dopamine and serotonin, were influenced by remoxipride. It is envisioned that PKPD clustering could serve as an initial framework for the development of mechanistic systems pharmacology models.

## Acknowledgements

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# SUPPLEMENTARY MATERIALS

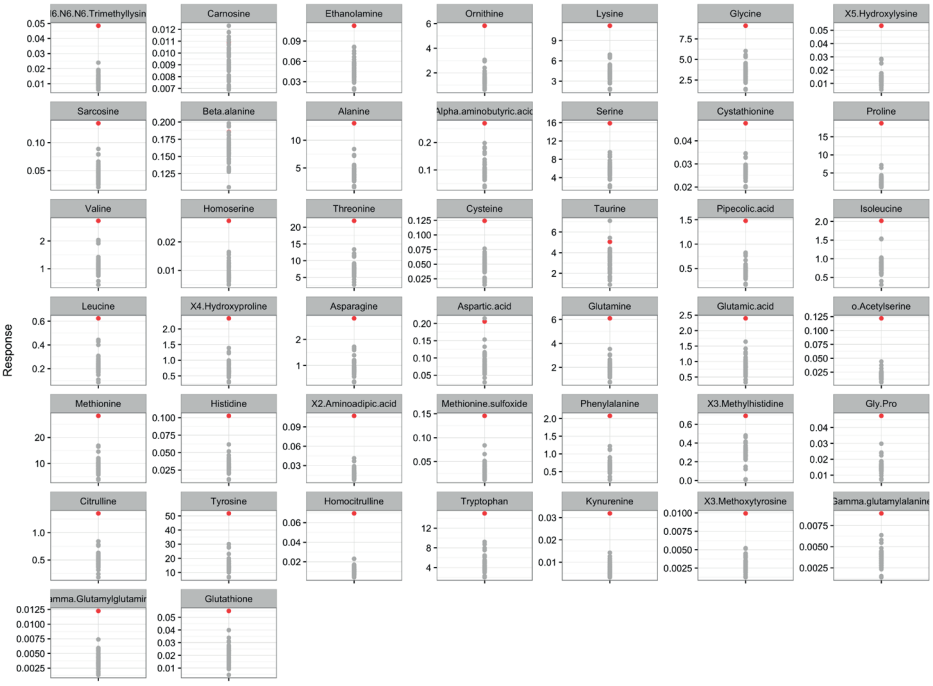



**Figure S1. Investigation of the main source of outliers. The red dot represents sample R26_T15, obtained from animal 26 at 15 minutes after administration of placebo. The response is deviated from the other responses in the placebo group (black dots), indicating that this is a sample specific, and not a treatment or time point specific outlier.**

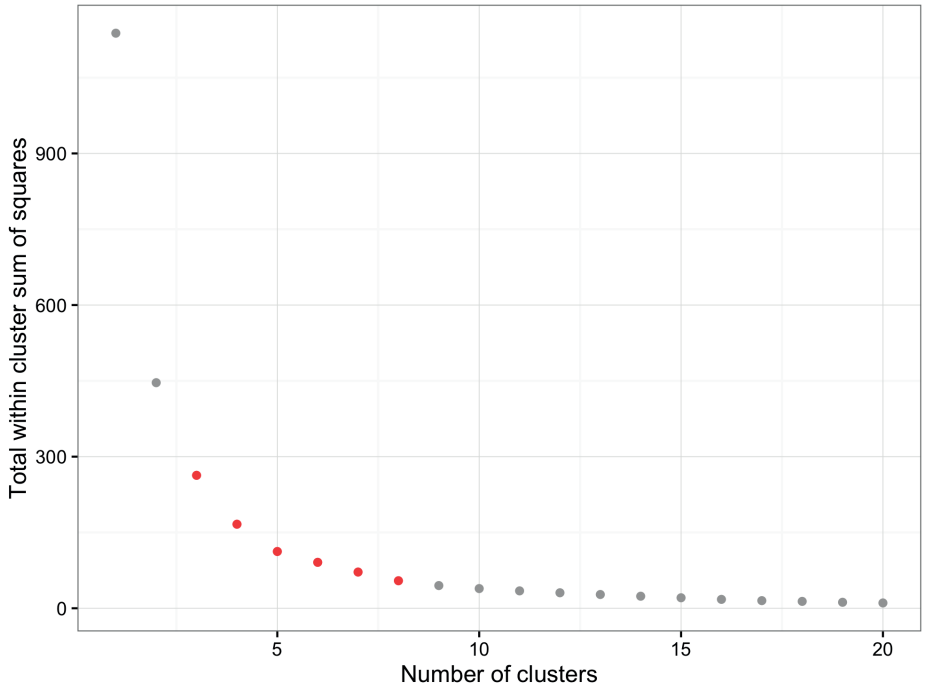



**Figure S2. Elbow plot visualizing the total within cluster sum of squares (WCSS) against the number of candidate clusters for the k-means clustering. The red dots show the 'elbow' of the WCSS, indicating the range comprising the optimal number of clusters.**

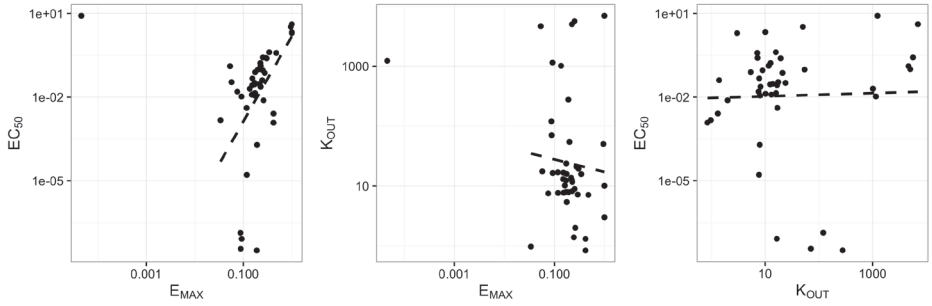



**Figure S3. Correlations between $E_{MAX}$, $EC_{50}$ and $k_{out}$ (dots). Linear regression was applied to the log-transformed parameter estimates as indicated by the dashed line. Kynurenine ($E_{MAX}$ < 0.001) was excluded from the linear regression analysis since it highly skewed the regression.**

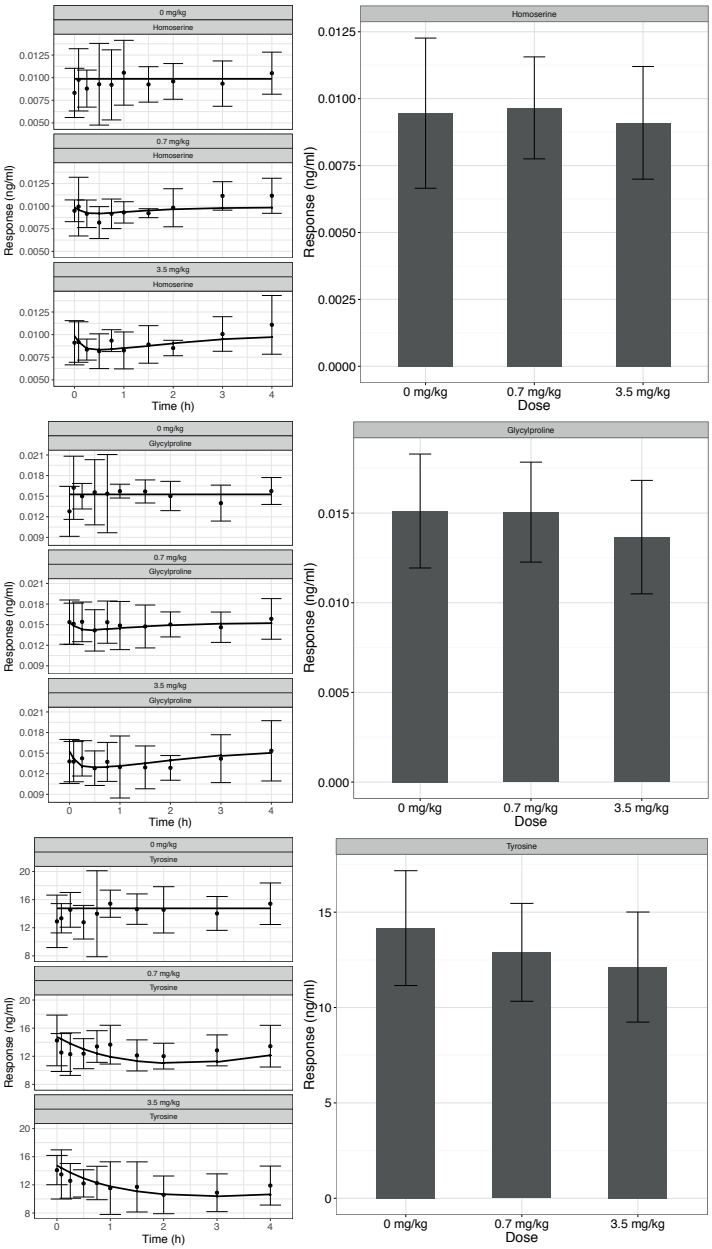



**Figure S4. Dose responses for homoserine (A), glycylproline (B), and tyrosine (C), with the longitudinal responses per dose (left, 1) and the dose response (right, 2). Homoserine (A) is identified by PKPD clustering, but not by PLS-DA. No dose response is visible if the data is pooled per dose group (A2), but a longitudinal decrease from baseline is observed (A1). Glycylproline (B) is identified by PLS-DA, but not by PKPD clustering. A lower response is observed in the highest dose group (B2), but this does not appear as a longitudinal response (B2). Tyrosine (C) is identified by PLS-DA, but not by PKPD clustering. A dose response is observed (C2), which also appears as a longitudinal response (C1).**

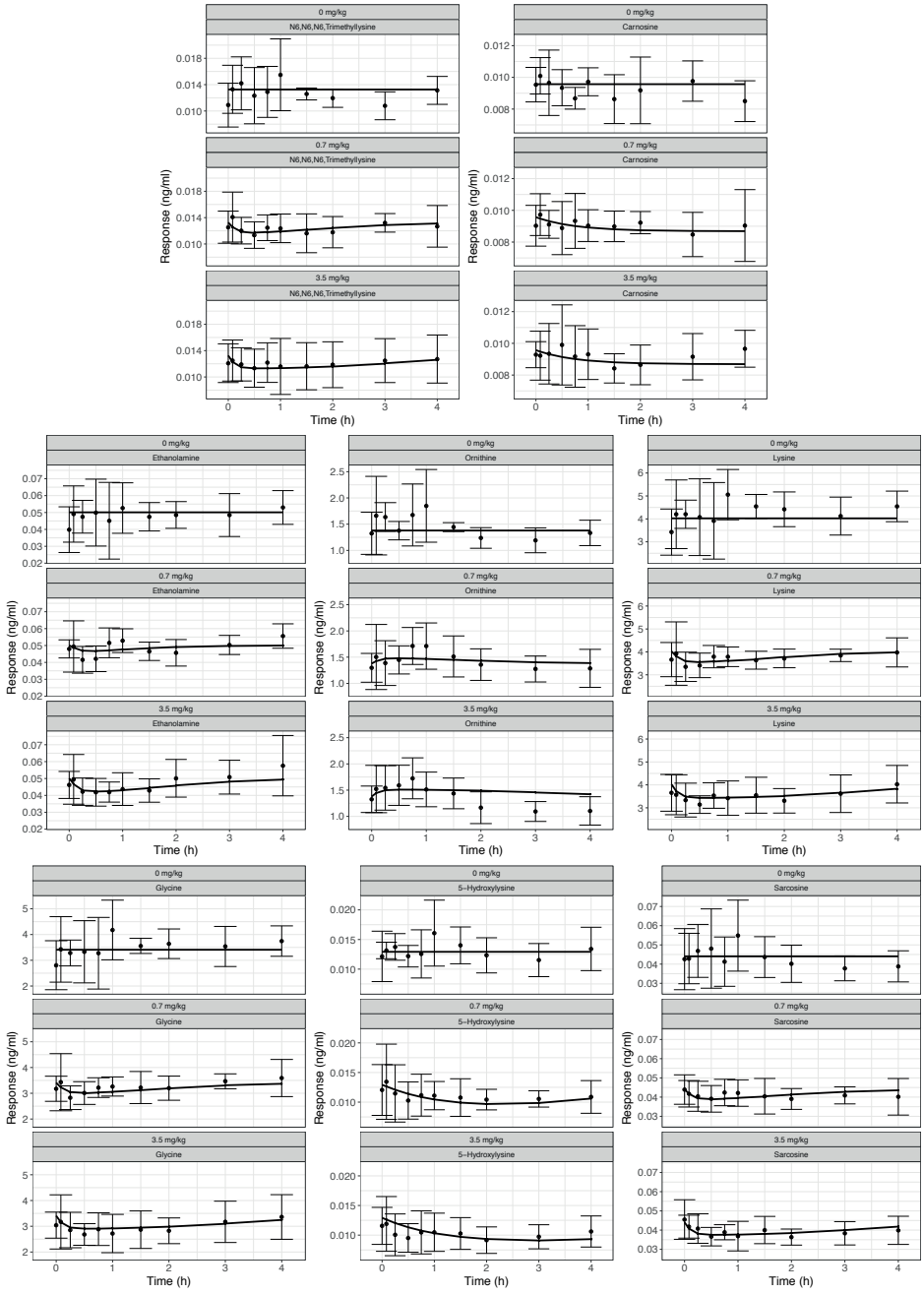



**Figure S5. Goodness-of-fit for each single metabolite. Predicted response is indicated by the black solid line, whereas the observed data is indicated by the dots (mean) and error bars (+/- standard deviation).**

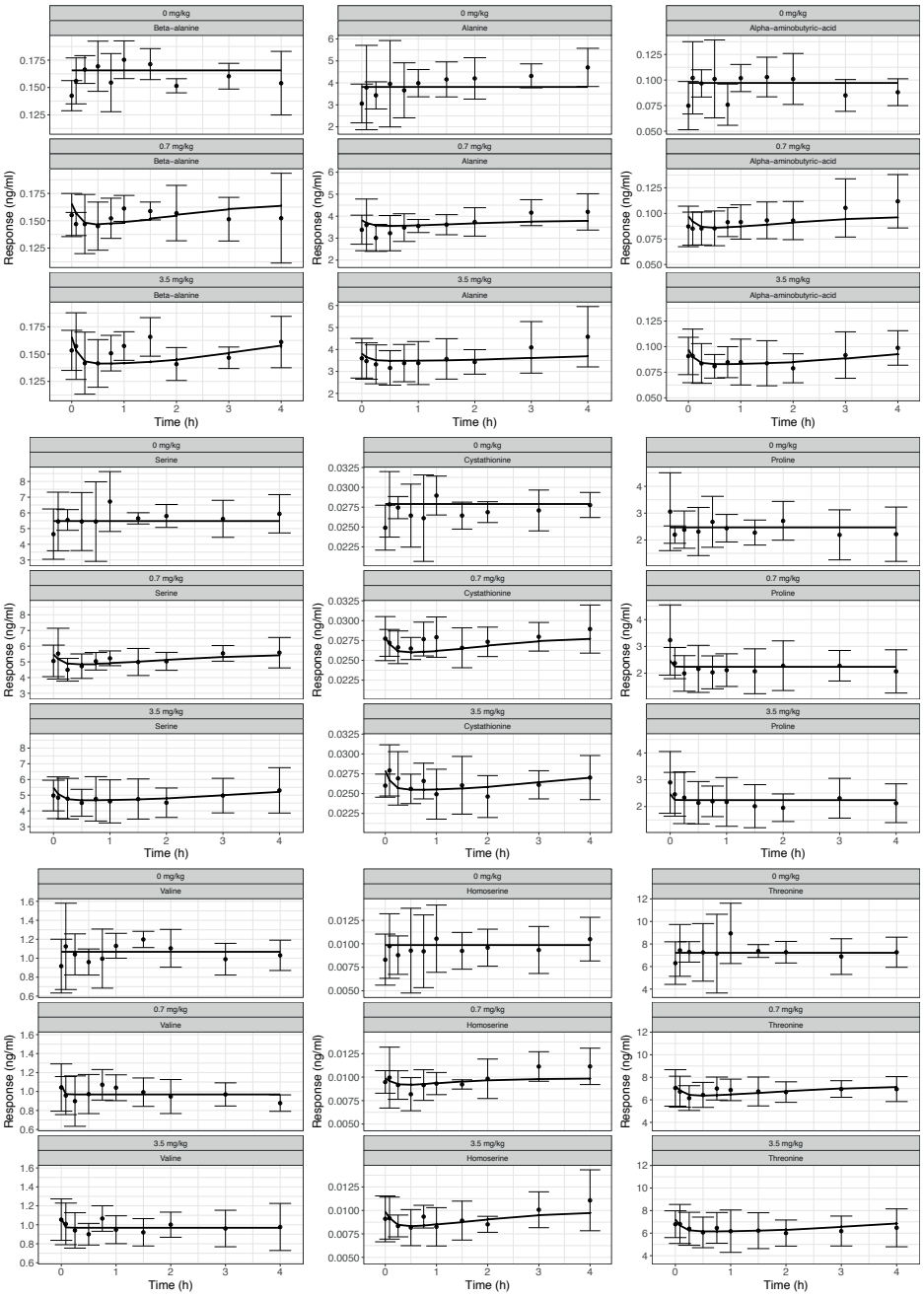



**Figure S5. Goodness-of-fit for each single metabolite. Predicted response is indicated by the black solid line, whereas the observed data is indicated by the dots (mean) and error bars (+/- standard deviation).**

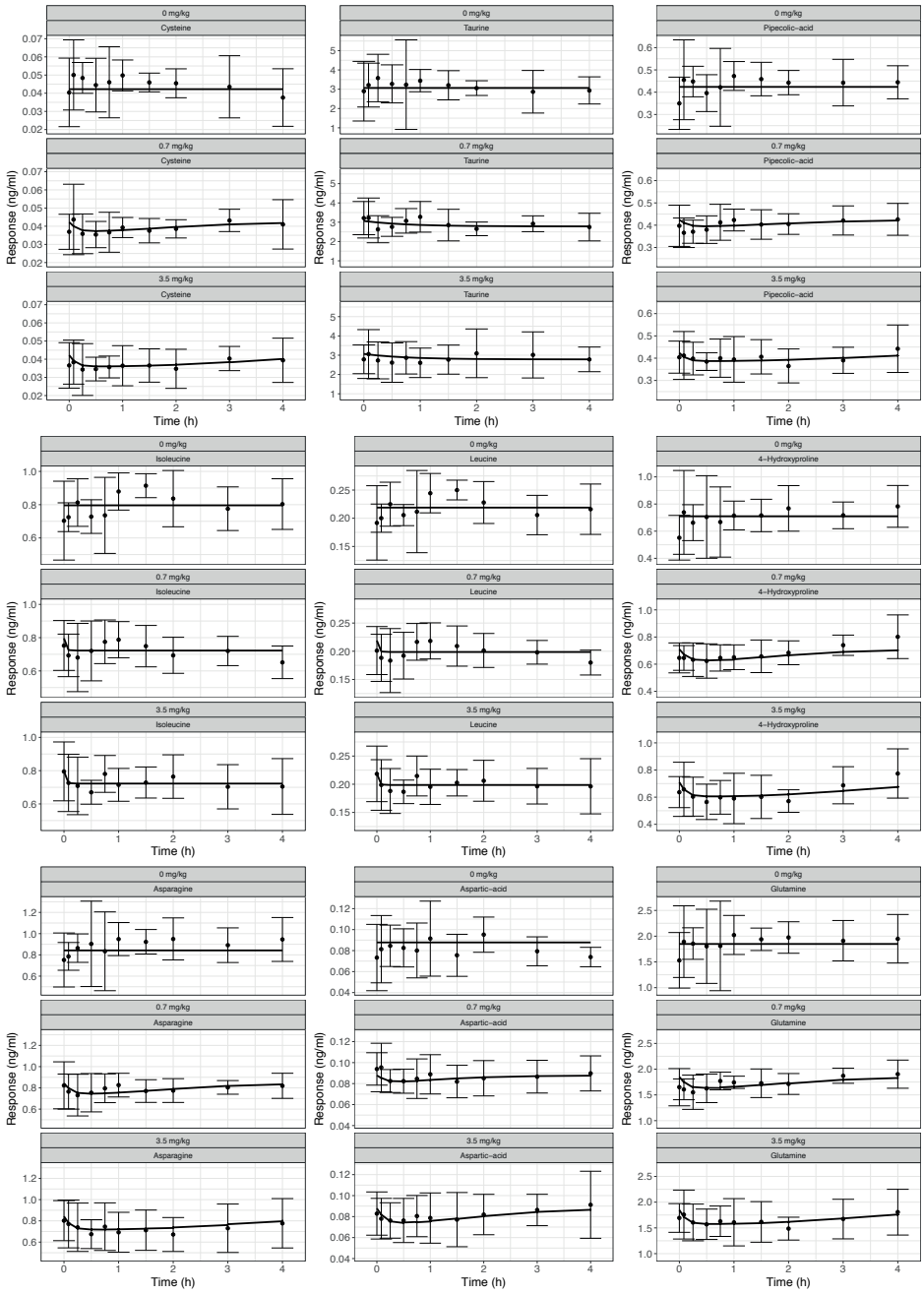



**Figure S5. Goodness-of-fit for each single metabolite. Predicted response is indicated by the black solid line, whereas the observed data is indicated by the dots (mean) and error bars (+/- standard deviation).**

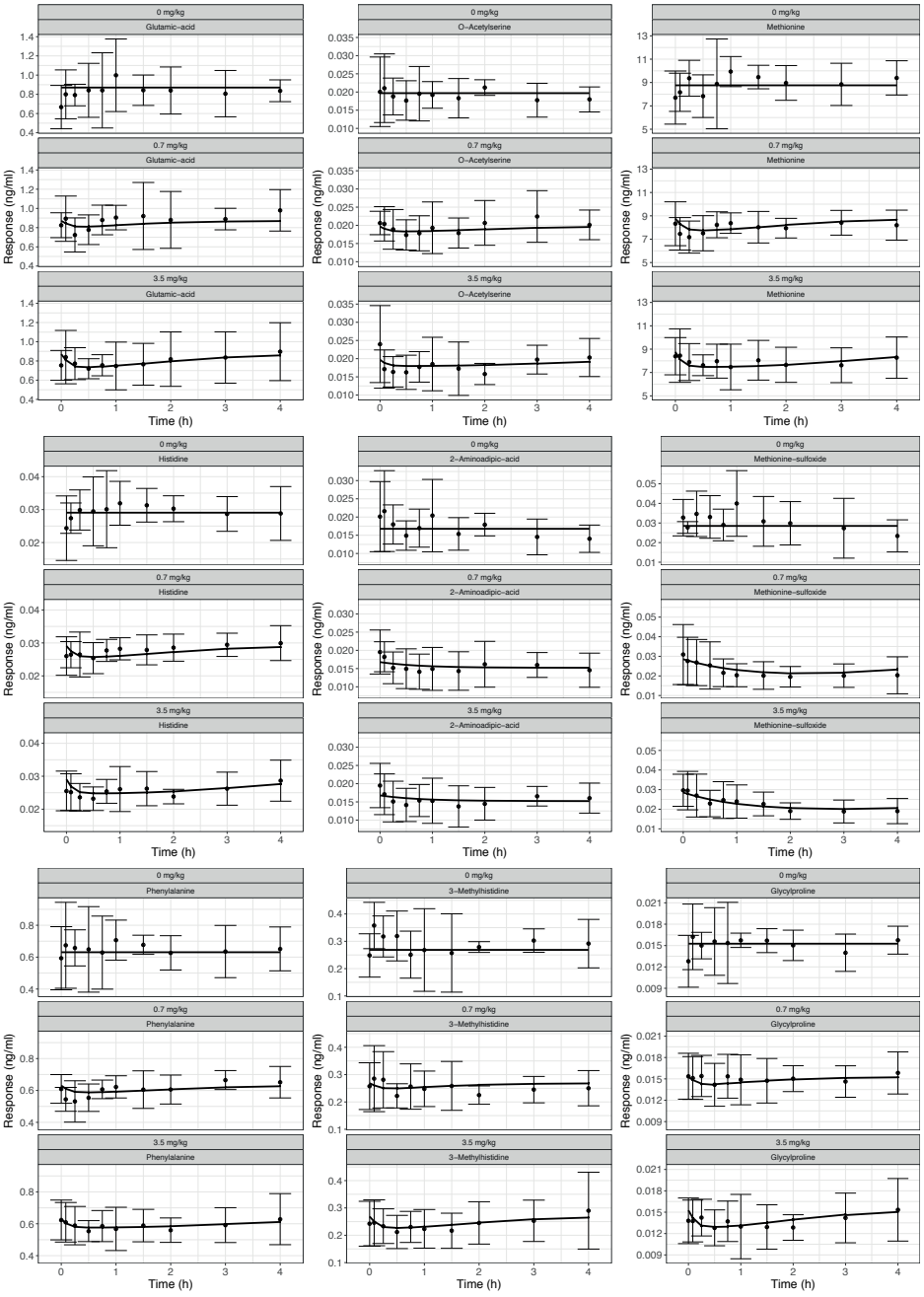



**Figure S5. Goodness-of-fit for each single metabolite. Predicted response is indicated by the black solid line, whereas the observed data is indicated by the dots (mean) and error bars (+/- standard deviation).**

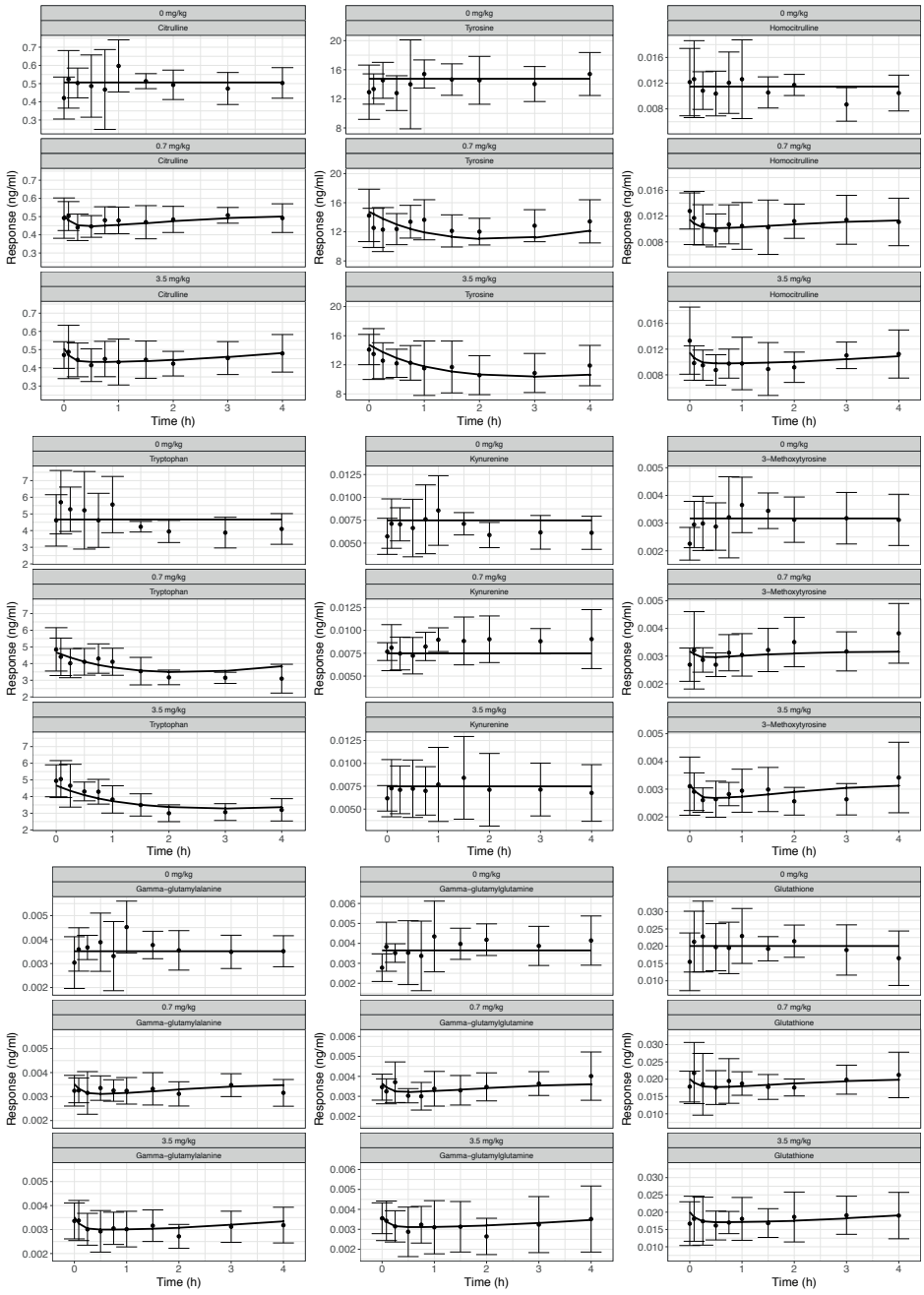



**Figure S5. Goodness-of-fit for each single metabolite. Predicted response is indicated by the black solid line, whereas the observed data is indicated by the dots (mean) and error bars (+/- standard deviation).**

# CHAPTER 7

## BLOOD-BASED BIOMARKERS OF QUINPIROLE PHARMACOLOGY: MULTIVARIATE PK/PD AND METABOLOMICS TO UNRAVEL THE UNDERLYING DYNAMICS IN PLASMA AND BRAIN

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## Abstract

A key challenge in the development of CNS drugs is the availability of drug target specific blood-based biomarkers. As a new approach, we applied multivariate pharmacokinetic/pharmacodynamic (PK/PD) analysis in $brain_{ECF}$ and plasma simultaneously after 0, 0.17 and 0.86 mg/kg of the dopamine $D_{2/3}$ agonist quinpirole (QP) in rats. We measured 76 biogenic amines in plasma and $brain_{ECF}$ after single and 8-day administration, to be analyzed by multivariate PK/PD analysis. Multiple concentration-effect relations were observed with potencies ranging from 0.001 – 383 nM. Many biomarker responses propagated over the blood-brain-barrier. Effects were observed for dopamine and glutamate signaling in $brain_{ECF}$, and branched-chain amino acid metabolism and immune signaling in plasma. Altogether, we showed for the first time how multivariate PK/PD could describe a systems-response across plasma and brain, thereby identifying potential blood-based biomarkers. This concept is envisioned to provide an important connection between drug discovery and early drug development.

**Keywords:** Metabolomics, systems pharmacology, PK/PD, CNS drug development, dopamine agonists

# Introduction

One of the key challenges in central nervous system (CNS) drug development is the discovery of blood-based biomarkers that reflect the central response (1,2). Such biomarkers enhance the evaluation of the proof of pharmacology of CNS drugs, which is crucial for successful drug development (3). It is particularly important to dynamically evaluate the biomarker responses in relation to the systems pharmacokinetics (PK) of the drug, given that the interaction between PK and pharmacodynamics (PD) typically is non-linear and time-dependent (4,5).

While currently biomarker discovery is nowadays typically driven by the known pharmacological mechanisms, metabolomics fingerprinting is not limited to these pathways. Metabolomics analysis has revealed multiple new biochemical pathways in relation to drug responses (6–11).

One of the techniques being useful in CNS biomarker discovery is intracerebral microdialysis. It is a well-established technique that has been successfully applied to study drug concentrations as well as drug response biomarkers in brain extracellular fluid ($brain_{ECF}$) to evaluate CNS PK and PD (12–14). Therefore, microdialysis is the method of choice to dynamically evaluate a metabolomics fingerprint in brain extracellular fluid ($brain_{ECF}$) simultaneously upon CNS drug treatment. Such dynamical evaluation would improve the quantitative insights into systems-wide responses (i.e. changes in biomarker concentrations), thereby shifting CNS drug development from an empirical towards a mechanistic discipline (15,16).

In an earlier study we have already shown that a multivariate (PK/PD) evaluation of a metabolomics response in plasma reveals multiple dynamics underlying a systems response upon treatment with remoxipride (17). In the current study we set out to extend this methodology with a simultaneous evaluation of a metabolomics response in both plasma and $brain_{ECF}$, using the dopamine $D_{2/3}$ receptor agonist quinpirole (QP) as paradigm compound. Overall, the purpose is to provide insight into the systems-wide biochemical responses of CNS drugs, combined with PKPD modeling as a new approach to discover blood-based biomarkers of central responses.

# Methods

## *Animals, surgery and experiment*

*Animals* – Animal studies were performed in agreement with the Dutch Law of Animal Experimentation and approved by the Animal Ethics Committee in Leiden, the Netherlands (study protocol DEC12247). For details on animals, surgery and experiment, we refer to (18).

*Surgery* – In short, male Wistar rats (n=44) underwent surgery while anesthetized, to receive cannulas in the femoral artery and vein for blood sampling and drug administration, respectively. The microdialysis probe guides (CMA/12) and their dummy probes were implanted in the caudate putamen in both hemispheres. The probes (CMA/12 – Elite 4 mm) were placed 24 hours before experiment.

*Experiment* – The animals were subjected to an experiment on two days with 7 days in between. On the days of experiment, the rats were randomly assigned to receive 0 mg/kg (n=12), 0.17 mg/kg (n=16) or 0.86 mg/kg (n=16) QP. Microdialysate samples were collected from -200 to 180 minutes (20-minute interval, 1.5 µl/min, 120 min. equilibration time). Blood samples were taken at -5, 5, 7.5, 10, 15, 25, 45, 90, 120 and 180 minutes and centrifuged to separate the plasma (1000 x g, 10 min, 4°C). Samples were stored at -80°C until analysis. Between the experiment days, the same doses were administered subcutaneously.

## *Chemical analysis of the samples*

*Targeted monoamine + metabolite analysis* – A selection of plasma and microdialysate samples collected on experiment day 1 were analyzed by BrainsOnline (Groningen, The Netherlands). The samples were delivered on dry ice and stored at -80°C until analysis. Monoamines and their metabolites (serotonin, 5-hydroxy indoleacetic acid, dopamine, 3,4-hydroxyphenylacetic acid, homovanillic acid, glutamate and glycine) were analyzed employing SymDAQ derivitization (19,20). Data were calibrated and quantified using the Analyst™ data system (Applied Biosystems, Bleiswijk, The Netherlands) to report concentrations of the analytes.

*Untargeted biogenic amine analysis* – The biogenic amines were analyzed in microdialysate and plasma samples of experiment day 1 and 8 according to a previously described method (21). Amino acids and amines were derivatized by an Accq-tag derivatization strategy. Plasma samples (5 µL) were reduced with TCEP (tris(2-carboxyethyl)phosphine) and deproteinated by MeOH. Microdialysate samples (30 µL) were only reduced with TCEP. The samples were dried under vacuum while centrifuged (9400xg, 10 min, Room Temperature), and reconstituted in borate buffer (pH 8.8) with with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) derivatization reagent. The reaction mixtures were injected (1 µL) into an UPLC-MS/MS system, consisting of an Agilent 1290 Infinity II LC system, an Accq-Tag Ultra column, and a Sciex Qtrap 6500 mass spectrometer. The peaks were assigned using Sciex MultiQuant software, integrated, normalized for their internal standards, and corrected for background signal. Only compounds with a QC relative standard deviation ($RSD_{QC}$) under 30% were reported to assure quality of the data.

## *Data analysis*

*Pharmacokinetic model* – The PK model has been published previously and described the free QP concentrations in plasma and brain$_{ECF}$ with QP doses ranging from 0.17 to 2.14 mg/kg (18).

*Pharmacodynamic models* – A PD model was developed for each single metabolite (hereafter called biomarkers) using a population approach in NONMEM® version 7.3.0 with subroutine ADVAN13. The inter-individual variability around the parameters and the residual error were described by an exponential distribution (suppl. Equation 1, 2). A combination of submodels was evaluated for each single biomarker consisting of i) a straight baseline, an exponential decay, or a linear slope model; ii) a linear or a sigmoid $E_{MAX}$ concentration response model; iii) a transit or no transit compartment model; and iv) a turnover or a pool model (Suppl. equations 4 - 7). In addition, a model with no drug response function was evaluated (Suppl. equation 8) The models were selected on basis of the objective function value ($\chi^2$-test, $p < 0.05$), the condition number, successful convergence and visual evaluation of goodness-of-fit plots.

*Exploration of target site* – For biomarkers showing a response in either plasma or brain$_{ECF}$, the site with the response was identified as effect target site. In case a biomarker showed a response both in plasma and brain$_{ECF}$, two PD models were developed with either the plasma biomarker response driving the brain$_{ECF}$ biomarker response or vice versa. The link between the response in plasma and brain$_{ECF}$ was described by a linear or a non-linear brain transport model following Michaelis Menten kinetics (Suppl. Equation 9). The Aikaike Information Criterium (AIC) of the 'brain$_{ECF}$ target site model' was subtracted from that of the 'plasma target site model' to calculate the ΔAIC for selection of the target site model. A negative ΔAIC indicated plasma as target site of effect, while a positive ΔAIC suggested brain$_{ECF}$ as target site of effect.

*Clustering* – The longitudinal biomarker responses were simulated for their determined target site and subsequently clusters of the dynamical pharmacological responses were identified in plasma and brain$_{ECF}$ using k-means clustering. The number of clusters was selected in two steps. First an elbow plot, depicting number of clusters against within cluster sum of squares, was used to identify a range of potential number of clusters. Second, for each potential number of clusters a PK/PD cluster model was developed describing the cluster responses. The AIC was used to select the model with the optimal number of clusters. Subsequently, a step-wise parameter sharing procedure was applied as previously described (17). In short, a single parameter (e.g. $EC_{50}$) was estimated for multiple clusters and evaluated by the change in OFV ($\chi^2$-test, $p < 0.05$) to determine whether this was statistically different from a model with separate parameters. If no difference was found, the shared parameter was kept in the model.

*Significance score calculation* - The cluster-based model was compared to a model with no drug effect model included, i.e. assuming no effect of QP. A significance score was calculated by the change in OFV corrected for the degrees of freedom with a Bonferroni-corrected significance threshold of $\alpha = 0.01$ (Suppl. equation 10). A significance score > 0 reflects a significant effect of QP on a biomarker response.

*Effect of eight-day QP administration*

Basal biomarker levels (t = 0) in both $brain_{ECF}$ and plasma at experiment day 1 and experiment day 8 were compared using two-way ANOVA with interaction between dose and experiment day. Tukey honest significant different test was used for posthoc analysis. $Brain_{ECF}$ basal biomarker levels were averaged per animal, given that there were 4-6 baseline samples for each animal. For the biomarkers that revealed a significant change with experiment day, a covariate analysis was performed in the single biomarker models by estimating a separate baseline parameter per combination of treatment group and experiment day. Only if the covariate analysis revealed a difference, the effect was considered significant.

# Results

## *Exploration of target site of effect*

The metabolomics data revealed 23 biomarkers primarily responding to QP in plasma, and 15 biomarkers primarily affected by QP in the brain (Table I, Figure 1). DL-3-aminobutyric acid and serotonin could only be measured in plasma, while L-glutamine could only be measured in $brain_{ECF}$. From all the biomarkers that reflected an effect of plasma QP, 19 showed a net transport to the $brain_{ECF}$. Inversely, 5 biomarkers exhibited a net transport from $brain_{ECF}$ into plasma, potentially leading to secondary responses in plasma. The inter-compartmental transport rates between plasma and $brain_{ECF}$ of many biomarkers were described by non-linear Michaelis-Menten kinetics (Table I).

**Table I. Overview of biogenic amines and their target site that showed a response upon QP treatment. The Delta Akaike Information Criterium (ΔAIC) indicates the target site (see methods). Also, the type of brain transport is indicated (yes, no or not available (N.A.)). PàB and BàP stand for plasma-to-brain and brain-to-plasma, respectively. Only biomarkers presented in black showed a significant response in the cluster models.**

| Biomarker | Target site | ΔAIC | Brain transport |
|---|---|---|---|
| | ***Targeted approach (BrainsOnline)*** | | |
| **Dopamine** | $Brain_{ECF}$ | - | No |
| **DOPAC** | $Brain_{ECF}$ | - | No |
| **HVA** | $Brain_{ECF}$ | - | No |
| **Glycine** | Plasma | -56.216 | Yes – $NonLin_{PàB}$ |
| **5-HIAA** | Plasma | - | No |
| **L-Glutamic acid** | Plasma | - | No |

**Table I. (continued)**

| Biomarker | Target site | ΔAIC | Brain transport |
|---|---|---|---|
| | ***Untargeted approach (BMFL)*** | | |
| **L-Phenylalanine** | Plasma | -75.811 | Yes – $NonLin_{BaP}$ |
| **L-Valine** | Plasma | -73.682 | Yes – $NonLin_{BaP}$ |
| **L-Methionine sulfoxide** | Plasma | -55.917 | Yes – $NonLin_{PaB}$ |
| **Taurine** | Plasma | -48.638 | Yes – $NonLin_{BaP}$ |
| **S-Methylcysteine** | Plasma | -46.564 | Yes – Linear |
| **L-Alpha-aminobutyric acid** | Plasma | -40.634 | Yes – $NonLin_{PaB}$ |
| **L-Asparagine** | Plasma | -37.597 | Yes – $NonLin_{BaP}$ |
| **L-Alanine** | Plasma | -35.086 | Yes – $NonLin_{PaB}$ |
| **Gamma-L-glutamyl-L-alanine** | Plasma | -33.872 | Yes – $NonLin_{PaB}$ |
| **L-Threonine** | Plasma | -31.734 | Yes – Linear |
| **L-Methionine** | Plasma | -24.946 | Yes – Linear |
| **L-Histidine** | Plasma | -24.715 | Yes – Linear |
| **L-Arginine** | Plasma | -24.469 | Yes – $NonLin_{PaB}$ |
| **L-Isoleucine** | Plasma | -13.582 | Yes – $NonLin_{BaP}$ |
| **Glycine** | Plasma | -12.572 | Yes – Linear |
| **Homocysteine** | Plasma | -10.954 | Yes – Linear |
| **L-Serine** | Plasma | -8.129 | Yes – Linear |
| **Citrulline** | Plasma | -5.407 | Yes – $NonLin_{BaP}$ |
| **L-Leucine** | Plasma | -2.462 | Yes – $NonLin_{BaP}$ |
| **DL-3-aminoisobutyric acid** | Plasma | - | N.A. |
| **Histamine** | Plasma | - | No |
| **L-Glutamic acid** | Plasma | - | No |
| **L-Homoserine** | Plasma | - | No |
| **Methionine sulfone** | Plasma | - | No |
| **Serotonin** | Plasma | - | N.A. |
| **L-Proline** | $Brain_{ECF}$ | 41.574 | Yes – $NonLin_{BaP}$ |
| **N6,N6,N6-Trimethyl-L-lysine** | $Brain_{ECF}$ | 27.282 | Yes – $NonLin_{BaP}$ |
| **Hydroxylysine** | $Brain_{ECF}$ | 8.103 | Yes – Linear |
| **L-Lysine** | $Brain_{ECF}$ | 4.747 | Yes – $NonLin_{BaP}$ |
| **L-4-hydroxy-proline** | $Brain_{ECF}$ | 1.111 | Yes – $NonLin_{BaP}$ |
| **Homocitrulline** | $Brain_{ECF}$ | 0.261 | Yes – $NonLin_{BaP}$ |
| **3-Methoxytyramine** | $Brain_{ECF}$ | - | No |
| **5-Hydroxy-L-tryptophan** | $Brain_{ECF}$ | - | No |
| **Cystathionine** | $Brain_{ECF}$ | - | No |
| **Gamma-aminobutyric acid** | $Brain_{ECF}$ | - | No |
| **L-2-aminoadipic acid** | $Brain_{ECF}$ | - | No |
| **L-Glutamine** | $Brain_{ECF}$ | - | N.A. |
| **L-Tryptophan** | $Brain_{ECF}$ | - | No |
| **L-Tyrosine** | $Brain_{ECF}$ | - | No |
| **Ornithine** | $Brain_{ECF}$ | - | No |
| **Putrescine** | $Brain_{ECF}$ | - | No |
| **Sarcosine** | $Brain_{ECF}$ | - | No |

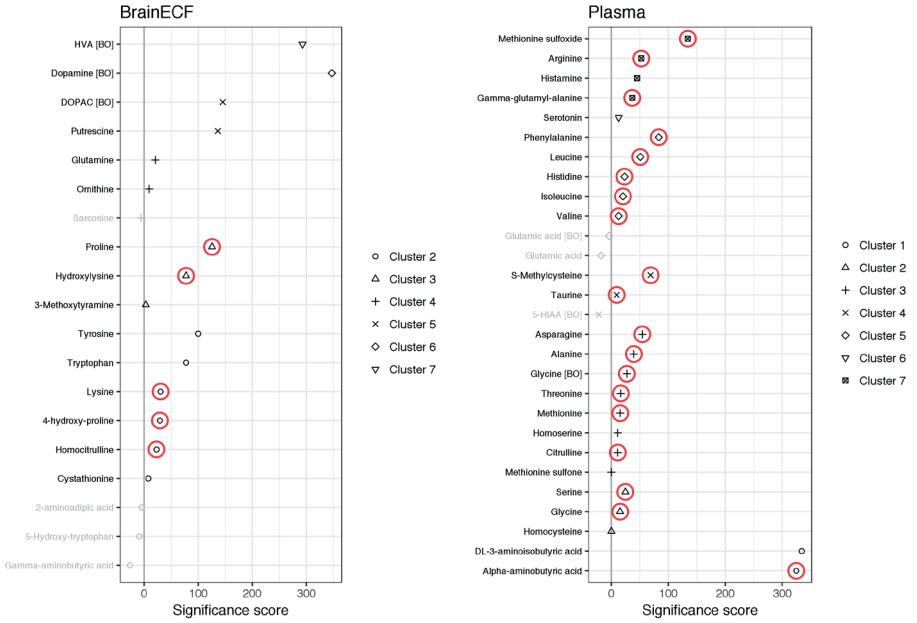



**Figure 1. Significance score of responding metabolites in brain$_{ECF}$ (left) and plasma (right) indicating their potential as a biomarker of the QP systems effect. The grey line marks the significance threshold; metabolites right of the line were significantly affected by QP. The red circles indicate the metabolites that distribute from brain$_{ECF}$ to plasma and vice versa. *Cluster 1 was excluded for brain$_{ECF}$ since no effect was observed. [BO] refers to the amines analyzed by BrainsOnline.**

## *Clustered response patterns in brain$_{ECF}$ and plasma*

A total of 7 clusters of dynamical biomarker responses in brain$_{ECF}$ was selected (Table II). Using parameter sharing, it was observed that the biomarkers responded with either a high or a low potency ($EC_{50}$ = 0.01 nM or $EC_{50}$ = 122 nM, Table III, Figure 2). The turnover of these biomarkers was low (0.031 $min^{-1}$ – 0.056 $min^{-1}$) or high (0.13 $min^{-1}$ – 0.44 $min^{-1}$). The responses in plasma were also separated into 7 clusters (Table II) described by models with transit compartment models (cluster 1 & 4), pool models (cluster 5 & 6) and turnover models (cluster 2, 3 & 7) (Table III). A wider variety of potency parameter estimates were identified in plasma as compared to brain$_{ECF}$: 0.01 nM, 17.2 nM, and 113 - 383 nM (Table III, Figure 2). Moreover, the direction of response was both up (cluster 1 & 4) and down (cluster 2, 3 & 5-7). The responses in brain$_{ECF}$ and plasma were well described by the cluster-PKPD models (Figure 3, suppl. Figure 2).

## *Effect of QP on the dopamine pathway*

Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), the key constituents of the dopamine pathway, were decreased in brain$_{ECF}$ upon QP treatment. Whereas the *in vivo* potency was found to be similar for these biomarkers (122 nM), the

**Table II. Determination of optimal number of clusters in plasma and brain$_{ECF}$ using the Akaike Information Criterium (AIC). In bold the selected number of clusters.**

| Plasma | | Brain$_{ECF}$ | |
|---|---|---|---|
| # clusters | **AIC** | # clusters | **AIC** |
| 4 | 65500.76 | 6 | 78140.64 |
| 5 | 64991.03 | **7** | **76518.12** |
| 6 | 64966.79 | 8 | 76523.49 |
| **7** | **64876.42** | 9 | 78319.55 |
| 8 | 66314.62 | 10 | 76535.81 |

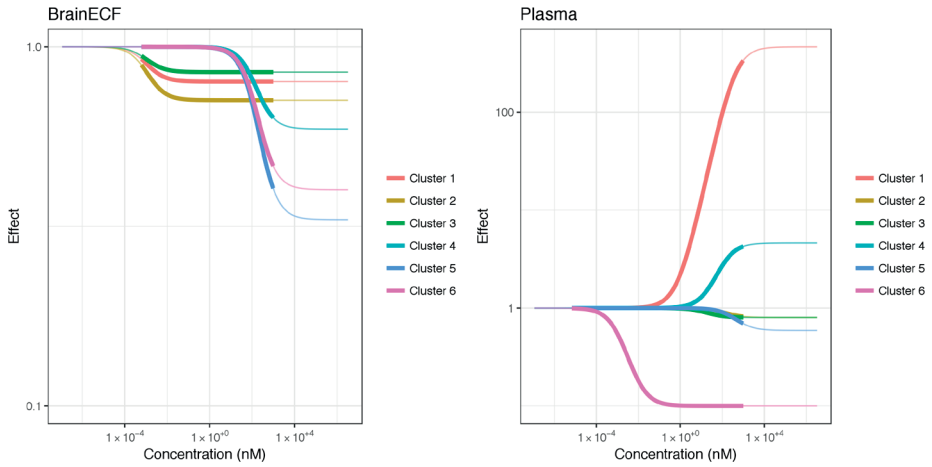



**Figure 2. An overview of the concentration-effect relations that underlie the systems responses in brain$_{ECF}$ (left) and plasma (right). Thick line parts represent the range of observed biomarker concentrations *Cluster 1 was excluded for brain$_{ECF}$ since no effect was observed.**

maximal inhibition values (DA: 67%, DOPAC: 41%, HVA: 60%) and the turnover rates (DA: 0.44 min$^{-1}$, DOPAC: 0.13 min$^{-1}$, HVA: 0.031 min$^{-1}$) were different (Table III, Figure 2). No responses of QP treatment were observed for DA and HVA in plasma, while DOPAC could not be measured in plasma due to assay lower limit of detection of 50 nM.

**Table III. Parameter estimates of the cluster models. RSE: relative standard error.**

| Plasma | | Brain$_{ECF}$ | |
|---|---|---|---|
| **Parameter** | **Estimate (RSE)** | **Parameter** | **Estimate (RSE)** |
| | *Cluster 1** | | |
| $E_{MAX}$ (%) | 4650 (41.1%) | | |
| $EC_{50}$ (nM) | 383 (54.3%) | | |
| $k_{out}$ (min$^{-1}$) | 0.035 (42.3%) | | |
| $k_{transit}$ (min$^{-1}$) | 0.044 (33.1%) | | |

| | | | |
|---|---|---|---|
| $n_{transit}$ | 8.3 (19.2%) | | |
| | *Cluster 2* | | |
| $I_{MAX}$ (%) | -20 (30.1%) | $I_{MAX}$ (%) | -20 (6.1%) |
| $IC_{50}$ (nM) | 113 (98.5%) | $IC_{50}$ (nM) | 0.001 (fix) |
| $k_{out}$ ($min^{-1}$) | 0.057(38.3%) | $k_{out}$ ($min^{-1}$) | 0.056 (27.9%) |
| | *Cluster 3* | | |
| $I_{MAX}$ (%) | -20 (30.1%) | $I_{MAX}$ (%) | -29 (7.1%) |
| $IC_{50}$ (nM) | 17.2 (50.6%) | $IC_{50}$ (nM) | 0.001 (fix) |
| $k_{out}$ ($min^{-1}$) | 0.11 (12.2%) | $k_{out}$ ($min^{-1}$) | 0.13 (13.3%) |
| | *Cluster 4* | | |
| $E_{MAX}$ (%) | 363 (67.5%) | $I_{MAX}$ (%) | -15 (13.5%) |
| $EC_{50}$ (nM) | 113 (98.5%) | $IC_{50}$ (nM) | 0.001 (fix)) |
| $k_{out}$ ($min^{-1}$) | 9.58 (104%) | $k_{out}$ ($min^{-1}$) | 0.14 (32.7%) |
| $k_{transit}$ ($min^{-1}$) | 0.0052 (46.8%) | | |
| $n_{transit}$ | 1.79 (17.9%) | | |
| | *Cluster 5* | | |
| $I_{MAX}$ (%) | -41 (14.6%) | $I_{MAX}$ (%) | -41 (9.0%) |
| $IC_{50}$ (nM) | 339 (32.8%) | $IC_{50}$ (nM) | 122 (51.4%) |
| $k_{out}$ ($min^{-1}$) | 0.11 (12.5%) | $k_{out}$ ($min^{-1}$) | 0.13 (13.3%) |
| $k_{rel}$ ($min^{-1}$) | 0.018 (27.5%) | | |
| | *Cluster 6* | | |
| $I_{MAX}$ (%) | -90 (0.3%) | $I_{MAX}$ (%) | -67 (4.9%) |
| $IC_{50}$ (nM) | 0.001 (fix) | $IC_{50}$ (nM) | 122 (51.4%) |
| $k_{out}$ ($min^{-1}$) | 0.10 (18.4%) | $k_{out}$ ($min^{-1}$) | 0.44 (47.9%) |
| $k_{rel}$ ($min^{-1}$) | 0.35 (19.6%) | | |
| | *Cluster 7* | | |
| $I_{MAX}$ (%) | -41 (6.4%) | $I_{MAX}$ (%) | -60 (9.3%) |
| $IC_{50}$ (nM) | 17.2 (50.6%) | $IC_{50}$ (nM) | 122 (51.4%) |
| $k_{out}$ ($min^{-1}$) | 0.060 (13.5%) | $k_{out}$ ($min^{-1}$) | 0.031 (28.9%) |

*** Cluster 1 was excluded for $brain_{ECF}$ since no dose-response was observed. Consequently, parameter estimates were not informative.**

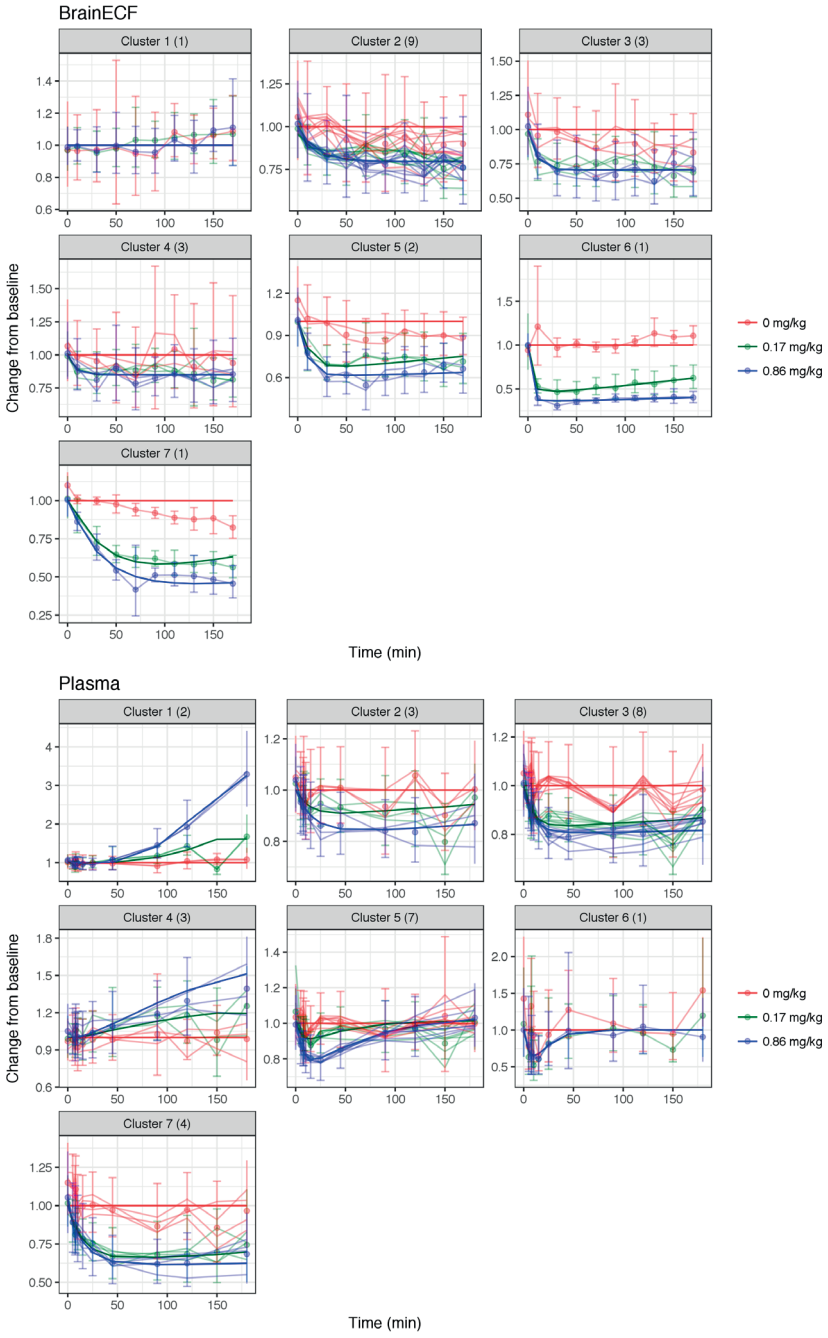



**Figure 3. Goodness-of-fit of the cluster responses as change from baseline in brain$_{ECF}$ (top) and plasma (bottom). Dots and error bars mark the geometric mean +/- standard deviation of the observed cluster responses, light lines represent the geometric mean of the single metabolite responses and dark lines show the predicted cluster responses. The facet labels show the number of metabolites between the parentheses.**

### *Effect of QP on other pathways in brain$_{ECF}$*

In brain$_{ECF}$ QP was found to interact with the polyamine metabolism (ornithine, putrescine), the proline metabolism (proline, L-4-hydroxyproline), neurotransmitter precursors (tryptophan, tyrosine), and lysine metabolism (lysine, hydroxylysine) (Table I, Figure 1).

### *Effect of QP on metabolic pathways in plasma*

The systemic response on amino acid metabolism in plasma indicated interactions between QP and the branched chain amino acid (BCAA) metabolism (leucine, isoleucine, valine), neurotransmitter synthesis (phenylalanine), serine-glycine-threonine metabolism (serine, glycine, threonine), and histamine metabolism (histidine, histamine) (Table I, Figure 1). Furthermore, alpha-aminobutyric acid and DL-3-aminoisobutyric acid strongly responded to QP treatment (Table I, Figure 1).

### *Effect of eigth-day QP administration on basal biomarker levels*

Eight-day QP administration did not result in significant changes in basal brain$_{ECF}$ biomarker levels, but showed a significant change in plasma levels of alpha-aminobutyric acid and DL-3-aminoisobutyric acid after 0.17 mg/kg ($p < 0.05$), but not after 0.86 mg/kg QP ($p > 0.05$) (Figure 4). However, including the interaction between treatment and day as a covariate in the PK/PD models for these biomarkers did not result in a significant improvement of the model ($p > 0.05$), potentially related to the lack of a dose-response relation.

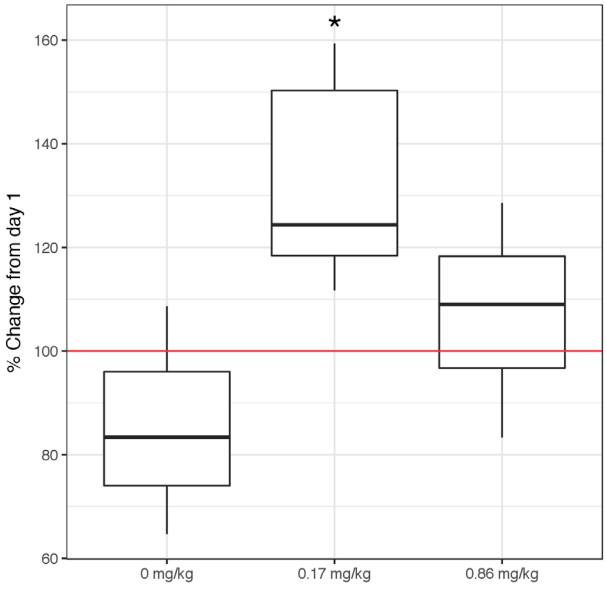



**Figure 4. Relative change of L-Alpha-aminobutyric acid levels in plasma after 8-day administration as compared to a single administration. * denotes a significant effect with $p < 0.05$.**

## Discussion

In this study we aimed for combining metabolomics in $brain_{ECF}$ and plasma with multivariate PK/PD modeling to obtain insight into the systems response, as well as to explore the target site of effect upon CNS drug administration. By integrating time-resolved metabolomics analysis with multivariate PK/PD, we revealed the diverse dynamical responses of biogenic amines and amino acids in $brain_{ECF}$ and plasma upon administration of the $D_{2/3}$ agonist QP. Indeed, the quantitative characterization of the system-wide biomarker responses showed a variety of *in vivo* potency and maximal response values in both $brain_{ECF}$ and plasma. Additionally, the unique evaluation of time-resolved metabolomics in both $brain_{ECF}$ and plasma revealed a few potential blood-based biomarkers reflecting effects in $brain_{ECF}$. Interestingly, it was also observed that many biochemical responses of QP have their main origin in the periphery rather than in the $brain_{ECF}$. Finally, our study showed no response of eight-day administration on biogenic amine and amino acid levels.

### *Exploration of target site and identification of blood-based biomarkers*

It is a great challenge to identify blood-based biomarkers that reflect neurochemical responses in the brain. Often, these measurements are done at one time-point. In such case, however, correlations between plasma and $brain_{ECF}$ responses cannot reveal the causal relation. In the current study we were able to use the temporal delay between the $brain_{ECF}$ and plasma biomarker responses to identify the potential causal relation between them (i.e. the slowest response is likely a consequence of the quickest response via transport over the blood-brain barrier (BBB)). The BBB has multiple transport systems that transport biogenic amines and amino acids, for example, the large neutral amino acid transporter 1 (LAT1; for transport of e.g. glutamine, tyrosine, tryptophan), the cationic amino acid transporter 1 (CAT1; for transport of arginine and lysine), or the serotonin transporter (SERT; for transport of serotonin) (22,23). These transport systems exist at both the luminal and abluminal site of the BBB, whereby biogenic amines and amino acids can be transported from plasma to brain and vice versa. It is therefore likely that the parallel responses in plasma are, at least partially, explained by BBB transport. Interestingly, the number of biogenic amines transported from $brain_{ECF}$ to plasma was lower than those transported from plasma to brain (Table I). This observation it suggests first of all that, even if a drug does not cause a direct response in the brain (e.g. because of no exposure), biochemical responses may propagate from plasma to $brain_{ECF}$ and cause secondary responses. Second, the observed assymetry underlines the difficulty of finding blood-based markers reflective of drug responses in $brain_{ECF}$. 6 Potential blood-based biomarkers nevertheless reflected a response in $brain_{ECF}$ (Table I, Figure 1). Importantly, 5 of them showed non-linear transport over the BBB. It is advisable to take this non-linearity into account when evaluating blood-based biomarkers as a surrogate for an effect in $brain_{ECF}$. The blood-based biomarker response may be restricted by the maximal transport rate over the BBB and hence it

may affect the estimation of the maximal effect ($E_{MAX}$) parameter. Therefore, in order to understand the dynamics of the blood-based biomarker response in a clinical context, it is recommended to determine the relation between the plasma and $brain_{ECF}$ biomarker response preclinically similar to the current study.

### *The effects of eight-day QP administration*

Interestingly, while there was a significant response upon eight-day administration of QP in PK/PD parameters describing the neuroendocrine response (18), no significant impact on basal biomarker levels was identified in the current study, although dopamine, DOPAC and HVA were only analyzed for experiment day 1. A possible explanation could be that the biological systems that underlie the amino acid and biogenic amine responses have greater flexibility than the neuroendocrine system in adapting to perturbations such as QP administration.

### *The effects of QP on multiple pathways*

QP appeared to have an overall inhibiting response on multiple biogenic amine pathways. First of all, the dopamine metabolism in the $brain_{ECF}$ was inhibited, which could be explained by the response of QP on the $D_2$ autoreceptors located on the presynaptic neuron (24). Moreover, QP reduced peripheral phenylalanine concentrations, thereby lowering the brain levels of phenylalanine and tyrosine that constitute the basis of the dopamine metabolism. Second, although QP did not significantly affect cerebral glutamate levels, glutamate signaling may be inhibited by QP, given that glycine, serine, proline and putrescine levels in $brain_{ECF}$ were decreased, all acting as co-activator of the NMDA receptor (25,26).

Furthermore the reduction of the BCAA levels and the increase of DL-3-aminoisobutyric acid in plasma may both be associated with increased activity of the animals. BCAA levels were found negatively correlated with activity (27), while DL-3-aminoisobutyric acid was observed positively associated with activity (28). Indeed, QP does induce locomotion as measure of increased activity and movement (29), and the modified levels of BCAA and DL-3-aminoisobutyric acid in our study may be a reflection of that.

Finally, the reduction of histidine and histamine in plasma may reflect an inhibitory effect of QP on the immune system. Histamine is directly released from dendritic cells, macrophages and neutrophils upon production from histidine by the enzyme histidine decarboxylase (30). Interestingly, dopamine receptors are expressed in various immune cells such as dendritic cells, neutrophils and natural killer cells (31), indicating a potential mechanism through which QP may have influenced the histamine metabolism.

### *Some limitations of the current study*

Of course we are aware of some limitations of this study. First of all, while the results in our study strongly indicate a systems wide response for the $D_{2/3}$ receptor agonist QP, it should be confirmed by using other $D_2$ agonists whether the observed responses are related to dopaminergic activity, and to which receptor subtype they are related. Such analysis would give insights into drug-class specific system-wide responses. For example, a multivariate analysis of several antipsychotic $D_2$ receptor agonists showed large neurochemical and behavioral overlap of clozapine with 5-$HT_{2a}$ antagonists, but not haloperidol (32). Ultimately, the multivariate PK/PD approach may link *in vitro* and *in vivo* characterizations of drug-class related pharmacology by connecting the pattern of *in vivo* potencies to *in vitro* affinities.

Second, although the analytical platforms that have been used in the current study are well-developed with proven robustness (19,21), glycine measured by the targeted platform was described by cluster 3 dynamics, while the glycine response as analyzed by untargeted analysis was closer to the cluster 2 pattern (Figure 1). Inter-laboratory reproducibility is currently a topic of investigation, although early research suggests good robustness of metabolomics platforms towards this type of variation (33). An explanation could be non-linearity of the apparatus response given the fact that the untargeted analysis provided response ratios (analyte peak area/internal standard peak area), whereas the targeted analysis presented concentrations.

Third, although not only biogenic amines and amino acids are expected to respond to QP, we were limited by sample volume of the microdialysates. It would be valuable to extend the current approach with multiple platforms integrated to obtain a comprehensive insight into the system-wide effects of CNS drugs. Fortunately, the microdialysis-metabolomics technology is rapidly evolving, requiring lower sample volumes for metabolomics analysis (34,35). Furthermore, to counteract the high attrition rates in CNS drug development, it will be important to accurately monitor the pharmacology in early clinical drug development (3). Such monitoring needs accessible biomarkers that can be obtained from the blood, for example. The combined microdialysis-metabolomics technology is envisioned valuable and relatively low-cost to develop specific biomarker panels for CNS drugs (or drug classes).

Finally, all $brain_{ECF}$ measurements were made in the striatum. To gain insight into the higher hierarchy of the brain, the brain circuitry, it is essential to do measurements in multiple brain regions that are relevant to the drugs' mechanism of action. Indeed, CNS diseases and treatment responses are determined by the balance among signaling of multiple neurotransmitters in multiple regions (36–38). Addition of multiple brain regions

to a multivariate PK/PD model is therefore envisioned to further elucidate the systems pharmacodynamics of CNS drugs.

## Conclusion

CNS drug development is challenged by low success rates and high development costs. Biomarker-driven drug development is seen as a logical step to improve these success rates, and metabolomics holds great promise in this regard. It provides a relatively low-cost method to comprehensively screen for drug response biomarkers. In this study we showed for the first time how time-resolved metabolomics analysis in combination with multivariate PK/PD describes the diverse dynamical patterns in $brain_{ECF}$ and plasma in a pharmacologically meaningful manner to evaluate systems-wide CNS drug effects. Moreover, our approach also enables to explore the target site of effect, as well as to identify blood-based biomarkers that are reflective of drug responses in $brain_{ECF}$. Further application and development of this method is envisioned to provide an important connection between drug discovery and early drug development.

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# Supplement 1 – Equations

*Inter-individual and residual variability*

$$\vartheta_i = \vartheta_{pop} * e^{\eta i} \quad \text{(Eq. 1)}$$

$$Log(C_{obs\,,\,i\,,\,j}) = Log(C_{pred}\,,\,i\,,\,j) + \varepsilon_{i\,,\,j} \quad \text{(Eq. 2)}$$

$\boldsymbol{\vartheta_i}$ is the estimated parameter for individual i; $\boldsymbol{\vartheta_{pop}}$ is the estimated parameter for the population; $\boldsymbol{\eta_i}$ follows a normal distribution with mean 0 and variance $\omega^2$; $\boldsymbol{C_{obs,i,j}}$ is the observed concentration data point for individual i at timepoint j; $\boldsymbol{C_{pred,i,j}}$ is the predicted concentration for data point for individual i at timepoint j; $\boldsymbol{\varepsilon_{i,j}}$ follows a normal distribution with mean 0 and variance $\sigma^2$.

## *Baseline models*

*No pattern*

$$C_{MET\,,\,BSL} = BSL_{MET} \quad \text{(Eq. 3a)}$$

*Linear decay function*

$$C_{MET\,,\,BSL} = BSL_{MET} * (\,1 + s * time) \quad \text{(Eq. 3b)}$$

*Exponential decay function*

$$C_{MET\,,\,BSL} = (\,BSL_{MET} - BSL_{min}\,) * e^{-\,kdec\,*\,time} + BSL_{min} \quad \text{(Eq. 3c)}$$

$\boldsymbol{C_{MET,BSL}}$ is the biomarker concentration given no drug response; $\boldsymbol{BSL_{MET}}$ is the biomarker concentration at baseline at time = 0; $\boldsymbol{s}$ is the slope of the change in baseline with time; $\boldsymbol{BSL_{min}}$ is the minimum level of the basal biomarker levels; $\boldsymbol{k_{dec}}$ is the rate of baseline biomarker decay with time.

## *Drug response models*

*Linear model*

$$E = slope * C_{QP} \quad \text{(Eq. 4a)}$$

*$E_{MAX}$ model*

$$E = \frac{E_{MAX} * C_{QP}}{EC_{50} + C_{QP}} \quad \text{(Eq. 4b)}$$

$\boldsymbol{E}$ is the magnitude of drug response; ***Slope*** is the parameter that determines the strength of the drug response; $\boldsymbol{C_{QP}}$ is the drug concentration at the target site, either plasma or $brain_{ECF}$; $\boldsymbol{E_{MAX}}$ is the maximal response; $\boldsymbol{EC_{50}}$ is the drug concentration at half maximal response.

## *Link models*

*No transit compartment model*

$$Tr = 1 \quad \text{(Eq. 5a)}$$

*Transit compartment model*

$$Tr = e^{ktr * time} * \frac{(k_{tr} * time)^{Ntr}}{e^{-Ntr} * \sqrt{2\pi} * N_{tr}^{Ntr+0.5})} \quad \text{(Eq. 5b)}$$

*Turnover model (effect on biomarker release)*

$$\frac{dC_{MET}}{dt} = k_{OUT} * C_{MET,BSL} * (1 + E * Tr) - k_{OUT} * C_{MET} \quad \text{(Eq. 6)}$$

*Pool model (effect on biomarker release)*

$$\frac{dC_{MET,pool}}{dt} = k_{OUT} * C_{MET,BSL} - k_{REL} * (1 + E * Tr) * C_{MET,pool} \quad \text{(Eq. 7a)}$$

$$\frac{dC_{MET}}{dt} = k_{REL} * (1 + E * Tr) * C_{MET,pool} - k_{OUT} * C_{MET,PL} \quad \text{(Eq. 7b)}$$

*No response*

$$C_{MET} = C_{MET,BSL} \quad \text{(Eq. 8)}$$

***Tr*** describes the time delay of response using a transit compartment model (1 = no delay); $\boldsymbol{k_{tr}}$ is the rate at which the response goes through the transit compartments; $\boldsymbol{N_{tr}}$ is the number of transit compartments; $\boldsymbol{C_{MET}}$ is the biomarker concentration in plasma or brain$_{ECF}$ ; $\boldsymbol{k_{OUT}}$ is the hormone turnover rate; $\boldsymbol{C_{MET,POOL}}$ is the biomarker concentration in the pool; $\boldsymbol{k_{REL}}$ is the biomarker release rate from the pool into plasma or brain$_{ECF}$.

### *Brain transport models*

$$k_{transp} = k_{transp} \quad \text{(Eq. 9a)}$$

$$k_{transp} = \frac{V_{max}}{k_m} + C_{MET,\,target} \quad \text{(Eq. 9b)}$$

$$k_{OUT,\,notTS} = k_{OUT,\,notTS} \quad \text{(Eq. 9c)}$$

$$k_{OUT,\,notTS} = \frac{V_{max}}{k_m} + C_{MET,\,notTS} \quad \text{(Eq. 9d)}$$

$$\frac{dC_{MET,\,notTS}}{dt} = k_{transp} * C_{MET,\,target} - k_{OUT,\,notTS} * C_{MET,\,notTS} \quad \text{(Eq. 9e)}$$

$\boldsymbol{k_{transp}}$ is the transport rate over the blood-brain-barrier from the target site to the other compartment; $\boldsymbol{V_{max}}$ the maximal rate with $\boldsymbol{k_m}$ being the concentration at 50% of the maximal rate; $\boldsymbol{k_{OUT,notTS}}$ is the elimination rate from the compartment that is not the target site compartment.

### *Significance score calculation*

$$Significance\ score = OFV_{ref} - OFV_{test} - inv.\chi^2\left(1 - \frac{\alpha}{n_{biomarker}},\, df\right) \quad \text{(Eq. 10)}$$

$\boldsymbol{OFV_{ref}}$ is a model with no drug effect included and $\boldsymbol{OFV_{test}}$ is a model with the drug effect included. The $\boldsymbol{inv.\chi^2}$ calculates a penalty for additional parameters (***df***) in the drug effect model on basis of the significance threshold ($\boldsymbol{\alpha}$) divided by the total number of biomarkers ($\boldsymbol{n_{biomarker}}$), i.e. bonferroni-correction.

# Supplement 2 – Elbow plots

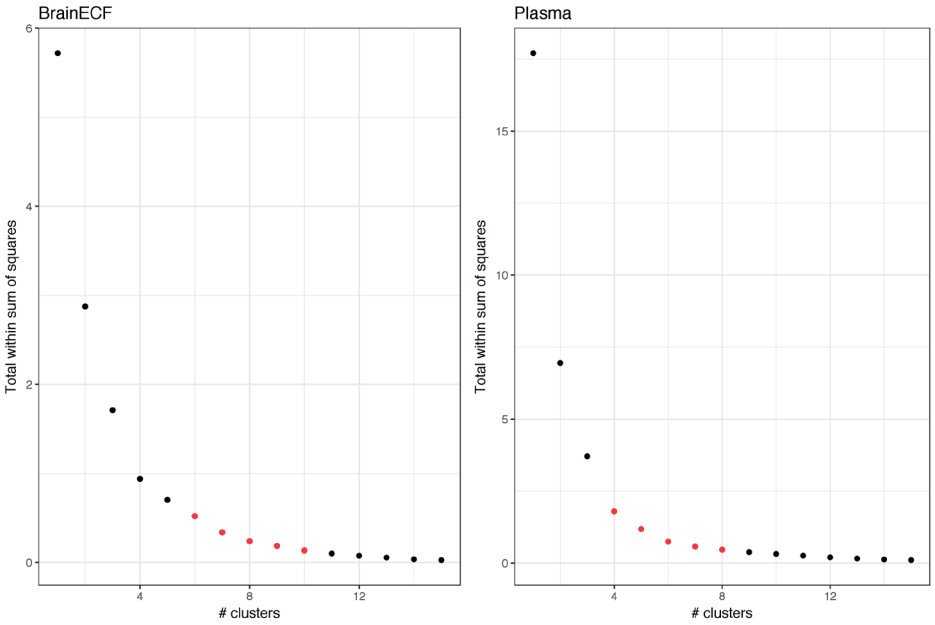



**Figure S1. Elbow plots for the clustering of brain$_{ECF}$ (left) or plasma (right) responses. The elbow plot shows the balance between the number of clusters and the total variation that is explained by the clusters. The 'elbow' in this figure marks the point where adding another cluster does not further decrease the total unexplained variation, and is used to define the optimal number of clusters. While this is not always very clear from an elbow plot, a series of cluster numbers were selected, marked by the red dots, to subsequently be evaluated in a PK/PD cluster model.**

**Supplement 3 – Goodness-of-fit single biomarkers**

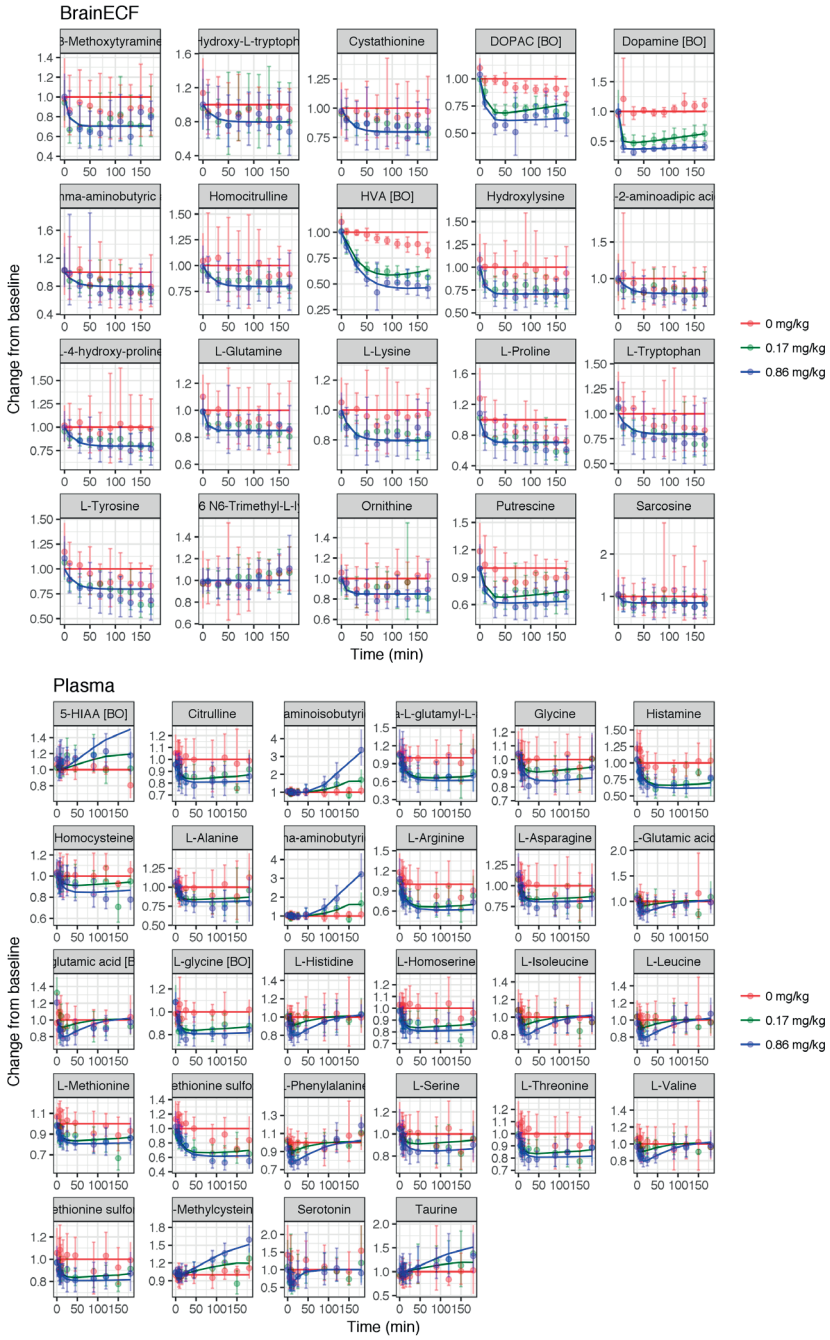



**Figure S2. Goodness-of-fit of the cluster models on the baseline corrected single metabolite levels in brain$_{ECF}$ (top) and plasma (bottom). Dots are the geometric means per time point and dose, while the errorbars mark the geometric standard deviation. The lines represent the model-based predictions for 0 mg/kg (red), 0.17 mg/kg (green) and 0.86 mg/kg (blue).**

# CHAPTER 8

## GENERAL DISCUSSION AND CONCLUSION

Diseases of the Central Nervous System (CNS) decrease the quality of life of millions of people worldwide (1–3). A lot of time, effort and resources are therefore put into the development of CNS drugs, while the success rates are low. For example, in the period from 2003 – 2011, almost 400 CNS drugs entered phase I clinical development, while less than 10% of them received market approval (4). The main reasons for these low success rates are the lack of understanding of the complexity of the brain, the presence of the blood-brain-barrier limiting drug penetration, CNS mediated side effects, and the lack of good biomarkers that represent the interaction of the drug with neurophysiological systems (5–7).

Systems pharmacology aims to integrate multiple biological systems for the evaluation pharmacological effects to improve the understanding and the prediction of drug effects (8–12). While several examples show the merits of systems pharmacology (13,14), they are driven by a priori insights into detailed pharmacological knowledge. This is not always available during early drug development. As an alternative, the pharmacometabolomics approach in combination with multivariate statistical methods provides an unbiased and data-driven way to evaluate the system-wide drug effects at the level of biochemical pathways (15–17). However, in order to understand and extrapolate the typically non-linear drug effects, one needs to quantify the relation between drug dose and response using pharmacokinetic/pharmacodynamic (PK/PD) modeling (18–20). In other words, it will be important to divert from a fully empirical approach towards a mechanistic approach, without loosing the unbiased and data-driven properties of pharmacometabolomics. Additionally, given the limited access to the brain in terms of sampling, it is important to discover blood-based biomarkers that represent drug effects in the brain. In this thesis, we therefore asked two questions:

1. How can we quantify the relation between drug dose and the dynamic systems response *in vivo*?
2. How can we obtain blood-based markers that represent central drug effect?

### *Section I – General introduction*

As an initial step, in **Chapter 2**, these questions were placed in the context of translational pharmacology of CNS drugs with particular focus on interspecies scaling. On one hand, metabolomics enables comprehensive evaluation of interspecies differences at the biochemical level (21–23). On the other hand, mechanistic PK/PD modeling in combination with allometric and physiology-based scaling is used to extrapolate drug responses from one species to another (18,19). Moreover, mechanistic PK/PD modeling can potentially be applied to describe the relation between neurological drug effects and blood-based biomarkers, as will be discussed below. PK/PD-metabolomics modeling was proposed as an integration of PK/PD and pharmacometabolomics with the potential to increase understanding and

extrapolative ability during translational drug development (Figure 1). In **Chapter 3**, a systematic search was performed in PubMed to investigate the pathways involved in dopaminergic drug effects, as well as the availability of blood-based biomarkers related to these pathways. A multitude of pathways appeared to be associated with dopaminergic drug effects. This included the neurotransmitter, the nitric oxide and the kynurenine pathway in the brain, as well as neuroendocrine and energy pathway responses in the periphery. Although this may partially be attributed to the lack of selectivity of dopaminergic drugs (24,25), also selective drugs appeared to perturb multiple pathways (26–28). Additionally, we found no studies describing the relation between drug effects in the brain and blood-based biomarkers, except for prolactin. Moreover, pharmacological effects were typically evaluated in a static manner, with no quantification of the dynamics underlying the dose response relation. On basis of these two chapters, we identified three goals an integrative PK/PD-metabolomics method needs to fulfill in order to answer our questions:

- Longitudinal measurement of a systems biomarker response with multiple dose levels included
- Simultaneous evaluation of drug concentrations and drug response biomarkers in plasma and the brain
- Integration of PK/PD principles into pharmacometabolomics data analysis

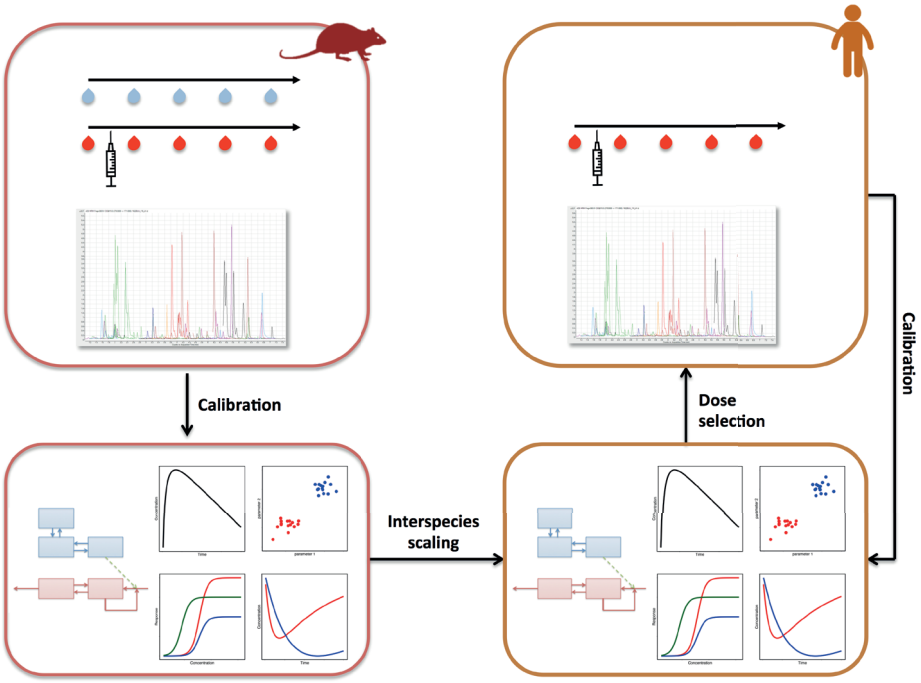



**Figure 1. Proposed workflow of PK/PD metabolomics in interspecies scaling Modified from (56) with permission of Taylor and Francis.**

### *Section II – The dynamical neuroendocrine systems response to study dopamine $D_2$ drug effects*

One way to discover blood-based biomarkers is to evaluate the neuroendocrine systems response to CNS drug administration (Figure 2). The neuroendocrine system is a connection between the neural system and the endocrine system, composed of the hypothalamus, the pituitary and the distant endocrine organs. Neural projections from the hypothalamus to the pituitary, for example the tuberoinfundibular dopamine (TIDA) neurons, are controlled by neurochemicals, such as dopamine, serotonin or acetylcholine. These neurons then release signal (e.g. dopamine) into the pituitary to regulate the release of hormones (e.g. prolactin) from the anterior pituitary into the circulation (29). Additional to this mechanism, hypothalamic neurons can also release neuropeptides (e.g. oxytocin) directly into the circulation from their end-feet located in the posterior pituitary. As one of the blood-based biomarker strategies depicted in Figure 2, this principle has been used to evaluate dopaminergic drug effects with prolactin as blood-based biomarker (30–33).

Although widely applied with proven applicability in biomarker-driven drug development, a single-biomarker approach has limitations. As we have seen, dopaminergic drugs exhibit multiple effects on the neuroendocrine system. To anticipate a broader *in vivo* pharmacological profile, we set out for a multi-biomarker approach that can reflect the dynamic endocrine systems response to dopamine drug administration. In **Chapter 4** we investigated the feasibility of a dynamical neuroendocrine systems response upon administration of the dopamine $D_2$ antagonist remoxipride. Interestingly, only adenocorticotropic hormone (ACTH) and prolactin showed a response, while brain-derived neurotropic factor (BDNF), follicle-stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH), thyroid stimulating hormone (TSH) and oxytocin remained unaffected by remoxipride (Table I). The number of neuroendocrine biomarkers responding to the dopamine $D_2$ agonist quinpirole was also low as we showed in **Chapter 5**, although now also GH and TSH responded in addition to ACTH and PRL (Table I). Considering that the dopaminergic system is biologically connected to more pituitary hormones and neuropeptides than those identified in our studies, for example FSH and LH (34), it is likely that the underlying biological networks are resilient to dopaminergic perturbation by remoxipride and quinpirole. Also, the absence of remoxipride effect on GH and TSH release suggests no basal $D_2$ receptor activation by endogenous dopamine. Considering the higher affinity of quinpirole to the $D_2$ receptor relative to endogenous dopamine, it is indicated that only high levels of $D_2$ receptor activation influence the release. Further studies with multiple dopamine agonists and antagonists are required to validate this series of neuroendocrine markers that can evaluate pharmacological perturbation of the dopaminergic system.

**Table I. Overview of neuroendocrine responses after remoxipride and quinpirole administration**

| | Remoxipride | Quinpirole |
|---|---|---|
| **Alpha MSH** | NA | 0 |
| **Beta Endorphin** | NA | 0 |
| **Neurotensin** | NA | 0 |
| **Orexin A** | NA | 0 |
| **Oxytocin** | 0 | 0 |
| **Substance P** | NA | 0 |
| **ACTH** | + | + |
| **BDNF** | 0 | 0 |
| **FSH** | 0 | 0 |
| **GH** | 0 | - |
| **LH** | 0 | 0 |
| **PRL** | + | - |
| **TSH** | 0 | - |

*NA: not measured*

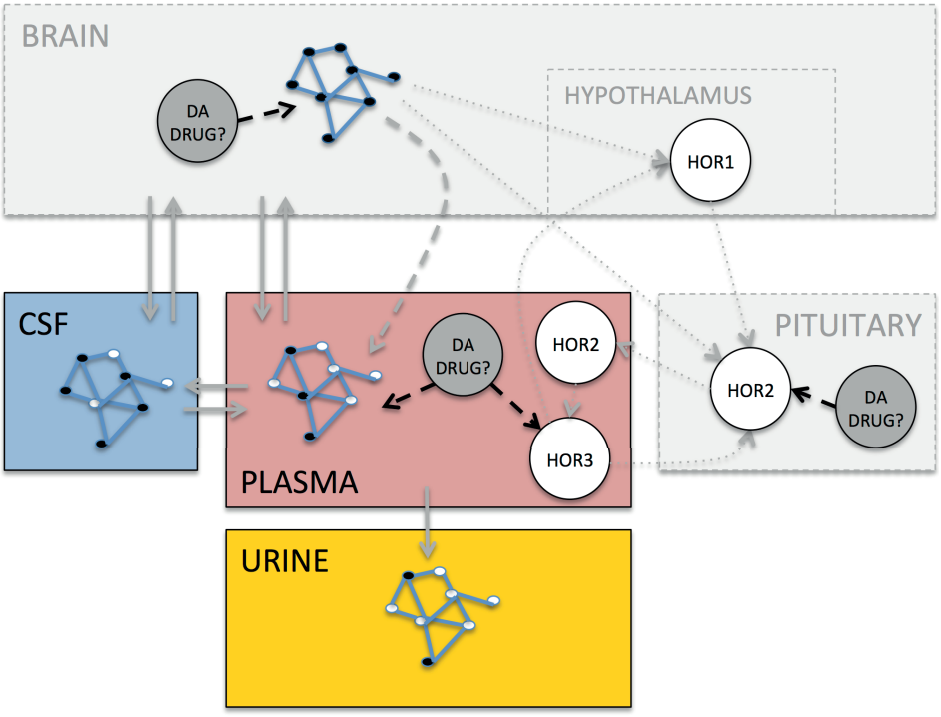



**Figure 2. Different physiological mechanisms through which blood-based biomarkers may be related to pharmacological effects in the brain. Modified from (57) with permission of Springer.**

An important question is how the hormone-specific potencies are to be interpreted. Two factors influence the potency: receptor affinity and signal transduction efficiency (35). For remoxipride, prolactin was assumed to be the 'gold standard' as biomarker for $D_2$ receptor activation. While ACTH was found controlled by dopamine in a $D_2$ specific manner, remoxipride would inhibit its release following this mechanism (36). Interestingly, an increase of ACTH release was observed in **Chapter 4**. Therefore, ACTH likely represents an off-target effect. Concretely, it was concluded that the effect was possibly mediated via the adrenergic receptor, given the ratio of potencies $EC_{50,ACTH}/EC_{50,PRL}$ being similar to the ratio of receptor affinities $k_{i,\alpha 2}/k_{i,D2}$ (37) (**Chapter 4**). While this is an example of the affinity driving the differences in potency estimates, **Chapter 5** shows how signal transduction efficiency determined the differences in potency estimates with quinpirole. Indeed, we could well describe the ACTH, GH, prolactin and TSH responses assuming equal affinity ($k_A$), but different maximal effect ($E_M$) and signal transduction efficiency ($\tau$) (Equation 1, Figure 3, **Chapter 5**). Interestingly, $\tau$ could be assumed being related to $D_2$ receptor expression on the hormone releasing 'troph' cells in the pituitary (Equation 2), showing that the signal transduction efficiency can be driven by the characteristics of a specific biological subsystem.

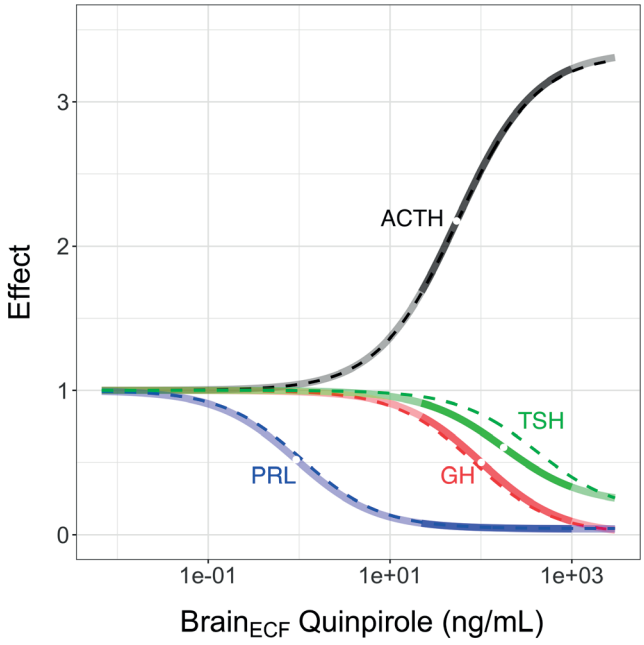



**Figure 3. Simulated concentration-effect relation profiles between quinpirole and ACTH, PRL, GH and TSH (solid lines) fitted by equation 1 and 2 (dashed lines)**

$$E = \frac{E_m * \tau * C}{k_A + (1 + \tau) * C} \quad (1)$$

$$\tau = \tau_0 * e^{slp * receptor\ expression} \quad (2)$$

Additional to single-administration biomarker responses, **Chapter 5** also presented the effects of longer-term quinpirole administration. This is potentially important for drugs with intended chronic use, amongst which antiparkinson $D_2$ agonists. Indeed, the effect of $D_2$ agonists may be subject to sensitization or tolerance as was shown in rats (38). The basal levels of ACTH and prolactin were changed after 8-day administration relative to a single administration. Moreover, not only the basal levels were changed, but also the potency of quinpirole affecting the TSH release. Thus, homeostatic feedback mechanisms may cause changes in basal biomarker levels, as well as the responsiveness of biomarkers to the drug. Interestingly, the biogenic amine and amino acid responses were not changed with longer-term quinpirole administration (**Chapter 7**). This suggests that the systems related to biogenic amines and amino acids are more resilient than the neuroendocrine system to longer-term quinpirole treatment.

As a final remark on using the neuroendocrine system to identify blood-based biomarkers of pharmacological action in the brain, we would like to discuss the topic of target site of drug action. The pituitary is not protected by the blood-brain-barrier, and hence, it is exposed to plasma drug concentrations. While we could statistically identify brain extracellular fluid ($brain_{ECF}$) as target site for the effect of remoxipride on prolactin, also confirmed by others (31), this was not possible for ACTH. Neither could we draw such conclusion for the effect of quinpirole on ACTH, GH, prolactin and TSH. In case of quinpirole, we assumed that with 5 times higher concentrations around the $D_2$ receptor in the brain as in plasma (i.e. $Kp,uu = 5$), the brain influence would be dominant. However, we acknowledge that the hormone release likely is influenced both at the level of the hypothalamus and the pituitary. The current lack of simultaneous drug and dopamine concentration data in both tissues hampers the development of a model describing a two-level influence of the drug. However, the collection of this type of data appears possible; others have performed microdialysis sampling in the hypothalamus (39) and the pituitary (40), enabling the quantitation of dopamine and drug levels in both tissues.

Altogether, these chapters included longitudinal measurement of a neuroendocrine systems biomarker, after multiple doses of a $D_2$ antagonist and a $D_2$ agonist. By applying a PK/PD evaluation to these data, we gained quantitative insights into the neuroendocrine response to dopaminergic drugs and relate those to drug-specific and system-specific pharmacological characteristics.

### *Section III – The dynamical biochemical systems response to study dopamine $D_2$ drug effects*

In section III the multi-biomarker approach was extended from a neuroendocrine platform with up to 15 hormones and neuropeptides to a metabolomics platform containing 76

amino acids and biogenic amines (41). Several statistical methodologies had been developed dealing with time-resolved high-dimensional (e.g. metabolomics) data. Clustering, for example, is a useful method to identify the main longitudinal patterns in a multivariate dataset (42,43). However, in our experiments we intended not only to include the dimension of time, but also the dimension of dose and sampling site (i.e. plasma and $brain_{ECF}$), in a parallel study design. A multivariate method that can include multiple dimensions, such as time, dose and sampling site, ANOVA-simultaneous component analysis (ASCA) was developed (44). It has been applied to study the effects of dose and time on a metabolomics response in osteoporosis arthritis guinea pigs receiving different dose levels of vitamin C. While this method filled the gap of taking into account underlying study design factors in multivariate statistics, it did not apply very well to our data. In contrast to the guinea pig data, our sampling times are very close and unevenly spaced. Therefore, the successive data points are correlated in a non-linear manner. Treating these time points as factor would limit the identification of the underlying dynamics. More importantly, none of the existing methods dealing with multivariate dynamical patterns integrates pharmacological principles into the data analysis. Therefore, we set out to integrate PK/PD principles into multivariate data analysis. In **Chapter 6**, we measured time-resolved biogenic amine and amino acid patterns upon administration of several remoxipride dose levels. Then, using a three-step approach of i) fitting a turnover model to each single biomarker; ii) clustering the metabolites on basis of the pharmacological parameter estimates; and iii) fitting a turnover model to each cluster of biomarkers, we identified 6 different PK/PD patterns in the data. The *in vivo* potency values related to the clusters were estimated to be 0.0027, 0.019 or 0.12 µM, indicating multiple pathways involved in remoxipride pharmacology. Although we cannot indicate whether these differences were related to off-target effects or differences in signal transduction efficiency, either way this PK/PD-metabolomics model provided a way to define a therapeutic range on basis of a systems response. Furthermore, from the 44 analytes that could be robustly analyzed, 18 were identified as potential biomarker for further validation. While this was a step forward shifting the fully empirical multivariate statistical methods towards a mechanistic modeling approach, the PK/PD-metabolomics method lacked one important feature. No information was included on the biomarker responses in the brain. Unfortunately, at the time, the measurement of biogenic amine and amino acid response in $brain_{ECF}$ by means of intracerebral microdialysis appeared not robust enough. In **Chapter 7**, however, after optimization of the microdialysis method for biogenic amine and amino acid analysis, simultaneous biomarker measurements in $brain_{ECF}$ and plasma were included. Using model comparison metrics, the target site of action related to the individual biomarkers was identified. The clustering step, now both on the $brain_{ECF}$ and the plasma response, was different from that applied in **Chapter 6**. While the remoxipride responses could all be described by turnover models, a larger variety of models, including pool models, turnover models and transit compartment mod-

els, was needed for the quinpirole responses. Therefore, parameter-based clustering not being possible, the clustering was based on simulated biomarker patterns. This clustering approach was proven successful by a good fit of the cluster patterns, as well as the single metabolite patterns by the cluster-based PK/PD model. From this chapter, there are three important conclusions to draw. First of all, even considering that the plasma quinpirole concentrations are 5 times lower than those in $brain_{ECF}$, there are multiple effects observed with plasma as target site. Second, while these biomarker responses originate in other tissues than the brain, most of them are propagated to the brain via transport over the BBB by various transporters. Thus even a drug that does not penetrate the brain, might cause secondary responses in the brain (Figure 4). Third, although multiple biomarker responses were observed in $brain_{ECF}$, only a few of them were transported over the BBB into plasma as a potential blood-based biomarker (Figure 4).

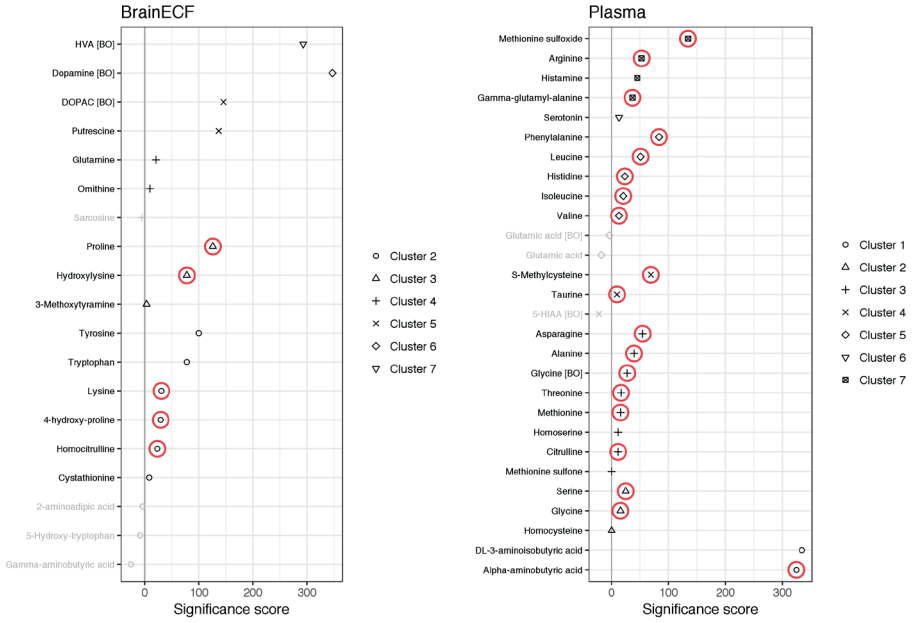



**Figure 4. Potential biomarkers of quinpirole effect in $brain_{ECF}$ (left) or plasma (right), positioned right from the vertical grey line. Red circles indicate the biomarkers that distribute over the blood-brain-barrier.**

Counter-intuitively, many of the amino acids and biogenic amines that decreased upon remoxipride treatment, were also inhibited by quinpirole. Since in both studies a control group receiving saline was included, the responses must be attributed to drug action. A possible explanation could be that the responses of either remoxipride or quinpirole are caused via another target than the $D_2$ receptor. Quinpirole has high affinity for the $D_2$ and the $D_3$ receptor. There is, however, no reason to believe that D3 receptor opposes D2

receptor, such that D3 receptor agonism leads to similar actions as D2 receptor antagonism. Alternatively, remoxipride possibly interacts with the adrenergic receptor at high concentrations (**Chapter 3**); however, in **Chapter 6** the highest dose was reduced by 50%. Interestingly, remoxipride has higher affinity as an antagonist for the sigma receptor than for the $D_2$ receptor (37,45,46). Sigma receptor ligands have been investigated in clinical trials for several indications, including diarrhea (agonists) and schizophrenia (antagonists) (47,48). Activation of the sigma receptor reduced the motility of the ileum (49). Antagonism of the sigma receptor, possibly leading to increased motility of the ileum, may lead to shorter intestinal transit time and the concordant reduction of amino acid uptake. Furthermore, the sigma receptor inhibits NMDA receptor sensitization by phosphorylation of the NR1 subunit (50). As an antagonist, remoxipride may thus have disinhibited NMDA receptor sensitization, with the reduction of the NMDA co-activators glycine and serine as a consequence of negative feedback. The reduction of biogenic amine and amino acid levels caused by remoxipride may thus be mediated via the sigma receptor. Although a definitive answer to this matter remains elusive, it underlines the need for extension of our work with other dopamine ligands to identify the responses that are specifically related to $D_2$ receptor interaction.

Altogether, in **Chapter 6** and **Chapter 7**, we have developed a methodology that accounts for the pharmacological principles underlying the relation between the drug dose and the systems biomarker response. Concretely, we could identify unique *in vivo* concentration-effect relations, target site of drug action, and potential blood-based biomarkers representing the systems response.

We have shown how the PK/PD-metabolomics method, in combination with serial blood and $brain_{ECF}$ sampling and multiple dose levels included, enables the identification of multiple concentration-effect relations and the concordant target site of drug action, and potential blood-based biomarkers that represent these pharmacological properties. Furthermore, as a step towards further mechanistic insight, in a targeted analysis on the neuroendocrine system, we could reveal the relation between drug response, signal transduction efficiency and $D_2$ receptor expression on the pituitary hormone secreting cells. This positions the PK/PD-metabolomics method in between the unbiased, yet empirical multivariate statistical methods and the mechanism-based quantitative systems pharmacology (QSP) approaches (Figure 5).

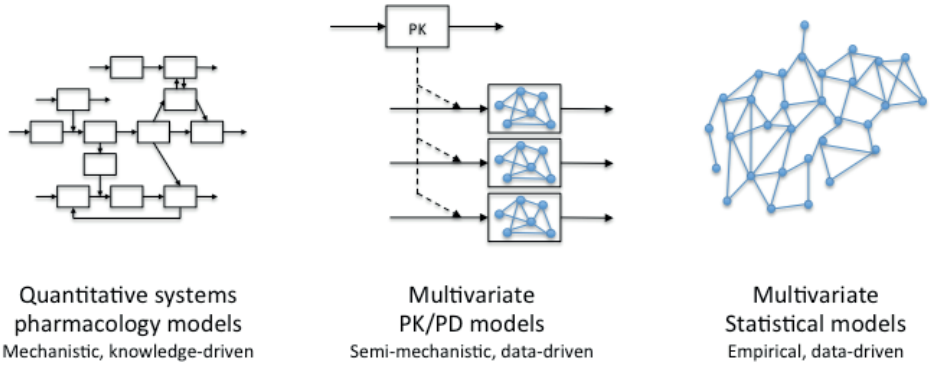



**Figure 5. Positioning of PK/PD-metabolomics models between quantitative systems pharmacology (QSP) models and multivariate statistical models**

### *Perspectives of the PK/PD-metabolomics method in CNS drug development*

CNS drug development is hampered by poor understanding of pharmacological mechanisms underlying the drug effects on one hand, and lack of (blood-based) biomarkers representing these mechanisms on the other hand. Given that insight into pharmacological mechanisms are strongly associated with the success in clinical drug development (20), there is a need for methodologies that enable early investigation of these mechanisms. The PK/PD-metabolomics method has the potential to increase early insights into pharmacological mechanisms in an unbiased and integrated manner. Metabolomics analysis can easily be added to the standard battery of analysis performed in (pre-)clinical studies. Currently, the metabolomics analysis of one sample costs between the 25 and 400 euros, depending on the sensitivity and the number of analytes. A hypothetical study with 50 subjects and 20 samples per subject will thus cost between 25,000 and 400,000 euros. In the context of the costs of late attrition, this is negligible (4).

PK/PD-metabolomics could have an advantage from early drug discovery to late drug development. During early drug discovery, drugs are typically selected on basis of their affinity to the target of interest. However, it is argued that the efficacy of many CNS drugs is related to multi-target affinity, rather than the selectivity for a single-target (24). Multivariate analysis of *in vitro* receptor affinity profiles of anti-Parkinson drugs revealed subclusters of the $D_2$ agonists, and it was suggested that other receptors were also involved in the efficacy of these drugs. PK/PD-metabolomics could provide the basis of the correlation between these *in vitro* receptor affinity profiles and the *in vivo* potency profiles to further investigate the relation between the *in vitro* binding fingerprint and the *in vivo* systems effects.

During early drug development, PK/PD-metabolomics will be of value for the discovery of biomarkers, as well as their characterization in terms of pharmacological parameters. Moreover, we have shown how blood-based biomarkers can be discovered with metabolomics analysis performed both in $brain_{ECF}$ and plasma. These biomarkers will be of great value for CNS drug development, given the limitations of human brain sampling. Ultimately, PK/PD-metabolomics could provide the basis of interspecies scaling of a systems-biomarker response as tool in the guidance of the first-in-human dosing regimen. The subsequent validation of the PK/PD-metabolomics model on human metabolomics data could create insights into interspecies differences relevant for drug development.

Finally, in this thesis inter-individual variation was mostly assumed not present given the standardized experimental design. However, inter-individual variation is a key element in precision medicine during clinical development (51). PK/PD-metabolomics is easily extended to describe personal drug responses in order to optimize dosing regimen in an individualized manner. For example, the reduction of TSH by $D_2$ agonists is known to exacerbate the clinical condition of hypothyroidism patients (52). Knowing the quantitative relation between $D_2$ agonist dose and the TSH response will enable personalized dosing guidance preventing an unacceptable reduction of TSH levels.

### *Further development of the PK/PD-metabolomics method*

While we have shown the potential of the PK/PD-metabolomics method, we would like to make a few recommendations for further research.

### *Application of the PK/PD-metabolomics method to other $D_2$ ligands and clinical validation for proof-of-concept*

This thesis has shown the development and feasibility of the PK/PD-metabolomics approach, which now is ready for further validation to generate a proof-of-concept. As we discussed earlier, the comparative results of the $D_2$ agonist quinpirole and the $D_2$ antagonist remoxipride appeared non-intuitive, possibly because non-dopaminergic responses are involved. Therefore, to ensure the discovery of dopamine system specific biomarkers, we recommend applying the PK/PD-metabolomics method to multiple $D_2$ ligands to reveal the overlapping systems biomarker responses. Furthermore, as we argued in **Chapter 2** the PK/PD-metabolomics method has potential to bridge the lack of mechanistic biomarkers that can be used across preclinical and clinical drug development. In **Chapter 7** we showed how a combination of microdialysis and PK/PD-metabolomics enables the identification of blood-based biomarkers. As visualized in Figure 1, an important future step will be the clinical validation of these biomarkers.

*Microdialysis in multiple brain locations to study regional responses in the brain*
In our experiments, microdialysate samples were collected from the caudate putamen to evaluate biomarker responses representing the function of striatal neurons. A higher level of complexity is presented in the form of brain circuitry that would allow for a regional evaluation of CNS pharmacology (7,14). An interesting next step will be to connect those circuits to the underlying biochemical processes. We recommend the simultaneous evaluation of biochemical responses in multiple brain regions, such as the caudate putamen, the prefrontal cortex or the nucleus accumbens, in order to evaluate the biochemistry related to the circuit functionality. Simultaneous microdialysis sampling in multiple brain regions has proven feasible for CNS PK studies (53).

Additionally, cerebrospinal fluid (CSF) sampling should be included in future PK/PD-metabolomics studies. CSF-based biomarkers have the advantage over blood-based biomarkers that they are not blocked by the BBB. Indeed, in our study with quinpirole, we found many $brain_{ECF}$ biomarkers not reflected in plasma. CSF might provide a good alternative.

*Application of PK/PD-metabolomics to measurements from multiple analytical platforms*
The PK/PD-metabolomics methodology was developed on basis of the biogenic amine and amino acid metabolomics platform. The choice of this platform was based on our expectation that many of these biomarkers would respond to dopamine ligands. Indeed, we identified many of them responding to remoxipride and quinpirole. However, many other biomarkers, for example, lipids and acylcarnitines, may also be included in the future to study the effects of CNS drugs on multiple biochemical pathways. Our analyses were limited to biogenic amines and amino acids because of limited microdialysate sample volume. Indeed, with microdialysis, there is a compromise between sample volume, time resolution and recovery of the biomarker into the microdialysate. Fortunately, developments at the microdialysis-metabolomics interface are continuously increasing, also focusing on improving the sensitivity of analytical methodology (54,55).

*Inclusion of sex differences in the study design*
While in our studies only male animals were included for purposes of standardization, there is clear evidence for the impact of sex on biological pathway functionality in disease and drug effect (56,57). Indeed, sex difference was one of the factors that limited the comparison of studies in **Chapter 3** and **Chapter 5**. As an example, the interaction between dopamine or dopamine agonists and the pituitary $D_2$ receptor is influenced by estrogen, that is expressed in much higher levels in females than in males (58,59). Given the importance of sex differences in disease and drug effect, it will be important to include sex as a variable in clinical and preclinical studies.

## General conclusion

We set out to quantify the relation between drug dose and the dynamic systems biomarker response, as well as to discover blood-based biomarkers that represent drug effects in the brain. To that end, we developed the PK/PD-metabolomics method for identification of the main PK/PD patterns in the data. These PK/PD patterns were described in terms of pharmacologically relevant parameters, such as $E_{MAX}$ and $EC_{50}$, enabling inter- and extrapolation of the systems biomarker response. For the neuroendocrine system, with more knowledge available on the physiological processes involved, we could obtain further mechanistic insights, relating signal transduction efficiency to $D_2$ receptor expression in the pituitary. Furthermore, with time-resolved metabolomics data available in both $brain_{ECF}$ and plasma, PK/PD-metabolomics enabled the identification of the target site of drug effect for the different biomarkers, as well as the discovery of blood-based biomarkers of drug effects in the brain. Being positioned between the general multivariate statistical methods and QSP models, PK/PD-metabolomics will be useful to provide quantitative pharmacological insights into the systems response of CNS drugs in a data-driven manner.

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# APPENDICES

# Nederlandse samenvatting

Ziekten van het centraal zenuwstelsel (*Central Nervous System; CNS*) verlagen de kwaliteit van leven van miljoenen mensen over de hele wereld. Er is veel tijd, moeite en energie gestoken in het ontwikkelen van *CNS* medicijnen, terwijl de successen beperkt zijn. Een belangrijke reden hiervoor zijn het beperkte begrip van de complexiteit van de hersenen, de aanwezigheid van de bloed-hersen-barrière die de penetratie van medicijnen in de hersenen tegenhoudt, bijwerkingen in het centraal zenuwstelsel, en de afwezigheid van goede biomarkers die de interactie tussen medicijnen en neurofysiologische systemen representeren.

Systeemfarmacologie heeft als doel om meerdere biologische systemen te integreren bij het evalueren van farmacologische effecten, om zo het begrip en de voorspelbaarheid van medicijneffecten te verbeteren. Veel systeem-farmacologische benaderingen zijn veelbelovend, echter, deze zijn veelal gebaseerd op gedetailleerde farmacologische voorkennis van het betreffende medicijn en biologische systeem. Dit is niet altijd het geval tijdens de vroege fase van medicijn-ontwikkeling. Met de zogenaamde *metabolomics*-benadering kunnen de farmacologische effecten gescreend worden op een data-gedreven manier om de systeem-effecten van medicijnen te evalueren op het niveau van biochemische mechanismen. Echter, om de typisch niet-lineaire relaties tussen medicijn dosering en effect te begrijpen en te extrapoleren, moeten deze relaties gekwantificeerd worden met behulp van farmacokinetisch/farmacodynamisch (PK/PD) modelleren. Het is daarom belangrijk om van een volledige empirische benadering te bewegen naar een mechanistische benadering zonder daarbij de data-gedreven eigenschappen van de *metabolomics*-benadering te verliezen. Bovendien, omdat de mogelijkheden het bemonsteren van het brein beperkt zijn, is het belangrijk om biomarkers te vinden die, aanwezig in het bloed, de medicijneffecten in het brein representeren. Dit proefschrift gaat daarom in op twee vragen:

1. Hoe kunnen we de relatie tussen medicijn dosering en de systeem-respons *in vivo* kwantificeren?
2. Hoe kunnen we biomarkers in het bloed identificeren die effecten in het brein representeren?

## Sectie I – Algemene introductie

Als eerste stap zijn deze vragen in **hoofdstuk 2** in de context geplaats van de vroege fase van *CNS* medicijnontwikkeling, namelijk die van de translationele farmacologie; de stap van dier naar mens. Hierbij ligt een speciale focus op de farmacologische schaling tussen diersoorten. Aan de ene kant maakt *metabolomics* het mogelijk om de biochemische verschillen tussen dieren grondig te onderzoeken. Aan de andere kant geeft mechanistisch PK/PD modelleren, in combinatie met technieken als allometrische en fysiologie-gebaseerde schaling, de mogelijkheid om medicijn-effecten te extrapoleren van de ene naar

de andere soort. Bovendien kan mechanistisch PK/PD modelleren gebruikt worden om de relatie tussen neurologische effecten en biomarkers in het bloed te beschrijven. Het zogenaamde PK/PD-*metabolomics* modelleren is voorgesteld als een integratie tussen PK/PD en *metabolomics* die de potentie heeft om het begrip en het voorspelbaar vermogen gedurende translationeel medicijnonderzoek te verbeteren. In **hoofdstuk 3** hebben we een systematische zoektocht in PubMed uitgevoerd om de biochemische mechanismen te onderzoeken die betrokken zijn bij dopaminerge medicijn-effecten, met daarin meegenomen de beschikbaarheid van biomarkers in het bloed die hieraan gerelateerd zijn. Een veelvoud aan biochemische mechanismen bleek geassocieerd met dopaminerge medicijneffecten, zoals de neurotransmitter, the *nitric oxide*, en de *kynurenine* systemen. Daarnaast bleken de neuro-endocriene en metabole systemen te reageren op toediening van dopaminerge medicijnen. Hoewel dit deels toe te schrijven is aan de beperkte selectiviteit van veel dopaminerge medicijnen, lieten ook de selectieve medicijnen een breed effectenpatroon zien. Met uitzondering van prolactine vonden we in deze literatuurstudie geen biomarkers die de relatie tussen medicijneffecten in het brein en biomarkers in het bloed beschreven. Bovendien liet deze studie zien dat farmacologische effecten over het algemeen statisch worden geëvalueerd (dosis-respons relatie op één tijdspunt), zonder de tijdsdynamiek mee te nemen die van groot belang is voor de dosis-effect relatie.

Op basis van deze twee hoofdstukken hebben we drie eigenschappen bepaald waaraan een PK/PD-*metabolomics* methode-ontwikkeling aan moet voldoen om onze vragen te beantwoorden:

1. Een longitudinale analyse van een systeem-biomarker respons met meerdere doseringsniveaus
2. Simultane analyse van medicijnconcentraties en medicijneffect in het bloed en de hersenen
3. Een integratie van PK/PD principes in multivariate analyse van *metabolomics* data

## Sectie II – De dynamische respons van het neuro-endocriene systeem om dopamine $D_2$ medicijneffecten te bestuderen

Een van de manieren om biomarkers in het bloed te vinden is om de respons van het neuro-endocriene systeem op toediening van *CNS* medicijnen te bestuderen. Het neuro-endocriene systeem verbindt het neurale systeem met het endocriene systeem, en bestaat uit de hypothalamus, de hypofyse en verschillende verder afgelegen endocriene organen. Neurale projecties vanuit de hypothalamus naar de hypofyse, zoals de tuberoinfundibulaire dopamine (TIDA) neuronen, worden aangestuurd door neuro-chemische stoffen zoals dopamine, serotonine en acetylcholine. Deze neuronen geven een signaal aan de hypofyse (door bijvoorbeeld dopamine uit te scheiden) om van daaruit de uitscheiding van hormonen (bijvoorbeeld prolactine) in het bloed te reguleren. Daarnaast

kunnen neuronen die hun cellichaam in de hypothalamus hebben ook neuro-peptiden (zoals oxytocine) uitscheiden vanuit hun eind-voeten die zich in de hypofyse bevinden. Het principe van deze neuro-endocriene mechanismen is al vaker gebruikt om dopaminerge medicijneffecten te bestuderen met behulp van prolactine als biomarker.

Hoewel het vaak toegepast is, met bewezen nut in biomarker-gedreven medicijnontwikkeling, heeft een *single*-biomarker benadering nadelen. Zoals we zagen in **hoofdstuk 3**, veroorzaken dopaminerge medicijnen een veelvoud aan effecten op het neuro-endocriene systeem. Anticiperend op een breder *in vivo* farmacologisch profiel, willen we een multi-biomarker benadering ontwikkelen dat de dynamische respons van het neuro-endocrine systeem op dopaminerge medicijntoediening beschrijft. In **hoofdstuk 4** hebben we de haalbaarheid van deze benadering onderzocht met behulp van de dopamine $D_2$ antagonist remoxipride. Interessant genoeg lieten allen *adrenocorticotropic hormone* (ACTH) en prolactine een respons zien, terwijl *brain-derived neurotropic factor*, follikelstimulerend hormoon, groeihormoon, luteïniserend hormoon, schildklier stimulerend hormoon en oxytocine niet reageerden op remoxipride. Het aantal neuro-endocriene hormonen dat een effect liet zien na toediening van de dopamine $D_2$ agonist quinpirole was ook laag, zoals we laten zien in **hoofdstuk 5**, hoewel nu ook groeihormoon en schildklier stimulerend hormoon een effect lieten zien. Overwegende dat het dopaminerge systeem biologisch verbonden is met meer neuro-hormonen en neuro-peptiden dan het aantal dat wij identificeerden in onze studies, is het waarschijnlijk dat de onderliggende biologische netwerken een bepaalde veerkracht hebben als het gaat om dopaminerge verstoring door remoxipride en quinpirole. Daarnaast suggereert de afwezigheid van het effect van de antagonist remoxipride op groeihormoon en schildklierhormoon dat er geen endogene dopaminerge stimulatie van deze hormonen plaatsheeft. Gegeven de hogere affiniteit van quinpirole voor de $D_2$ receptor, in vergelijking met endogeen dopamine, lijkt het waarschijnlijk dat alleen hoge mate van $D_2$ receptor activatie van invloed is op de uitscheiding van deze twee hormonen. Vervolgstudies met meerdere dopamine agonisten en antagonisten zijn nodig om de gevonden biomarkers te valideren.

Met behulp van PK/PD modelleren zijn de potenties (mate van activiteit van een medicijn) van quinpirole en remoxipride op de verschillende hormonen bepaald. Een belangrijke vraag is hoe deze geïnterpreteerd moeten worden. Er zijn twee factoren die de potentie van medicijnen beïnvloeden: receptor affiniteit (mate van bindingsvermogen aan de receptor) en signaal transductie efficiëntie (mate van doorgeven van een signaal na binding aan de receptor). In het geval van remoxipride laten we zien dat de receptor activiteit bepalend kan zijn voor verschillen in potenties tussen de verschillende biomarkers. Prolactine en ACTH lieten allebei een effect zien met een verschillende potentie voor remoxipride. Prolactine is welbekend als biomarker voor dopamine effecten. De ACTH respons betreft

waarschijnlijk een *off-target* effect, aangezien de verhoging van ACTH concentraties in het bloed niet verklaard kan worden met een dopamine effect – de ACTH concentraties zouden dan verlagen. Mogelijk kan dit effect verklaard worden met een adrenerg mechanisme, aangezien de ratio van de potenties ($EC_{50,ACTH}/EC_{50,PRL}$) vergelijkbaar is aan de ratio van de receptor affiniteiten ($k_{i,\alpha 2}/k_{i,D2}$) (**hoofdstuk 4**). Terwijl dit voorbeeld laat zien dat de receptor affiniteit bepalend kan zijn voor het verschil in potenties, laten we in **hoofdstuk 5** zien hoe signaal transductie de verschillen kan verklaren. Met behulp van vergelijking (1) hebben we de relatie tussen quinpirole concentraties en hormoon effecten beschreven. Hierin namen we aan dat de affiniteit ($k_A$) gelijk was bij ieder hormoon, terwijl het maximale effect ($E_m$) en de signaaltransductie efficiëntie parameter ($\tau$) verschillend waren tussen de hormonen. Onder deze aannames kon deze vergelijking de effecten van quinpirole op de hormoonspiegels goed beschrijven. Een interessante observatie in **hoofdstuk 5** was, bovendien, dat de signaaltransductie efficiëntie beschreven kon worden aan de hand van de dopamine $D_2$ receptor expressie op de hormoon-uitscheidende cellen in de hypofyse (vergelijking (2)). Dit laat zien dat de signaal transductie efficiëntie gedreven kan worden door karakteristieken van een specifiek biologisch sub-systeem.

$$E = \frac{E_m * \tau * C}{k_A + (1 + \tau) * C} \quad (1)$$

$$\tau = \tau_0 * e^{slp * receptor\ expression} \quad (2)$$

Naast het effect van eenmalige toediening van quinpirole op de hormoonspiegels, hebben we in **hoofdstuk 5** ook de effecten onderzocht van langdurige toediening. Dit kan belangrijk zijn voor medicijnen die bedoeld zijn om langdurig gebruikt te worden, zoals anti-parkinson $D_2$ agonisten. Inderdaad kan het effect van $D_2$ agonisten over tijd veranderen als gevolg van sensitisatie en tolerantie mechanismen, zoals eerder aangetoond in ratten. In onze studies zagen we de basale bloedspiegels van ACTH en prolactine veranderen na acht dagen van toediening. Bovendien veranderden niet alleen de basale spiegels, maar ook de potentie van quinpirole om schildklier stimulerend hormoon te beïnvloeden. Biologische terugkoppelingsmechanismen kunnen dus, als gevolg van langdurige toediening van quinpirole, zowel de basale biomarker spiegels als de farmacologische gevoeligheid van biomarkers veranderen. In tegenstelling tot de neuro-endocriene biomarkers, lieten de *biogenic amines* en aminozuren geen specifieke verandering zien als gevolg van langdurige toediening, zoals we in **hoofdstuk 7** laten zien. Dit suggereert dat de systemen onderliggend aan deze biomarkers veerkrachtiger zijn dan het neuro-endocriene systeem als het gaat om langdurige blootstelling aan quinpirole.

In de hoofdstukken 4 en 5 hebben we aangenomen dat de neuro-hormonen aangestuurd werden vanuit het brein. Aangezien de hypofyse niet beschermd wordt door de bloed-hersen-barrière, is deze ook blootgesteld aan medicijnconcentraties in het bloed. Hoewel

we in **hoofdstuk 4** op basis van model selectie criteria konden aantonen dat de prolactine respons op remoxipride waarschijnlijk hoofdzakelijk vanuit de hersenen aangestuurd wordt, konden we dit niet aantonen voor ACTH. Ook voor het effect van quinpirole op de verschillende hormonen konden we dit niet aantonen in **hoofdstuk 5**. In het geval van quinpirole namen we aan dat het effect op de neuro-endocriene hormonen hoofdzakelijk in het brein geïnitieerd werd, vanwege het feit dat de vrije medicijnconcentraties in het brein 5 keer hoger zijn dan in het bloed. De huidige afwezigheid van simultane medicijn en dopamine concentraties in hersenen en bloed, beperkt de mogelijkheden tot het ontwikkelen van den model dat de invloed van *CNS* medicijnen op beide niveaus tegelijk beschrijft.

Samenvattend beschrijven deze hoofdstukken studies die een longitudinale analyse van een neuro-endocriene systeem-biomarker bevatten met meerdere doseringsniveaus van een $D_2$ agonist en een $D_2$ antagonist. Door PK/PD modellering toe te passen op deze data kregen we kwantitatief inzicht in de neuro-endocriene respons op dopaminerge medicijnen, en konden we deze relateren aan medicijn-specifieke en biologisch systeem-specifieke farmacologische eigenschappen.

## Sectie III – De dynamische respons van biochemische systemen om dopamine $D_2$ medicijneffecten te bestuderen

In dit deel van het proefschrift hebben we de multi-biomarker benadering uitgebreid van een neuro-endocrien platform met 15 hormonen en peptiden naar een *metabolomics* platform met 76 aminozuren en *biogenic amines*. Er bestaan verschillende statische methoden om tijdsafhankelijke en multi-biomarker (bijv. *metabolomics*) data te analyseren. Zo is clusteren een gebruikelijke methode om de belangrijkste longitudinale patronen in een multivariate dataset te identificeren. In onze data, echter, wilden we niet alleen de tijdsdimensie, maar ook de dimensie van dosering en plaats van bemonstering (zoals bloed en brein) bestuderen. Al eerder is een multivariate methode ontwikkeld, *ANOVA-simultaneous component analysis* (ASCA), die dit mogelijk maakt. Met deze methode worden de onafhankelijke variabelen (tijd, dosering etc.) als categorische data behandeld. Hoewel dit goed werkt in het geval deze categorieën zich relatief lineair tot elkaar verhouden, wordt het problematisch wanneer dit niet het geval is. In onze data waren de monstertijden en de verschillende doseringen erg niet-lineair, waardoor deze methode het identificeren van de onderliggende dynamiek zou beperken. Belangrijker nog, geen van de bestaande methoden om multivariate dynamische patronen te analyseren integreerde farmacologische principes. Om die reden besloten we een methode te ontwikkelen die om kan gaan met niet-lineaire tijds- en doserings-patronen, en die PK/PD concepten integreert in de multivariate data analyse. In **hoofdstuk 6** hebben we tijdsafhankelijke *biogenic amine* en aminozuur patronen gemeten na toediening van verschillende doseringen

van de $D_2$ antagonist remoxipride. Vervolgens hebben we een drie-stappen benadering gebruikt – i) het beschrijven van alle individuele biomarkers met een *turnover model*; ii) het clusteren van de metabolieten op basis van hun farmacologische parameters; iii) het beschrijven van de clusters met een *turnover model*. Hiermee konden we zes verschillende PK/PD patronen identificeren, met *in vivo* potenties van 0.0027, 0.019 of 0.12 µM, wat suggereert dat er meerdere biochemische systemen beïnvloed worden door remoxipride. Hoewel we niet kunnen zeggen of deze verschillen gerelateerd zijn aan *off-target* effecten of verschillen in signaaltransductie efficiëntie, kunnen we met dit model wel iets zeggen over de therapeutische range op basis van een systeem-effect. Van de 44 metabolieten die we robuust genoeg konden meten, werden er 18 geïdentificeerd als potentiële biomarker voor verdere validatie.

Hoewel dit een stap voorwaarts was van een volledig empirische, data-gedreven methode, miste dit model nog een belangrijk element; er was geen component dat de biomarker respons in het brein beschreef. Helaas was toentertijd de methode om *biogenic amines* en aminozuren in de extracellulaire vloeistof van het brein (via microdialyse) nog niet robuust genoeg om te gebruiken voor onze modellen. Na optimalisatie van deze methode konden we in **hoofdstuk 7** *biogenic amines* en aminozuren simultaan meten in bloed en brein. Met onze modelleringsmethoden konden we nu de plaats van medicijnwerking bepalen; een belangrijke stap vooruit. Met betrekking tot deze uitbreiding van de methode doen we drie belangrijke observaties. Allereerst, hoewel de concentraties van quinpirole in het brein 5 keer hoger zijn, lijken veel effecten te ontstaan buiten het brein. Ten tweede blijken veel van deze effecten over de bloed-hersen-barrière heen te getransporteerd te worden om alsnog een respons in het brein te veroorzaken. Als derde, hoewel een veelvoud aan biochemische responsen in de hersenen waargenomen worden als gevolg van quinpirole toediening, worden maar een enkele van deze responsen over de bloed-hersen-barrière getransporteerd om vervolgens in het bloed potentieel te kunnen functioneren als biomarker.

Tegen de verwachting in, namen we een heel aantal aminozuren en *biogenic amines* waar die verlaagd waren na toediening van remoxipride (antagonist), maar ook na quinpirole (agonist). Mogelijk kan dit verklaard worden met *off-target* effecten. Zo heeft remoxipride hoge affiniteit voor de sigma receptor, en zou het op deze manier de opname van aminozuren kunnen reduceren. Verder onderzoek is echter nodig om de onderliggende verklaring te achterhalen. Bovendien benadrukt deze observatie het belang van uitbreiding van deze studies naar meerdere dopaminerge medicijnen. Hiermee is het mogelijk om te identificeren welke effecten specifiek zijn voor interactie met de dopamine receptor.

Kortom, in **hoofdstuk 6** en **hoofdstuk 7** hebben we een methode ontwikkeld dat de farmacologische principes die onderliggend zijn aan de relatie tussen medicijn dosering en biochemische systeem-respons te kwantificeren. Hiermee hebben we de *in vivo* concentratie-effect relaties, de plaats van werking, en potentiele biomarkers in het bloed bepaald.

## Perspectieven van de PK/PD-*metabolomics* methode in *CNS* medicijnontwikkeling

De PK/PD-*metabolomics* methode heeft potentie om de vroege inzichten tijdens het traject van medicijnontwikkeling te vergroten op een data-gedreven en integrale manier. *Metabolomics* analyse kan eenvoudig toegevoegd worden aan de standaard set van analyses die worden gedaan in (pre-)klinische studies op een relatief goedkope manier, afgezet tegen de kosten van een laat-klinische mislukking van een medicijn. In tegenstelling tot de huidige selectiecriteria van nieuwe medicijnen gedurende de vroege ontwikkeling, veelal op basis van affiniteit voor een enkele receptor, is het mogelijk beter om te selecteren op basis van een affiniteitsprofiel voor meerdere receptoren, zoals blijkt uit literatuur. PK/PD-*metabolomics* zou een manier kunnen zijn om de relatie tussen zo'n *in vitro* affiniteitsprofiel en de *in vivo* systeem-effecten verder te bestuderen om uiteindelijk te komen tot een model dat de systeem-effecten kan voorspellen op basis van een affiniteitsprofiel. Hoe dan ook zal PK/PD-*metabolomics* van waarde zijn voor het ontdekken van nieuwe biomarkers, samen met hun farmacologische karakterisering. Bovendien kunnen op deze manier al vroegtijdig potentiele biomarkers in het bloed geïdentificeerd worden in relatie tot hun respons in het brein. Dit is van grote waarde, gezien de beperkingen van bemonstering van het menselijk brein. Uiteindelijk kan PK/PD-*metabolomics* zo de basis vormen van de schaling van medicijneffecten van dieren naar mensen om zo richting te geven aan de doseringsbepaling in de *first-in-human* studies.

## Algemene conclusie

Het doel van deze studies was om een methode te ontwikkelen die de relatie tussen medicijndosering en de dynamische systeem-biomarker respons kan beschrijven, en die biomarkers in het bloed identificeert die iets vertellen over medicijneffecten in het brein. Hiervoor hebben we de PK/PD-*metabolomics* methode ontwikkeld die de belangrijkste biomarker patronen beschrijft in termen van farmacologisch relevante parameters zoals $E_{MAX}$ en $EC_{50}$. Voor het neuro-endocriene systeem, waar we veel *a priori* biologische kennis van hebben, konden we een model ontwikkelen om de verschillen in hormoonrespons te verklaren met behulp van receptor expressie niveaus van de $D_2$ receptor. De combinatie van deze responsen zijn daarmee uniek voor stimulatie van de $D_2$ receptor. Daarnaast hebben we laten zien dat met PK/PD-*metabolomics*, in combinatie met tijdsopgeloste *metabolomics* data in het brein en het bloed, de plaats van werking van medicijnen bepaald

kan worden, en biomarkers in het bloed gevonden kunnen worden die iets zeggen over effecten in het brein. Met haar positie tussen data-gedreven multivariate methodes en mechanistische systeem-farmacologie modellen, kan PK/PD-*metabolomics* kwantitatieve farmacologische inzichten geven in de systeem-respons van *CNS* medicijnen op een data-gedreven manier.

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## Curriculum Vitae

Willem van den Brink started his studies in Biopharmaceutical Sciences in 2007 at Leiden University. During his masters he did an internship at the department of Pharmacology at the LACDR applying clinical trial simulation to optimize clinical trial designs COPD drug development. Intrigued by the potential of modeling and simulation, he started an internship at the Dutch Medicines Evaluation Board, developing a model to predict C-cell carcinogenicity potential of GLP-1 receptor agonists. After finishing his masters in 2013, he came back at the LACDR to start his PhD research. During this period he worked on applying the principles of PK/PD modeling on multi-biomarker data to evaluate systems responses and identify blood-based biomarkers in early CNS drug development. Currently, he is employed at TNO within the group of Systems Biology, focusing on quantification of health status in diseased and healthy population for development of curative and preventive lifestyle/nutritional interventions.

# List of publications


1. **Brink WJ van den**, Berg D-J van den, Bonsel FE, Hartman R, Wong Y-C, Graaf PH van der, et al. (2018) Fingerprints of CNS drug effects: a plasma neuroendocrine reflection of D2 receptor activation using multi-biomarker PK/PD modeling. *Br J Pharmacol.* 175(19):3832-3843.
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Discovery and development of Central Nervous System (CNS) drugs is hampered by high attrition rates. One of the reasons is the lack of blood-based biomarkers that represent the interaction between the drug and the neurological systems of interest. Here, we present a systems-pharmacology approach that combines a multi-biomarker discovery approach (e.g. metabolomics) with pharmacokinetic/pharmacodynamic (PK/PD) modeling to reveal quantitative pharmacological characteristics that are relevant to dopaminergic drug action. Moreover, we set out to identify biomarkers that can be obtained from the blood as non-invasive sampling site.

In the first section of this thesis the methodology is introduced in the context of translational CNS drug development. Additionally, a systematic search is performed to available biomarkers of dopaminergic drug action. Then, in the second part, the multi-biomarker PK/PD approach is applied to biomarkers from the neuroendocrine system as connection between brain and blood. In the third section, the methodology is developed using the simultaneous, time-resolved metabolomics response in brain extracellular fluid and plasma.

By applying multi-biomarker PK/PD modeling we revealed quantitative pharmacological characteristics of dopaminergic drugs with regard to multiple biological processes. Moreover, we identified potential blood-based biomarkers of dopaminergic effect in the brain.

