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**MECHANISTIC MODELING OF ALPHA-SYNUCLEIN
AGGREGATION AND BRAIN PHARMACOKINETICS
FOR DRUG DEVELOPMENT IN NEURODEGENERATION**

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To my family and to everyone who helped clear my path to this
milestone—some by moving pebbles, others by pulling weeds, and some by
rolling away true boulders

ABSTRACT

Mechanistic modeling plays an increasingly important role in central nervous system (CNS) drug development, but its impact depends on how well models reflect the underlying biology and account for the limitations of available data. This thesis addresses these challenges through two distinct yet complementary research areas, focused on foundational and methodological aspects of quantitative systems pharmacology (QSP) and physiologically based pharmacokinetic (PBPK) modeling. The first part of the work focuses on the mathematical modeling of alpha-synuclein (aSyn) aggregation dynamics under *in vitro* conditions—a molecular process relevant to Parkinson’s disease (PD) and other synucleinopathies. Building on *in vitro* aggregation studies and chemical kinetic models, we develop a mechanistic model of aSyn accumulation that incorporates both pure and lipid-mediated aggregation pathways. This unified framework captures key features of *in vitro* aggregation mechanisms, including toxic oligomer structural changes, fibril-mediated secondary nucleation, and aSyn–lipid interactions. It enables the evaluation of PD therapeutic strategies targeting different steps of the aggregation cascade, with applications in QSP. The second part tackles specific methodological limitations in evaluating *a priori* predictions of brain drug distribution against experimental homogenate measurements. Building on the Rodgers & Rowland method and related tissue composition-based models, we propose a homogenate-specific metric that accounts for artifacts induced by tissue homogenization. This metric serves as a mechanistically appropriate endpoint for homogenate assays, provided brain lipid composition is well characterized. The analysis contributes to improving model evaluation practices and enhancing the reliability of *a priori* predictions of drug partitioning in intact brain tissue, with implications for PBPK modeling of CNS-active small molecules. Although these two projects differ in biological scale and modeling approach, both target issues in advancing mechanistic models in the CNS field. Rather than delivering ready-to-use preclinical tools, the aim is to promote more biologically grounded and interpretable modeling practices—either by improving the representation of critical pathological processes or by clarifying how model predictions relate to the data used to evaluate them. By building on and complementing existing frameworks, the models developed here foster more transparent, robust, and data-aligned modeling in neurodegeneration. Ultimately, through enhanced mechanistic understanding at both the molecular and pharmacokinetic levels, this work can support therapeutic strategy design for Parkinson’s disease, with implications for target characterization and brain exposure optimization.

SOMMARIO

La modellistica meccanicistica riveste un ruolo sempre più centrale nello sviluppo di farmaci per il sistema nervoso centrale, ma il suo impatto dipende dalla capacità dei modelli di riflettere accuratamente la biologia sottostante e di tenere conto delle limitazioni dei dati disponibili. Questa tesi affronta tali sfide attraverso due ambiti di ricerca distinti ma complementari, incentrati su aspetti concettuali e metodologici della *quantitative systems pharmacology* (QSP) e della *physiologically based pharmacokinetics* (PBPK). La prima parte del lavoro si concentra sulla modellizzazione matematica della dinamica di aggregazione dell'alfa-sinucleina, un processo molecolare rilevante per il morbo di Parkinson ed altre sinucleinopatie. Basandosi su studi di aggregazione *in vitro* e su modelli cinetici chimici, abbiamo sviluppato un modello meccanicistico dell'accumulo di alfa-sinucleina che integra sia il percorso di aggregazione pura sia quello mediato dai lipidi. Questo framework unificato cattura aspetti chiave dei meccanismi di aggregazione *in vitro*, tra cui le modifiche strutturali di oligomeri tossici, la nucleazione secondaria mediata da fibrille e le interazioni tra proteina e lipidi, consentendo la valutazione di strategie terapeutiche che agiscono a diversi stadi della cascata di aggregazione, con applicazioni nell'ambito QSP. La seconda parte affronta specifiche limitazioni metodologiche nella valutazione delle predizioni della distribuzione cerebrale dei farmaci rispetto ai dati sperimentali ottenuti da omogeneizzati di tessuto cerebrale. Sulla base del metodo di Rodgers & Rowland e di modelli basati sulla composizione tissutale, abbiamo proposto un parametro specifico che tiene conto degli artefatti introdotti dalla procedura di omogeneizzazione. Questa metrica rappresenta un riferimento meccanicisticamente coerente per l'interpretazione dei dati sperimentali, a condizione che la composizione lipidica cerebrale sia adeguatamente caratterizzata. L'analisi contribuisce a migliorare le pratiche di valutazione dei modelli e ad aumentare l'affidabilità delle predizioni della distribuzione dei farmaci nel tessuto cerebrale intatto, con implicazioni per la modellistica PBPK di *small molecules* attive nel sistema nervoso centrale. Sebbene i due progetti differiscano per scala biologica e approccio modellistico, entrambi affrontano temi utili per l'avanzamento della modellistica meccanicistica nell'ambito delle patologie neurodegenerative. Più che fornire strumenti preclinici pronti all'uso, l'obiettivo è promuovere pratiche modellistiche più interpretabili e più aderenti alla biologia—da un lato migliorando la rappresentazione dei processi patologici chiave, dall'altro chiarendo la relazione tra le predizioni modellistiche e i dati utilizzati per la loro validazione. A partire da framework esistenti, i modelli sviluppati in questa tesi favoriscono un approccio più trasparente, robusto e allineato ai dati, nel contesto delle malattie neurodegenerative. In definitiva, attraverso una comprensione meccanicistica più approfondita sia a livello molecolare sia farmacocinetico, questa tesi può contribuire alla progettazione di strategie terapeutiche per il morbo di Parkinson, con implicazioni per la caratterizzazione dei target e l'ottimizzazione dell'esposizione farmacologica cerebrale.

PUBLICATIONS & ONGOING WORK

The content of the dissertation is based on the following material:

1. **“Mechanistic models of α -synuclein homeostasis for Parkinson’s disease: A blueprint for therapeutic intervention”**

Righetti Elena, Antonello Alice, Marchetti Luca, Domenici Enrico, and Reali Federico*

Frontiers in Applied Mathematics and Statistics (2022)

[Righetti et al., 2022](#)

2. **“A mechanistic model of pure and lipidic α -synuclein aggregation for advancing Parkinson’s therapies”**

Righetti Elena*, Marchetti Luca, Domenici Enrico, and Reali Federico*

Communications Chemistry (2025)

[Righetti et al., 2025](#)

3. **“Evaluating a priori fraction unbound predictions against brain homogenate data: Accounting for the experimental bias”**

Righetti Elena, Petrov Aleksandr, Kloft Charlotte, Reichel Andreas, and Huisinga Wilhelm

PAGE conference (2025) poster - Manuscript in preparation: “Lost in homogenization: Reconciling *a priori* mechanistic predictions with experimental estimates of brain fraction unbound”

*Where you invest your love,
you invest your life*

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INTRODUCTION AND OVERVIEW

Mechanistic models can support central nervous system (CNS) research at multiple stages of the drug development pipeline. This thesis explores how such models can more accurately reflect biology at molecular and tissue levels and align with experimental data, thereby informing the design of therapeutic strategies for neurodegeneration.

1.1 CURRENT STATUS OF DRUG DEVELOPMENT IN NEURODEGENERATION

Neurodegenerative disorders—such as Alzheimer’s disease (AD) and Parkinson’s disease (PD)—are characterized by progressive neuronal dysfunction and lack effective disease-modifying therapies. Despite significant scientific progress, there are persistent therapeutic challenges. As of 2024, only 5% of the 60 active disease-modifying therapy clinical trials for PD have reached Phase 3, with the majority (58%) stalled in Phase 2 ([McFarthing et al., 2023](#)). AD shows a slightly more optimistic trajectory, with approximately 23% of 96 disease-modifying agents in Phase 3 ([Cummings et al., 2024](#)). Yet, across CNS indications, the clinical failure rate is above 90% ([Gribkoff et al., 2017](#); [Morofuji et al., 2020](#); [Geerts et al., 2020](#)). Many of these failures stem from pharmacokinetic, safety, or efficacy problems that become evident in clinical trials ([Fogel, 2018](#)). These issues reflect broader challenges, such as the limitations of animal models in recapitulating pathology, the difficulty of modeling human brain pharmacology, and the complex, multifactorial nature of neurodegenerative diseases. A particularly relevant example in CNS pharmacology is the pathological aggregation of α -synuclein (aSyn), which plays a central role in synucleinopathies, *i.e.*, PD, multiple system atrophy (MSA), and dementia with Lewy bodies (DLB). Although aSyn aggregation is a well-established hallmark for these diseases, only ten clinical trials have directly targeted this process ([McFarthing et al., 2023](#); [Bendetowicz et al., 2024](#); [Abdelnour et al., 2023](#)), with just one reaching Phase 3 ([H. Lundbeck A/S, 2024](#)). This limited progress underscores uncertainties at various scales ([Brundin et al., 2017](#); [Espay et al., 2020](#)). At the molecular level, critical questions remain: whether aSyn aggregation is a primary driver or a downstream event of disease progression ([Lashuel, 2020](#); [Calabresi et al., 2023](#)); which stage of the aggregation cascade represents the most promising therapeutic target ([Dehay et al., 2015](#); [Fields et al., 2019](#); [Jasutkar et al., 2022](#)); how to pinpoint the step at which a drug exerts its effect ([Iljina et al., 2017](#); [Perni et al., 2018](#); [Limbocker et al., 2021](#)); and why compound binding to aSyn so often fails to translate into measurable

changes in aggregation kinetics (Antonschmidt et al., 2022; Dominguez-Meijide et al., 2020; Fellner et al., 2016; Gamage et al., 2024). There is, therefore, a need for deeper mechanistic insight into both the aggregation process itself and the ways in which therapeutic agents modulate it. In particular, distinguishing how different binding modes and mechanisms of action alter aggregation may provide critical guidance for designing more effective therapies. While such insights cannot on their own overcome the translational barriers of neurodegeneration, they represent an essential step toward narrowing the persistent gap between mechanistic understanding at the molecular level, preclinical studies, and clinical outcomes (Obeso et al., 2017; Espay et al., 2020).

1.2 MECHANISTIC MODELING IN CNS DRUG DEVELOPMENT: OPPORTUNITIES AND LIMITATIONS

Quantitative modeling has become integral to drug development pipelines as model-informed drug development (MIDD), particularly through empirical modeling approaches, such as pharmacokinetic/pharmacodynamic and population pharmacokinetic models (Madabushi et al., 2022). However, more mechanistic approaches are currently gaining traction due to their systems perspective (Geerts et al., 2020; Bloomingdale et al., 2022b), yet remain underutilized in neurodegeneration, leaving substantial room for improvement.

Mechanistic modeling indeed offers a systematic framework to interpret biological complexity, integrate diverse data, and simulate drug behavior *in silico*. We focus on two key approaches:

- **Physiologically based pharmacokinetic (PBPK) modeling** relies on anatomical and physiological parameters to simulate how a drug is absorbed, distributed, metabolized, and excreted across organs. It can predict drug exposure in specific tissues, including the brain, by integrating drug properties (*e.g.*, lipophilicity, ionization) with tissue-specific features (*e.g.*, binding sites).
- **Quantitative systems pharmacology (QSP) modeling** integrates systems biology with pharmacology to capture system-wide drug responses by incorporating a broad range of data spanning molecular, cellular, tissue, and clinical levels. Originally developed to explain target–effector relationships and therapeutic response dynamics, QSP is increasingly being explored to support biomarker interpretation, mechanistic hypothesis testing, dose selection, and combination strategy design.

These models are particularly relevant in CNS drug development, where direct access to target tissues is limited, and complex, nonlinear interactions underlie both disease progression and treatment effects (Geerts et al., 2020; Bloomingdale et al., 2022b). However, their widespread adoption remains limited due to some key challenges: (i) need for extensive and high-quality

input data: sparse or fragmented biological datasets and unavailable measurements for all biological scales hinder accurate model parameterization and limit multi-scale validation (Azer et al., 2021); (ii) inherent model complexity: striking a balance between mechanistic depth and computational feasibility remains difficult, leading to models that are either overparameterized or oversimplified (Bloomingdale et al., 2021b); (iii) translational barriers: species differences and poorly characterized human-specific parameters limit the model translational power (Azer et al., 2021). Despite these limitations, recent years have seen growing regulatory engagement, reflected by initiatives such as the FDA Modernization Act 2.0 (U.S. Congress, 2022) and EMA’s Directive 2010/63/EU (European Parliament and Council of the European Union, 2010; Mehta et al., 2025). PBPK modeling is now routinely accepted by agencies such as the FDA and EMA (Paul et al., 2025), while QSP remains less standardized (Bai et al., 2021). In the “regulatory hope-hype lifecycle” described by Zineh (2019) and Gartner, Inc. (2019)—mapping the trajectory of emerging technologies through stages of hype, learning, and eventual integration, PBPK is nearing the productivity plateau, while QSP is climbing the slope of enlightenment—highlighting both its expanding promise and current regulatory limitations. Continued guidance and coordinated progress are therefore essential to fully integrate both approaches into drug development pipelines. Nonetheless, multiple successful applications exist in preclinical and clinical drug development and post-market analysis across CNS research areas, including neurodegeneration and psychiatry (Geerts et al., 2020; Mehta et al., 2025). A landmark example involves the 2015 FDA approval of a recombinant human parathyroid hormone for hypoparathyroidism: a QSP model proposed by Peterson et al. (2010) supported regulatory decisions for the optimal dosing regimen (Khurana et al., 2019). Moreover, a QSP/PBPK model combining synaptic propagation dynamics and microglia activation has elucidated possible reasons for anti-aSyn and anti-tau therapy clinical failures (e.g., prasinezumab) by demonstrating poor target engagement in synaptic clefts despite cerebrospinal fluid biomarker effects (Geerts et al., 2023b)¹.

1.3 THESIS CONTRIBUTIONS

Such *in silico* mechanistic modeling frameworks are only as robust as the biological principles that underpin them. Their predictive power and applicability depend heavily on the quality of the biological knowledge and data they incorporate. Specifically, robust mechanistic models require:

- clearly defined molecular pathways and system behaviors;
- reliable parameters supported by experimental data;
- explicit assumptions and constraints that define model scope.

¹ <https://www.certara.com/qsp-modeling-platforms-for-neuroscience>

Thus, foundational research is essential for both model construction and interpretation. This thesis lies at the intersection of basic research and applied QSP and PBPK modeling by contributing to some foundational and methodological underpinnings of mechanistic modeling for CNS drug development. Specifically, it addresses two areas: the molecular mechanisms of alpha-synuclein aggregation and the reliability of *in silico* predictions of unbound drug concentration in the brain.

(1) Developing a unified framework for α -synuclein aggregation. Existing mathematical models of aSyn aggregation, while insightful, are often tailored to specific experimental settings. Here, building on multiple chemical kinetic studies (Knowles et al., 2009; Iljina et al., 2016; Gaspar et al., 2017; Dear et al., 2024), we develop a unified model that incorporates pure and lipidic aSyn aggregation pathways, calibrated with data from *in vitro* aggregation assays. By capturing the influence of lipidic vesicles on aggregation kinetics and the relevant aggregation mechanisms, the model provides a virtual lab for exploring the effects of perturbations on oligomer formation and fibril growth, such as changes in lipid concentrations or the mechanism of action of inhibitory compounds. Our work relies on the hypothesis that the fundamental mechanisms of aSyn aggregation are preserved from *in vitro* to *in vivo* scenarios, although with different rates and concurring processes. Therefore, this framework can serve as a mechanistic module within QSP platforms targeting synucleinopathies.

(2) Accounting for the experimental bias in *a priori* predictions of brain fraction unbound. Mechanistic predictions of the unbound fraction of drug in brain tissue are often evaluated against data from homogenized brain samples. However, tissue homogenization disrupts key physiological features, introducing artifacts. We propose a correction metric, building on Rodgers & Rowland methods (Rodgers et al., 2005; Rodgers et al., 2006; Rodgers et al., 2007), to account for homogenate-specific effects such as cell membrane and pH compartmentalization disruption. We compare predictions to experimental data and analyze the impact of brain lipid composition on prediction accuracy. This work highlights both methodological limitations and opportunities to refine PBPK models for CNS drug distribution.

While preclinical and clinical applications are beyond the immediate scope of our work, this thesis lays useful groundwork for future MIDD efforts in neurodegeneration—providing a mechanistic module for QSP models of synucleinopathies (particularly Parkinson’s disease) and refining the evaluation of brain drug exposure in PBPK modeling for early-phase drug development.

OUTLINE

Each chapter is self-contained and describes an original contribution to the field, including relevant background, methodology, and results. The final chapter synthesizes the main findings and outlines future directions, offering a perspective on how the presented research can inform future mechanistic modeling efforts for Parkinson's disease and CNS drug development. Hereafter, we provide a summary of the contributions presented in the various chapters.

Chapter 2 - Mathematical modeling of α -synuclein homeostasis

Based on [Righetti et al. \(2022\)](#), this chapter reviews available mathematical models of α -synuclein homeostasis, with a focus on its aggregation and degradation pathways. It provides conceptual bases for mechanistic molecular modeling in Parkinson's disease and highlights key knowledge gaps, some of which are addressed in Chapter 3.

"Mechanistic models of α -synuclein homeostasis for Parkinson's disease: A blueprint for therapeutic intervention"

Righetti Elena, Antonello Alice, Marchetti Luca, Domenici Enrico, and Reali Federico

Frontiers in Applied Mathematics and Statistics (2022)

Chapter 3 - A mechanistic model of pure and lipidic α -synuclein aggregation

Based on [Righetti et al. \(2025\)](#), this chapter presents the main original contribution of this thesis. Building on chemical kinetics models, we propose a mathematical model of both protein-only and lipid-mediated α Syn aggregation that provides a unified framework for *in vitro* aggregation. The model enables *in silico* investigations of aggregation dynamics and pharmacological interventions at the molecular scale.

"A mechanistic model of pure and lipidic α -synuclein aggregation for advancing Parkinson's therapies"

Righetti Elena, Marchetti Luca, Domenici Enrico, and Reali Federico

Communications Chemistry (2025)

Chapter 4 - Accounting for experimental bias in mechanistic predictions of unbound drug fraction in brain homogenates

Based on an ongoing PBPK project, this chapter addresses experimental artifacts in predictions of brain fraction unbound by proposing a correction framework for comparing model predictions to homogenate-derived data. Using mechanistic modeling and sensitivity analysis, we show how tissue composition—especially lipid content—affects prediction accuracy.

“Evaluating *a priori* fraction unbound predictions against brain homogenate data: Accounting for the experimental bias”

Righetti Elena, Petrov Aleksandr, Kloft Charlotte, Reichel Andreas, and Huisinga Wilhelm

PAGE conference (2025) poster**“Lost in homogenization: Reconciling *a priori* mechanistic predictions with experimental estimates of brain fraction unbound”**

Righetti Elena, Petrov Aleksandr, Kloft Charlotte, Reichel Andreas, and Huisinga Wilhelm

Manuscript in preparation

Contribution to the above three chapters. I served as first author on all the projects presented in this thesis. The two published papers presented in Chapters 2 and 3 represent the core of my PhD research. Under the guidance of my supervisors, I led all stages of the project development: I reviewed the broader literature on mechanistic modeling of aSyn aggregation and its role in Parkinson’s disease, conceptualized the projects, formulated the research questions, and designed the modeling framework. I implemented the mathematical models, developed and calibrated the computational tools, analyzed and interpreted the results, and wrote the original manuscripts. My co-authors provided feedback on model assumptions, commented on draft versions of the manuscript, and contributed to the scientific discussion of the results. This research was presented, in the form of posters and oral communications, at several international conferences, including ECMTB 2024, PAGE 2023 and 2024, ACoP 2023, and AD/PDTM 2025. For the last project, presented in Chapter 4 and currently unpublished, I wrote the first draft of the chapter and revised it based on feedback from co-authors. The broader research direction on unbound CNS drug exposure in brain homogenate tissue was developed collaboratively, with ideas being iteratively discussed and refined over time. I derived the model equations for predicting the fraction unbound in homogenized brain and developed the methodology for addressing dilution effects in the experimental data. The notation and structure of the chapter follow that of a draft book chapter on PBPK modeling, co-authored by collaborators, with appropriate adaptations. This work was also presented as a poster at the PAGE 2025 conference. We plan to use this chapter as the basis for an original journal manuscript, with all listed co-authors contributing to its development.

MATHEMATICAL MODELING OF ALPHA-SYNUCLEIN HOMEOSTASIS

Chapter Abstract Parkinson's disease is the second most common neurodegenerative disorder worldwide, yet there is no disease-modifying therapy up to this date. The biological complexity underlying this disease hampers the investigation of the principal contributors to its pathogenesis. In this context, mechanistic models grounded in molecular-level knowledge provide virtual labs to uncover the primary events triggering Parkinson's disease onset and progression and suggest promising therapeutic targets. Multiple modeling efforts in Parkinson's research have focused on the pathological role of α -synuclein, a presynaptic protein that emerges from the intricate molecular network as a crucial driver of neurodegeneration. Here, we collect the advances in mathematical modeling of aSyn homeostasis, focusing on aggregation and degradation pathways, and discussing potential modeling improvements and possible implications in Parkinson's disease drug development.

This Chapter is based on the following work: Righetti et al., *Mechanistic models of α -synuclein homeostasis for Parkinson's disease: A blueprint for therapeutic intervention* (Righetti et al., 2022). As first author, my contributions included reviewing the literature on existing mechanistic models for Parkinson's disease, conceptualizing the manuscript structure, and writing the entire manuscript.

2.1 INTRODUCTION

Parkinson's disease (PD) is a debilitating neurodegenerative disorder with currently no disease-modifying therapy or cure (Poewe et al., 2017). The high prevalence of PD on the global scale and its exponential growth over the last few decades suggest the imminent outbreak of a non-infectious pandemic propelled by aging and industrialization as unconventional vectors of this rapidly growing neurological disorder (Dorsey et al., 2018). Given the enormous strain that a pandemic would put on the healthcare system and the resulting human and economic toll, it is no surprise that investigating PD pathogenesis has rapidly become a serious public health concern. Since 1817, when the "shaking palsy" was first described (Parkinson, 2002), considerable progress has been made in understanding PD, from the clinical symptoms to the molecular mechanisms. Such progress has yet to translate into effective treatments that can hamper or even reverse PD progression. The current gold-standard therapy (Levodopa or L-Dopa treatment) manages motor symptoms but cannot stop neurodegeneration (LeWitt et al., 2016). It counterbalances dopamine (DA) deficiency in the basal ganglia by relying on the pharmacological input of the DA precursor L-Dopa. Indeed, impairments in the dopaminergic pathway and the subsequent neuronal death in the *substantia nigra pars compacta* stand out as primary hallmarks of the disease (Poewe et al., 2017).

Human and molecular genetic research has soon uncovered PD multifactorial nature, thus unveiling a complex molecular network (Poewe et al., 2017; Blauwendraat et al., 2020). In addition to impairments in DA metabolism, numerous processes such as protein aggregation, defective degradation, neuroinflammation, and oxidative stress contribute to disease onset and progression through various feedback mechanisms (see Fujita et al. (2014) for a comprehensive disease map). The presynaptic amyloidogenic protein α -synuclein (aSyn) emerges from the intricate molecular landscape as a key driver of PD pathogenesis (Poewe et al., 2017). Here, we focus on two mechanisms governing its homeostasis, *i.e.*, the aggregation and degradation pathways. Figures 2.1A and 2.2A display the biological mechanisms involved in these pathways. Their link to PD neurodegeneration is supported by multiple experimental and genome-wide association studies (Blauwendraat et al., 2020; Gan-Or et al., 2015; Walden et al., 2017). Even though multiple studies have highlighted the significant contribution of these processes to PD neurodegeneration (Vekrellis et al., 2011; Navarro-Romero et al., 2020; Olanow et al., 2006), the interplay between the uncontrolled accumulation of aSyn aggregates and impairments in the degradation mechanisms is still unclear (Xilouri et al., 2013).

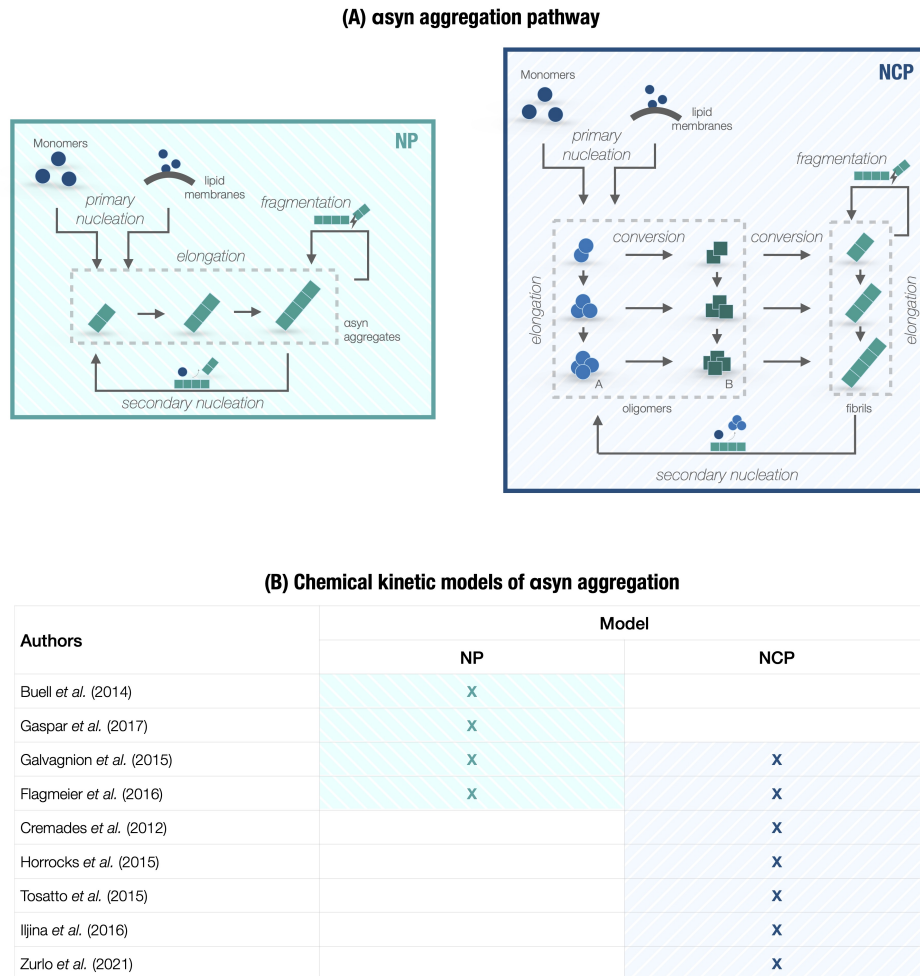
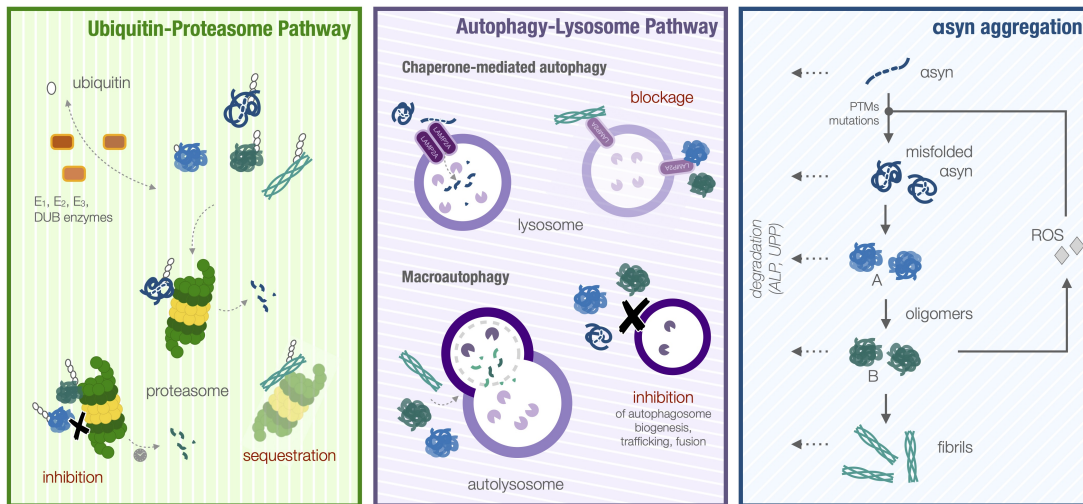


Figure 2.1: Chemical kinetic models of α syn aggregation. (A) A conceptual scheme of the PD molecular landscape underlying models in Section 2.2. The kinetic of α syn aggregation can be represented by either a nucleation-polymerization (NP) process or a nucleation-conversion-polymerization (NCP) process. The pathway includes homogeneous primary nucleation from monomers, heterogeneous primary nucleation catalyzed by lipid membranes, aggregate elongation by monomer addition, structural conversion between oligomeric species (type-A and type-B oligomers) and fibrillar species (if we consider NCP models), fibril fragmentation, and heterogeneous secondary nucleation on fibril surfaces. Reverse processes, such as monomer dissociation and reverse conversion, are not shown since they are negligible at the early aggregation steps. (B) Table collecting the chemical kinetic models of α syn aggregation considered in Section 2.2 that provide mechanistic insights into PD neurodegeneration. The studies are based on the master equation approach and focus either on NP or NCP models.

Given the intricate biological picture outlined so far, identifying the specific role of aSyn aggregation and degradation pathways would support therapeutic strategies currently under investigation (Fields et al., 2019), *e.g.*, small molecule inhibition, aSyn antibodies, and autophagy stimulation, or even pave the way for new pharmacological approaches. To this end, the experimental analysis calls for a QSP approach at the interface between pharmacological research and systems biology (Azer et al., 2021). As recently highlighted in Geerts et al. (2020), Abrams et al. (2020), Bloomingdale et al. (2021b), and Bloomingdale et al. (2022b), QSP models indeed hold a great potential in neuroscience as they provide a systems-level understanding of complex biological processes and insights into their response to drugs. Specifically, modeling efforts in PD research are moving toward multiscale QSP models that describe molecular, cellular, whole-brain, and organism levels to assess how the effects of a molecular perturbation can scale up to influence the clinical outcome (Stephenson et al., 2022). Starting from the molecular level, various mechanistic models falling within the systems biology framework have tackled PD complexity. As highlighted by previous studies on systems biology models for neurodegenerative disorders (Lloret-Villas et al., 2017; Bakshi et al., 2019), they have mainly focused on protein aggregation, synaptic transmission, apoptosis, oxidative stress, genetic components, and protein degradation. The number of models including degradation processes is likely to rise along with increasing evidence that supports the critical role of the protein clearance machinery in PD (Navarro-Romero et al., 2020; Klein et al., 2018).

Chapter outline With this in mind, in this chapter, we focus on mathematical models of aSyn aggregation and degradation by the ubiquitin-proteasome pathway, macroautophagy, and chaperone-mediated autophagy. We divide the mechanistic models into two groups: (i) single-pathway models of aSyn aggregation representing the chemical kinetics of the process, *i.e.*, **chemical kinetic models** (see Section 2.2); (ii) multiple-pathway models of aSyn homeostasis focusing on the interplay between aSyn aggregation and degradation mechanisms, *i.e.*, **degradation models** (see Section 2.3). Section 2.4 presents limitations, potential improvements, future directions, and applications for PD therapeutic strategy design.

(A) α syn aggregation and degradation pathways(B) Models of α syn aggregation and degradation

Authors	Degradation pathway			Aggregation pathway
	UPP	ALP	Generic	
Sneppen <i>et al.</i> (2009)	X (*)			X
Pigolitti <i>et al.</i> (2013)			X (*)	X
Proctor <i>et al.</i> (2007)	X			X
Proctor <i>et al.</i> (2010)	X			X
Cloutier <i>et al.</i> (2012)	X (*)			X
Raichur <i>et al.</i> (2006)	X			X
Ouzonoglou <i>et al.</i> (2014)	X	X		X
Sass <i>et al.</i> (2009)	X	X		X
Büchel <i>et al.</i> (2013)	X	X		X
Kuznetsov & Kuznetsov (2016)			X (*)	X
Kuznetsov & Kuznetsov (2022)	X (*)	X (*)		X

Figure 2.2: Models of α syn aggregation and degradation. (A) A conceptual scheme of the PD molecular landscape underlying models in Section 2.3. A high-level representation of α syn aggregation (light-blue box) is integrated into a more complex network involving the ubiquitin-proteasome pathway (UPP) and the autophagy-lysosome pathway (ALP) (green and purple boxes, respectively), reactive oxygen species (ROS) production, and the corresponding feedback loops. The aggregation process involves α syn misfolding, the conformational change between type-A and type-B oligomers, the conversion from oligomers to fibrils, and degradation of α syn species. Factors triggering α syn misfolding can be post-translational modifications (PTMs), α syn mutations, and high ROS levels. UPP degradation, including ubiquitination and proteasome-mediated degradation, is impaired by fibrils sequestering the proteasome and oligomers inhibiting proteasomal activity either by proteasome overload or directly due to their inherent toxicity. Pathogenic α syn also interferes with ALP components (Xilouri *et al.*, 2016). Fibrils and oligomers can block CMA degradation by obstructing LAMP2A receptors and inhibiting their assembly (Cuervo *et al.*, 2004; Martinez-Vicente *et al.*, 2008; Xilouri *et al.*, 2009). Macroautophagy is the main degradation route for α syn aggregates. In return, misfolded α syn and its aggregates can exert an overall inhibitory effect on macroautophagy and hamper its activity by interfering with autophagosome formation, maturation, and trafficking, in addition to lysosomal fusion and transport (Wong *et al.*, 2015; Wong *et al.*, 2017). (B) Table collecting the models considered in Section 2.3. The term Generic indicates a generic degradation mechanism, either proteasome or lysosome-mediated. The models denoted by the symbol (*) incorporate degradation mechanisms either as pseudo-first-order reactions or enzymatic reactions; the remaining models are characterized by a more detailed representation of the degradation pathways.

2.2 MECHANISTIC MODELS OF ASYN AGGREGATION KINETICS

Protein aggregation and misfolding are central features in the molecular network of neurodegeneration (Chiti et al., 2017). It is therefore no surprise that a large part of the modeling effort targeting PD investigates the chain of microscopic events in aSyn aggregation. These studies fall into a broader line of research devoted to uncovering the generality of amyloid formation by focusing on the chemical and physical aspects of this phenomenon (Dobson, 2003; Knowles et al., 2009). Here, we analyze mechanistic models that extend the formalism of chemical kinetics to the aggregation process; we thus refer to them as **chemical kinetic models** (see Figure 2.1). These models adopt a master equation approach building on the pioneering work by Oosawa & Kasai (Oosawa et al., 1962) to provide a mathematical formulation of each molecular step in the process and derive the corresponding rate constants. To gain novel biological insights and formulate new scientific queries, *in silico* models evolve along with increasingly advanced experimental procedures in a virtuous cycle of model calibration and validation against *in vitro* quantitative measurements (Chiti et al., 2017).

2.2.1 ASYN MISFOLDING AND AGGREGATION AND PD NEURODEGENERATION

Soluble aSyn is an aggregation-prone protein with an intrinsically disordered conformation that prevents its spontaneous self-assembly (Gracia et al., 2020). Many factors can trigger aSyn misfolding and accumulation, such as high aSyn concentrations, genetic mutations, increased reactive oxygen species (ROS) levels, and interaction with lipid membranes (Hijaz et al., 2020). The process initiates upon structural changes that expose the highly amyloidogenic region of the protein to the cytosol. Misfolded monomers slowly aggregate into oligomeric species, thus creating nucleation seeds. In return, seeding-competent oligomers can rapidly accumulate and elongate by monomer addition forming protofibrils and insoluble fibrils (Figure 2.1A). Multiple aSyn species eventually gather into amyloid inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs), which represent one of PD's main molecular hallmarks (Spillantini et al., 1997).

A considerable body of research points to the harmful effect of aSyn aggregation and its crucial contribution to PD neurodegeneration. For instance, the *SNCA* gene, encoding for aSyn, was the first gene associated with PD (Polymeropoulos et al., 1997). Still, there is no agreement on the specific mechanisms of toxicity. Seeding and spreading ability and inherent toxicity are the functional and structural factors that determine the overall toxicity of aggregates (Gracia et al., 2020; Froula et al., 2019). According to *in vitro* and *in vivo* experimental evidence, the most cytotoxic species are oligomers with

a partially folded structure similar to mature fibrils (Hijaz et al., 2020; Froula et al., 2019; Cremades et al., 2017). Due to their conformation, some oligomeric species can create pores within the cell membrane, thus enhancing its permeability and dysregulating calcium homeostasis (Alam et al., 2019). They can also impair mitochondrial function and proteolytic machinery. On the other hand, small fibril fragments have been identified as major-spreading agents in PD because of their high seeding efficiency and internalization ability (Hijaz et al., 2020). According to one of the leading hypotheses for aSyn propagation, pathogenic species are assumed to spread in a prion-like manner (Brundin et al., 2010). In this scenario, they are released into the extracellular space and endocytosed by neighboring neurons, where they recruit non-native monomers, acting as seeds for further aggregation. In experimental models, this process leads to the propagation of protein aggregates across interconnected brain regions over time (Chung et al., 2019). In humans, patterns of pathology observed in post-mortem brains (e.g., Braak staging, Braak et al. (2002)) provide a snapshot of different disease stages, which is often interpreted as temporal progression and contribution to PD neurodegeneration (Poewe et al., 2017). However, this inferred propagation may reflect either direct prion-like spread of pathogenic aSyn, or sequential vulnerability of interconnected regions, or other systemic processes that result in the apparent expansion of aggregation (e.g., inflammation fueling). In parallel, considerable progress has been made in understanding the kinetics of aSyn misfolding and aggregation. There is no agreement, however, on many aspects of this mechanism. Chemical kinetic models can provide valuable insights in understanding, for example, the relative contribution of different microscopic events to the process, the structural and functional features of aSyn aggregate species (Cremades et al., 2017), and the relationship between aSyn aggregation and LB formation and maturation (Hardenberg et al., 2021; Shahmoradian et al., 2019).

THE MASTER EQUATION APPROACH

The aggregation pathway of a generic amyloidogenic protein can be represented by a minimal set of fundamental reactions, regulating the transition from soluble monomers to fibrils through various intermediate states. As shown in Figure 2.1A, we can differentiate between primary reactions, *i.e.*, homogeneous primary nucleation and elongation, and secondary reactions, *i.e.*, heterogeneous secondary nucleation and fibril fragmentation (Cohen et al., 2011b; Meisl et al., 2016). Homogeneous nucleation initiates the aggregation process by combining monomers into newly formed aggregates that, in turn, elongate by monomer addition. By integrating these reactions, fibril fragmentation and heterogeneous nucleation on aggregate surfaces multiply the aggregate number in a positive feedback loop with elongation, thus ensuring self-replication. Their relative contribution to the aggregation process

depends on the specific protein considered (Knowles et al., 2009). Primary and secondary reactions can incorporate more complex events, such as interaction with lipid membranes and multistep nucleation. The master equation approach (Oosawa et al., 1962; Michaels et al., 2014) translates the reaction network into an infinite system of coupled non-linear differential equations describing, in probabilistic terms, the time course of the species involved, from monomers to filaments identified by their monomer count.

The main goal of this approach is to find an analytical solution for the system to describe its reaction time course. As a result, the analytical solution can be fitted to experimental data to identify kinetic rate constants and provide mechanistic insights into the aggregation process. Since the master equation formulation is often analytically and numerically intractable, specific approximation methods can be employed to reduce the system to two differential equations describing the time evolution of aggregate number and mass concentrations, *i.e.*, readily available observables. Multiple efforts have channeled into a formal analysis of this minimal closed system. Knowles and colleagues (Knowles et al., 2009; Cohen et al., 2011b; Cohen et al., 2011a) proposed fixed-point approaches to derive explicit solutions for the entire time course of aggregates. All the identified parameters had a clear physical meaning related to the underlying molecular events. Furthermore, analytical solutions exhibited scaling laws used in model fitting to determine the importance of primary and secondary reactions (Michaels et al., 2016). For instance, in the case of breakable fibrils (Knowles et al., 2009), the authors found that the reaction time course mainly depended on a parameter combining elongation and fragmentation rate constants. By analyzing the relationship between this parameter and the observables, such as half-time and maximal growth rate, the authors showed that even small perturbations in the fragmentation reaction significantly affected the aggregation process. This procedure was implemented in a global fitting tool for chemical kinetic models of *in vitro* protein aggregation (AmyloFit) (Meisl et al., 2016).

Model parsimony and data accuracy are essential requirements for the fitting protocol. A parsimonious model can be fitted to a wide range of experimental data acquired for different initial monomer concentrations and in the presence and absence of preformed aggregate seeds. Techniques for measuring aggregate species vary from fluorescent dyes (*e.g.*, thioflavin T) to mass spectrometry for aggregate mass concentration and single-molecule fluorescence for oligomer concentrations (Meisl et al., 2016; Cremades et al., 2012; Perrett et al., 2014). With improvements in experimental approaches come recent advances in the mathematical framework, including the investigation of stochastic aggregation kinetics (Michaels et al., 2018), the spatial dependence of protein aggregation (Cohen et al., 2014), and the nature of oligomeric species (Cremades et al., 2017; Dear et al., 2020b).

2.2.2 CHEMICAL KINETIC MODELS OF ASYN AGGREGATION

A significant number of chemical kinetic models address aSyn aggregation and its implications in PD.

Nucleation-polymerization (NP) models. NP models describe aggregation as fibril formation by monomer addition, which follows a nucleation event and escalates by secondary reactions. This process is represented by a characteristic sigmoidal growth curve, identified by a lag phase, a growth-phase, and a plateau (Knowles et al., 2009). By relying on the kinetic theory of aggregation that revolves around the master equation approach, multiple studies have combined experimental analysis with these mechanistic models to investigate underlying microscopic events and the effect of varying experimental conditions.

Buell et al. (2014) focused on the effect of pH on aSyn aggregation in the presence and absence of preformed fibril seeds. Homogeneous and heterogeneous nucleations and fragmentation were negligible in purely monomeric solutions at neutral pH. Adding preexisting aggregates to the solution enhanced the aggregation rate and shortened the lag phase of the sigmoidal growth curve. To obtain the elongation rate constant, the authors used a minimal model involving only an elongation reaction saturating at high monomer concentrations (*i.e.*, two-step elongation). On the other hand, a mildly acidic pH uncovered a much faster aggregation, that is, a longer lag phase and a more rapid growth phase, due to either surface-catalyzed nucleation or fibril fragmentation. Building on Buell et al. (2014), Gaspar et al. (2017) determined the pH-dependent role of these two events by relying on the theoretical analysis of a model including primary and secondary reactions. According to an experimental analysis supported by a global fitting procedure (Meisl et al., 2016), the autocatalytic nature of aggregation was mainly determined by secondary nucleation of monomers on fibril surfaces. These studies provided insightful information on physiological mechanisms; for example, they showed that even a slight pH change associated with different intracellular environments may enhance the production of toxic aggregates.

Small lipid vesicles can alter aSyn aggregation as much as pH levels (Galvagnion et al., 2015). Upon interaction with lipid membranes, the protein adopts a stable α -helical conformation (Fusco et al., 2014). Galvagnion et al. (2015) showed that an imbalance between free and membrane-bound aSyn states can trigger amyloid formation by enhancing heterogeneous primary nucleation on lipid surfaces. To confirm and quantify these results, the authors employed an NP model including this nucleation reaction, two-step elongation, and the conversion between intermediate species (Cremades et al., 2012). Relying on Buell et al. (2014) and Galvagnion et al. (2015), Flagmeier et al. (2016) investigated how mutations in aSyn influence the fate of amyloid fibrillation.

The authors performed three experiments to separately analyze initiation, elongation, and multiplication steps for all aSyn variants. Each experiment was associated with a mechanistic model: the NP model from [Galvagnion et al. \(2015\)](#) for lipid-induced aggregation; and linear polymerization models for strongly and weakly seeded aggregation processes at neutral and mildly acidic pH. Experimental and theoretical results agreed that the rate of surface-catalyzed nucleation on lipid membranes and fibril surfaces could vary by several orders of magnitude across different mutations.

Nucleation-conversion-polymerization (NCP) models. The NP models are coarse-grained to the extent that they do not explicitly account for aSyn intermediate species. The modeling choice mainly relates to the absence of accurate measurements of oligomer concentrations in the aggregation process. Oligomers are indeed transient and highly heterogeneous species. The development of new methods to report their levels has motivated the transition to NCP models ([Dear et al., 2020b](#); [Garcia et al., 2014](#)). These models include the structural interconversion from disordered clusters to oligomers with a partially formed fibrillar structure, eventually growing into fibrils.

Multiple studies on aSyn aggregation have provided experimental and theoretical support to this updated kinetic theory ([Horrocks et al., 2015](#); [Tosatto et al., 2015](#); [Iljina et al., 2016](#); [Zurlo et al., 2021](#)). Single-molecule Förster Resonance Energy Transfer (FRET) experiments can distinguish two oligomeric populations associated with low and high FRET levels. Type-A oligomers are early-formed, disordered, highly degradable, and mildly toxic species, whereas type-B oligomers are later-forming, more compact, degradation-resistant, and ROS promoting species ([Cremades et al., 2012](#); [Iljina et al., 2016](#)). Both oligomeric species appear as required subsequent steps in the aggregation process ([Dear et al., 2020a](#)). As displayed in Figure 2.1A, NCP models tailored for aSyn aggregation include primary nucleation, which generates type-A oligomers from monomers, and the structural conversion from type-A to type-B oligomers and then fibrils ([Cremades et al., 2012](#); [Iljina et al., 2016](#)). Both fibrillar and oligomeric species increase in size by monomer-dependent elongation. In this reaction network, the conformational change between oligomeric species emerges as a crucial step for initiating aSyn aggregation. Upon fitting the model to experimental data, [Cremades et al. \(2012\)](#) showed that the corresponding kinetic rate is slow, thus providing a sufficient lag time for the protein quality control machinery to hamper the pathological process. A potential therapeutic strategy may employ molecular chaperones or small molecules to regulate this reaction. A follow-up study ([Iljina et al., 2016](#)) used the NCP model to quantify seeding ability and conditions for type-B oligomers and fibrils, with implications on prion-like spreading. Model predictions showed that fibrils were more prone to seed aggregation, whereas oligomers contributed mostly to the increase of oxidative stress.

The studies reported above focused on the early stages of aSyn aggregation; thus, they did not require full-time analytical solutions. To extend the analysis to the entire time course, [Dear et al. \(2020b\)](#) provided a general mathematical framework for NCP models. The authors proposed a reaction network with a single oligomeric state. Such a coarse-grained model is suitable to avoid overfitting in the analysis of experimental data on total oligomer concentration that, as such, did not account for differences between oligomeric populations, *e.g.*, see [Zurlo et al. \(2021\)](#). Moreover, the model is instrumental for classifying oligomers according to theoretically defined metrics: persistence or half-life, abundance, and productivity, *i.e.*, the propensity to form fibrils rather than dissociate. The authors proved that oligomers were more prone to dissociation than fibrillation as a universal feature. Focusing on aSyn oligomers, results showed that they were very kinetically stable, more persistent than monomers, relatively abundant, and highly productive when compared to other proteins. Overall, chemical kinetic models contribute to a detailed mechanistic understanding of aSyn aggregation by combining theoretical results and *in vitro* data acquired through advanced techniques. By exploring a wide range of *in silico* experimental conditions, these models return coherent predictions that can be useful for studying PD. Further investigation in this direction should focus on whether and how these results apply to *in vivo* settings to provide insights into the mechanisms of neurodegeneration and, possibly, inform therapeutic strategy design ([Sinnige, 2022](#)).

2.3 MECHANISTIC MODELS OF ASYN AGGREGATION AND DEGRADATION PATHWAYS

Given the central role of degradation in regulating aSyn homeostasis, multiple models have investigated this process in relation to PD neurodegeneration, including models solely focused on aSyn aggregation and degradation or *miscellaneous* models that include additional pathogenic processes (*e.g.*, ROS production and defective dopamine metabolism). These degradation models offer a heterogeneous picture in terms of specific mechanisms they consider, granularity, and modeling approaches ([Bakshi et al., 2019](#)) (see Figure 2.2). The biological processes can be represented by varying degrees of detail, and the modeling formalism can range from ordinary differential equations (ODEs) and algebraic equations to stochastic simulations, partial differential equations, or flux balance analysis ([Lloret-Villas et al., 2017](#); [Bakshi et al., 2019](#)).

2.3.1 DEFECTIVE PROTEIN DEGRADATION MACHINERY AND PD NEURODEGENERATION

The proteolytic machinery combined with the molecular chaperone system ensures the renewal of functional components and regulates the fate of altered

proteins to avoid the build-up of intracellular damage. In PD and other neurodegenerative diseases, degradation efficiency declines with age and oxidative stress (Szweda et al., 2002), thus increasing misfolded aSyn and aggregate levels. On the other hand, aSyn aggregation can hamper the proteolytic machinery causing the accumulation of other cellular components (Tai et al., 2008). The interplay of aSyn aggregation and impairment of degradation in multiple negative feedbacks poses a causality dilemma (Ciechanover et al., 2003): which pathological process comes first? There is still no clear answer. Degradation defects and aSyn aggregation can be either primary or secondary events of neurodegeneration (Xilouri et al., 2013; Scrivo et al., 2018). The investigation of their role in PD is even more challenging due to interfering factors such as increased oxidative stress and mitochondrial dysfunctions (Poewe et al., 2017; Szweda et al., 2002). Systems biology models can help in elucidating these contributions by providing *in silico* benchmarks to assess these hypotheses.

The ubiquitin-proteasome pathway (UPP). UPP is the dominant clearance route for mislocated, misfolded, damaged, and short-lived proteins. Target substrates are selectively tagged by polyubiquitin chains through multiple enzymatic reactions and then transferred to the proteasome. Ubiquitinating and deubiquitinating enzymes such as PARKIN and UCH-L1 play a central role in modulating protein degradation in PD, as indicated by UPP-related familial cases (Walden et al., 2017; Tai et al., 2008). For instance, UCH-L1 maintains a stable pool of monomeric ubiquitin; an imbalance of this pool can lead to poor substrate labeling with consequent impaired proteasomal degradation. Furthermore, the presence of LBs as a PD molecular hallmark corroborates the correlation between proteasomal dysfunctions and PD pathogenesis. Indeed, LBs mainly consist of ubiquitin-tagged aSyn aggregate, ubiquitinated proteins, and UPP components (Tai et al., 2008; Kuzuhara et al., 1988; Shults, 2006). According to multiple studies (Olanow et al., 2006; McNaught et al., 2001), their composition suggests a potential cytoprotective role for LBs, which may act as sinks for poorly degraded proteins to reduce the toxic effect of protein accumulation and slow down neurodegeneration. Still, it is unclear whether these cytoplasmic inclusions represent a harmful or defense molecular mechanism for the neuron. UPP dysfunctions and aSyn aggregation create a negative feedback loop. Genetic and age-related impairments in UPP may hamper the proper degradation of misfolded aggregate-prone aSyn. On the other hand, aSyn species refractory to proteasomal degradation, such as mutant aSyn forms, oligomers, and fibrils, can inhibit proteasomal activity by sequestering the proteasome. In addition, the interaction with oligomers and oxidatively damaged aSyn may directly alter proteasome subunits, as shown in the green box in Figure 2.2A.

The autophagy-lysosome pathway (ALP). ALP removes long-lived proteins, lipids, and dysfunctional organelles, such as aging mitochondria (*i.e.*, mitophagy),

thus maintaining neuronal homeostasis. It includes three degradation mechanisms: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. Experimental findings indicate that both UPP and ALP degradation of aSyn can occur (Webb et al., 2003). The autophagic mechanisms are involved in physiological aSyn turnover and aSyn aggregates degradation (see the purple box in Figure 2.2A). Specifically, wild-type aSyn monomers are degraded mainly by CMA (Cuervo et al., 2004), which relies on molecular chaperones to recognize a specific motif on the target substrate. Upon chaperone binding, the cargo interacts with the membrane receptor LAMP2A, which assembles into multimeric translocation complexes. The substrate then enters the lysosome lumen, where it is rapidly degraded. CMA also clears ALP and UPP-related proteins, such as LRRK2, UCH-L1, DJ-1, and GCase (Hou et al., 2020). Furthermore, macroautophagy represents the main clearance route for aSyn aggregates due to the inaccessibility of the CMA degradation motif (Scrivo et al., 2018). This degradation mechanism consists in the formation of autophagosomes, *i.e.*, double-membrane vesicles surrounding the cargo and fusing with lysosomes to allow hydrolase-mediated degradation in the lysosomal lumen. In selective macroautophagy, specific autophagosome membrane receptors target protein aggregates. The link between impaired autophagy and PD neurodegeneration conceals a complex interaction network between aSyn aggregates and the lysosomal proteolytic machinery. Growing evidence supports the negative effect of aSyn aggregation on autophagy acting on multiple levels (Scrivo et al., 2018; Xilouri et al., 2016), as displayed in Figure 2.2A. At the same time, defective ALP cannot handle harmful aSyn species, thus leading to their accumulation. Such defects can originate from various sources, such as increased oxidative stress, aging, and dysfunctional proteins related to genetic PD forms, namely, LRRK2, DJ-1, and GBA (Blauwendraat et al., 2020), and with a physiological role in ALP.

2.3.2 DEGRADATION MODELS

UPP models. Relying on experimental evidence pointing to the essential contribution of UPP to neurodegeneration (McNaught et al., 2004; Cuervo et al., 2010), Sneppen et al. (2009) provided a minimal theoretical model of the interplay between impairments in proteasomal degradation and aSyn fibrillation. The proteasome ensured protein homeostasis by keeping the amount of aSyn aggregates low. On the other hand, insoluble aSyn fibrils sequestered this proteolytic machinery and formed a slowly-degraded complex, thus reducing proteasome function. Computational simulations of this ODE system supported by stability analysis showed a bifurcation behavior. When the fibril level exceeded a particular threshold related to proteasome production capacity, the double-negative feedback broke aSyn homeostasis in favor of a pulsatile oscillatory regime characterized by spikes and long periods of low

proteasome concentrations. As a result, the system alternated between the hypothetical recovery of proteolytic activity and the uncontrolled accumulation of harmful aggregates and fibrils. Several oscillations eventually built up toxicity over decades, thus explaining the slow progression of PD neurodegeneration. The authors therefore associated proteasome overload and the subsequent oscillatory regime with the onset of sporadic PD phenotype. In a follow-up study (Pigolotti et al., 2013), the stochastic analysis of the core negative feedback confirmed and generalized the above phenomenon for the whole protein clearance machinery. The bifurcation behavior characterized by a *healthy* homeostatic state and an oscillatory *disease* state indeed emerged for both UPP and ALP degradation. In addition, stochastic simulations captured dynamical differences between the two degradation systems, modeled as enzymatic reactions slowly reducing aggregate concentrations. Lysosomes were assumed to be more efficient but less abundant than proteasomes. Fibril-mediated sequestration of the proteasome therefore led to regular and almost deterministic oscillations, whereas lysosome overload resulted in irregular fluctuations.

In contrast to the high-level representation of the UPP in Sneppen et al. (2009) and Pigolotti et al. (2013), Proctor et al. (2007) and Proctor et al. (2010) proposed a detailed description of this pathway by including mono- and polyubiquitination, proteasome binding, ATP-dependent degradation, and proteasome inhibition by protein aggregates. These studies were devoted to the mathematical modeling of the aging process, which contributes to the decline of proteolytic activity and the build-up of oxidative stress due to flawed mitochondria (Kirkwood et al., 2003; Mc Auley et al., 2017). Both models indeed investigated the role of the proteasomal degradation pathway in age-related neurodegeneration. In Proctor et al. (2007), the authors first presented a stochastic model for aggregation and proteasomal degradation of a generic protein under normal homeostasis and aging. The authors then tailored the system to assess the UPP role in aSyn aggregation during PD neurodegeneration (Proctor et al., 2010). The final model included additional modules representing aSyn metabolism and UCH-L1 deubiquitination activity in proteasomal degradation. This stepwise approach relied on the idea that the molecular landscape of neurodegeneration can be arranged in building blocks, as most biological processes are assumed to be modular (Lloret-Villas et al., 2017; Hartwell et al., 1999). Both general and specific models proved to be functional for investigating the negative feedback of protein aggregate formation and UPP impairment. Employing the more general model (Proctor et al., 2007), the authors explored the impact of various experimental procedures, *e.g.*, shutting down a ubiquitin-protein ligase or the proteasome activity. The results suggested that depleted ubiquitin pools might contribute to proteostasis impairment. In addition, relying on the model tailored for PD (Proctor et al., 2010), the authors analyzed the effects of both mutated and oxidatively damaged UCH-L1. Stochastic simulations indicated that proteasome inhibition might not be the triggering

event for neurodegeneration but rather an exacerbating factor accelerating aSyn aggregation and inclusion formation. Both models were validated against data gathered in *ad-hoc* designed experiments, in a virtuous cycle of model refinement and *in vitro* experiments.

UPP and ROS models. Since oxidative stress and impairments in UPP degradation are interconnected, multiple studies have analyzed their synergistic effect on aSyn homeostasis. [Cloutier et al. \(2012b\)](#) provided a mathematical model accounting for the interplay between aSyn aggregation, ROS production, and proteasome-mediated degradation. Given the variety of molecular processes involved, the authors were able to evaluate the impact of multiple risk factors on aSyn metabolism. For instance, perturbations of system parameters related to oxidative stress and protein clearance capacity reproduced the high variability of LB concentration in PD forms ([Cloutier et al., 2012b](#); [Foltynie et al., 2002](#)). Moreover, supported by experimental verification ([Finnerty et al., 2013](#)), deterministic simulations of the model uncovered a bistable process whereby the system irreversibly switched from a healthy to a disease state, identified by high misfolded aSyn and ROS levels, in response to toxin exposure, genetic mutations, and aging. On the other hand, the model could not recapitulate the oscillatory regime due to decreased levels of available clearance machinery obtained in [Sneppen et al. \(2009\)](#) and [Pigolotti et al. \(2013\)](#). To identify the primary mechanisms determining the bifurcation behavior, the authors reduced the system to focus only on the double-positive feedback between aSyn misfolding and ROS production ([Cloutier et al., 2012a](#)). This minimal version retained the bistable behavior, thus associating PD onset with a switch-like transition in response to enhanced ROS concentrations. In line with [Sneppen et al. \(2009\)](#) and [Pigolotti et al. \(2013\)](#), the central idea remained that neurodegeneration results from the transition between two dynamical behaviors.

[Raichur et al. \(2006\)](#) also presented a model targeting the interplay between oxidative stress, aSyn aggregation, and UPP dysfunctions. Compared to [Cloutier et al. \(2012b\)](#), this system displayed a more granular description of the UPP pathway, from ubiquitination and proteasome recognition to degradation. The close molecular detail of ROS production and UPP degradation provided in the model inevitably hampered the theoretical analysis. On the other hand, the explicit representation of proteasome-mediated degradation enabled the authors to explore different scenarios of UPP-related genetic predisposition: mutations in *UCH-L1*, *PARKIN*, and *DJ-1* genes, combined with age-related oxidative stress. These familial PD forms determined the accumulation of ubiquitinated proteins, leading to a more rapid aSyn aggregation than sporadic forms. Furthermore, the synergistic combination of ROS production and UPP-related genetic mutations fueled aSyn aggregation. These results pointed to therapeutic strategies involving restored UPP functionality or enhanced ALP activity in combination with antioxidant treatments.

UPP and ALP models. The modeling picture outlined so far focuses on the ubiquitin-proteasome pathway, while the autophagy-lysosome pathway is under-represented despite its critical role in aSyn turnover; when considered, autophagic clearance has often been incorporated as a pseudo-first-order reaction. This is not the case of [Ouzounoglou et al. \(2014\)](#) who provided a detailed representation of selective macroautophagy, CMA, and proteasome-mediated degradation, focusing on the inhibitory feedback between aSyn oligomers and the CMA receptor LAMP2A. Upon model calibration and validation against aSyn overexpression data from human neuroblastoma cells, the authors performed stochastic simulations to explore different interventions with neuroprotective potentials, such as shutting off DA production, increasing LAMP2A levels, and reducing aSyn synthesis. The resulting predictions of increased cell viability were in agreement with experimental evidence and literature ([Xilouri et al., 2009](#); [Vekrellis et al., 2009](#)).

To provide valuable insights into the role of aSyn degradation in neurodegeneration, a mechanistic model should integrate ALP and UPP degradation mechanisms, ideally accounting for the crosstalk between these pathways. In this direction, [Sass et al. \(2009\)](#) proposed a model targeting aSyn aggregation, UPP and ALP degradation, and DA metabolism and based on the biochemical system theory. A granular representation of these mechanisms and their interactions facilitated the analysis of specific system perturbations in both disease and treated states (*e.g.*, enhanced aSyn fibrillation and aggregation rate). Including DA metabolism in the model also enabled the authors to simulate impairment of DA vesicle packaging and available treatments related to its shortage, such as L-Dopa therapy combined with monoamine oxidase inhibitors. Increasing model complexity even further, [Büchel et al. \(2013\)](#) presented an extended model of a dopaminergic neuron including the highest number of molecular components among the available PD models of aSyn homeostasis ([Lloret-Villas et al., 2017](#)). The model described eleven biological processes, including DA metabolism and transport, aSyn aggregation, oxidative stress, UPP and ALP clearance mechanisms, and specific details on PARKIN and DJ-1 proteins. Here, the modeling approach adopted was crucial to tackle model complexity. The authors employed flux balance analysis to investigate the steady-state behavior of the model rather than deterministic or stochastic analysis based on mass action kinetics. The qualitative prediction of multiple *in silico* experiments pointed to increased ROS, neurotoxin MPTP, and aSyn levels as crucial factors in PD neurodegeneration.

aSyn degradation and axonal transport models. The degradation models analyzed so far do not account for the spatial dependence of aSyn aggregation and degradation mechanisms. However, aSyn homeostasis and disease progression may strongly depend on intraneuronal protein propagation. Along this line, [Kuznetsov et al. \(2016a\)](#) and [Kuznetsov et al. \(2016b\)](#) investigated aSyn

axonal transport and how its perturbation between the somatic and synaptic compartments affected the aggregation and degradation processes. By relying on a two-compartment ODE model, the authors predicted axonal death due to aSyn accumulation at the synaptic terminal, pointing to defective protein clearance machinery as the primary cause for aSyn aggregation onset compared to reduced aSyn production and transport (Kuznetsov et al., 2016b). Differences in the degradation of aSyn monomers and aggregates were determined by assuming distinct half-lives. Furthermore, building on recent findings on the absence of aSyn fibrils in LB inclusions (Shahmoradian et al., 2019), the authors proposed a mathematical model of aSyn aggregation, degradation, and transport to test the hypothetical autocatalytic nature of LB formation and its impact on protein accumulation (Kuznetsov et al., 2022). Here, the authors assumed spatial-dependent proteasomal and autophagic degradation mechanisms. The study pointed to the central role of the autophagic clearance pathway in LB formation and PD progression; defective autophagy may favor the aggregation of membrane-bound organelles catalyzed by aSyn aggregates and, thus, LB formation. Most degradation models do not differentiate between aSyn fibrillation and LB formation, whereas chemical kinetic models do not even include these cytoplasmic inclusions. The detailed implementation of LB formation and maturation would allow for the investigation of LB neuroprotective role (McNaught et al., 2001; Kuznetsov et al., 2022).

The wide variety of degradation models reflects PD multifactorial nature. Such a modeling effort has provided multiple insights into the interplay between aSyn aggregation and protein degradation by spanning various levels of molecular detail, from coarse-grained to highly granular representations. Minimal models have been used to uncover dynamical features of disease progression (e.g., Sneppen et al. (2009), Pigolotti et al. (2013), and Cloutier et al. (2012a)). More detailed models (e.g., Proctor et al. (2010) and Ouzounoglou et al. (2014)) have proven to be suitable for testing the effects of potential treatments. Overall, degradation models move toward an integrative representation of PD pathogenesis. Future work may focus on the crosstalk between UPP and ALP pathways, the interplay between autophagy and apoptosis in PD neurodegeneration (Tavassoly et al., 2015), and the integration of other control systems for protein homeostasis such as the molecular chaperone system (Proctor et al., 2005).

2.4 DISCUSSION

Mechanistic modeling grounded in molecular-level knowledge is a powerful tool for tackling PD biological complexity. By exploring the intricate interaction network of neurodegeneration through *in silico* experiments, mathematical models can investigate the key drivers of PD pathogenesis, discover promising targets, and even simulate the effect of potential single or combination

therapies. Given the pivotal role of aSyn homeostasis in PD, this review has provided a collection of available mathematical models of aSyn aggregation and degradation pathways. Chemical kinetic models focus on a single molecular pathway and rely on a unifying kinetic theory that translates into the consistency of modeling formalism (*i.e.*, master equations approximated to ODE systems). In contrast, degradation models describe multiple biological processes and adopt a wide range of approaches to analyze the dynamical features of the system. The latter are modeled as deterministic and stochastic simulations based on mass action kinetics; yet, there are some exceptions, such as flux balance analysis, mainly related to the complexity of the reaction network considered. Stochastic approaches (Gillespie, 1977; Wilkinson, 2018) have been employed to analyze biological processes with low molecular counts and capture their inherent fluctuations. Future investigation may explore the role of intrinsic noise in PD onset and progression since it can unpredictably affect the system behavior, for example, by triggering the transition from a healthy to a disease state leading to PD onset (Bakshi et al., 2019; Cloutier et al., 2012b).

Integrating chemical kinetic and degradation models. Both chemical kinetic and degradation models include a representation of aSyn aggregation. As shown in Figures 2.1A and 2.2A (light-blue box), chemical kinetic models account for each molecular step separately, whereas degradation models employ macro-variables for different aSyn aggregate species according to their size and functional features. The master equation approach returns parameters that physically relate microscopic events to macroscopic observables. On the other hand, a higher-level representation of the process enables the targeting of additional pathways and the investigation of their interplay in the molecular network. A modeling opportunity lies at the intersection of the two groups: degradation models can be integrated with a chemical kinetic representation of the aggregation process. In this way, they can benefit from the kinetic rate constants derived from fitting to the wide variety of *in vitro* data available. At the same time, chemical kinetic models can be extended to include UPP and ALP pathways, converging toward an *in vivo* representation. These integrated models can be employed to determine the relative importance of each microscopic event in relation to degradation mechanisms (*e.g.*, the structural interconversion between type-A and type-B oligomers), thus suggesting specific target species and reactions. Recent studies have moved a few steps toward model integration (Pigolotti et al., 2013; Thompson et al., 2021). Here, clearance mechanisms are approximated by pseudo-first-order reactions to facilitate theoretical analysis. Further investigation should focus on an explicit representation of the degradation processes, especially the ALP pathway, considering the growing interest in the role of autophagy in neurodegeneration (Gan-Or et al., 2015; Klein et al., 2018; Senkevich et al., 2020). However, this choice translates into an increased complexity of the system itself. Future work should

favor an integrative representation of pathological mechanisms that balances high granularity and analytical or computational tractability (Ribba et al., 2017).

In vitro/in vivo translation. Integrating chemical kinetic and degradation models is part of the modeling efforts currently chasing the mechanistic representation of aSyn aggregation *in vivo*. To this end, both groups of models would benefit from calibration and validation against measurements of aggregate levels in living systems. However, the availability of this quantitative information is limited by technical difficulties in monitoring the time evolution of aggregates and addressing the heterogeneous and elusive nature of oligomers (Sinnige, 2022). As a result, chemical kinetic models have been trained mainly on *in vitro* data. Among the limitations of using this type of data is the unclear relevance of the results on a disease time scale, moving from the few hours or days of test-tube experiments to the decades of PD progression (Bakshi et al., 2019). In addition, *in vitro* aggregation assays typically rely on initial protein concentrations that far exceed aSyn levels detected intracellularly and in post-mortem brain extracts from PD patients and controls (Banks et al., 2020; Hannestad et al., 2020; Paleologou et al., 2009). On the other hand, concentrations used *in vitro* are comparable to those estimated *in vivo* at presynaptic terminals (Bodner et al., 2009; Westphal et al., 2013; Wilhelm et al., 2014; Perni et al., 2017). Such a potential discrepancy in aSyn concentrations may hamper translatability due to the strong dependence of aggregation rates on initial protein levels (Sinnige, 2022; Meisl et al., 2020). Nevertheless, studying aggregation mechanisms *in silico* can bridge *in vitro* and *in vivo* settings. For example, *in vitro* studies paired with chemical kinetic models showed that adding lipid vesicles to the solution accelerates *in vitro* aggregation, which occurs at lower bulk concentrations (Buell et al., 2014; Gaspar et al., 2017). This result may suggest a similar mechanism in living systems, given aSyn intracellular localization at presynaptic terminals and its physiological role in vesicular trafficking. Moreover, according to a recent perspective paper (Sinnige, 2022), the late onset of amyloid formation in sporadic PD may emerge from the intrinsic properties of aggregation kinetics, specifically from a combination of dominant secondary processes and prion-like spreading. Age-related defects in protein degradation may also work in concert with secondary events in the aggregation process to drive amyloid formation. Qualitative similarities between *in vitro* and *in vivo* aggregation mechanisms support this scenario. Indeed, biophysical features of amyloid formation detected in test-tube experiments seem to be preserved in living systems (Sinnige et al., 2020; Sinnige, 2022). On the other hand, a quantitative analysis of the aggregation kinetics *in vivo* is lacking. Current studies focus on the applicability of *in vitro* molecular-level mechanistic studies to living systems and the related modeling limitations and opportunities, as extensively discussed in Meisl et al. (2022). Molecular models combining the kinetic of aSyn aggregation and multiple biological pathways underlying PD neurodegeneration can assist *in vitro* to *in vivo* translation (Cassotta et al., 2022).

Modulating the protein aggregation pathway. PD research efforts seek to provide a solid foundation for therapeutic strategy design in PD. Not only can mechanistic models of aSyn homeostasis identify candidate target mechanisms, but they can also support the development of various anti-aggregation treatments currently under study. In this context, chemical kinetic models provide a theoretical framework for analyzing the inhibitory effect of multiple compounds on the aggregation process. Employing a nucleation-polymerization model that accounts for interactions with a new general component, [Michaels et al. \(2020\)](#) identified different regimes of inhibition related to specific combinations of kinetic rate constants and binding affinities, thus optimizing the inhibitor efficacy of the compound. Furthermore, a follow-up study simulated the impact of therapeutic interventions on key oligomeric features by tuning specific rate constants ([Michaels et al., 2022](#)). This analysis suggested that inhibiting the primary nucleation event that dominates lipid-induced aSyn aggregation strongly reduces the peak and overall concentration of the oligomeric population. As shown in [Perni et al. \(2018\)](#), this mechanism of action characterizes the natural aminosterol compound known as trodusquemine, which interferes with aSyn aggregation by blocking its interaction with lipid membranes and thus inhibit lipid-induced primary nucleation and fibril-mediated secondary nucleation. Also, it can suppress aggregation-related cytotoxicity by hampering oligomer interaction with cell membranes ([Limbocker et al., 2020](#)). In addition to small molecules, potential therapeutic strategies targeting different aSyn species and reactions include peptides and peptidomimetics, antibodies, and molecular chaperones ([Fields et al., 2019](#)). In this respect, chemical kinetic studies have recently investigated the mechanism of action of specific molecular chaperones ([Arosio et al., 2016](#); [Cohen et al., 2015](#)) and antibodies ([Linse et al., 2020](#)) on amyloid beta aggregation in Alzheimer's disease (*e.g.*, Brichos and Aducanumab, respectively); the results were in good agreement with clinical outcomes. These studies should be extended to Parkinson's disease, *e.g.*, supporting clinical trials that involve anti-aSyn antibodies such as prasinezumab and cinpanemab.

Targeting protein degradation pathways. Inhibiting specific molecular steps of the aggregation pathway is not the only possible intervention targeting impaired aSyn homeostasis. Genetic-based strategies reducing protein synthesis have been proposed to avoid aggregation in the first place, such as employing antisense oligonucleotides, RNA interference, and β 2-adrenoreceptor agonists ([Fields et al., 2019](#)). However, side effects may arise from protein down-regulation due to aSyn physiological role in presynaptic vesicle trafficking. Mechanistic models should be employed to determine a critical range for aSyn expression levels, facilitating the transition from preclinical to clinical trials. However, currently available models do not include a genetic level representation; thus, transcription and translation inhibitions can be simulated only by

varying the rate constant associated with aSyn production. Another strategy consists in modulating UPP and ALP degradation mechanisms to hamper aSyn aggregation and PD neurodegeneration. Autophagy can be enhanced selectively or as a whole. An overall ALP enhancement driven by chemical compounds such as rapamycin and trehalose has limited applicability in PD since it may interfere with essential physiological processes and the homeostasis of proteins other than aSyn (Scrivo et al., 2018). The same limitations hold for therapeutic strategies focusing on overall UPP degradation (Dantuma et al., 2014). In contrast, selective targeting of specific molecular steps in proteasomal degradation, CMA, and macroautophagy may be a promising strategy (Scrivo et al., 2018). UPP-related candidate treatments include stimulating ubiquitination and regulating the activity of chaperone systems. In addition, selective ALP activators may act by modulating LAMP2A assembly and translocation, enhancing autolysosome fusion, and targeting lysosomal function. Ideally, mechanistic models can support the development of current and future interventions of this type. However, further research is needed to reach this goal. Most degradation models analyzed here lack a granular representation of autophagic pathways that would allow the simulation of these selective strategies. Still, they can provide insightful information on the role of UPP and ALP degradation pathways in neurodegeneration as the first step toward *in silico*-aided therapeutic strategies against PD.

Toward multiscale QSP models for PD. The points discussed above highlight the need for multiscale QSP models able to link molecular mechanisms to cellular and tissue-level events and, finally, clinical manifestations. These models would leverage the large amount of available data associated with neuroimaging techniques and disease-rating scales for motor symptoms (MDS-UPDRS) and cognitive impairment (MoCa and MMSE) (Poewe et al., 2017; Roberts et al., 2016). This type of data has already been employed by quantitative models that address the heterogeneity of the PD clinical picture by accounting only for physical symptoms (Sarbaz et al., 2016). In addition to quantitative tools supporting PD diagnosis, a mechanistic understanding of the molecular processes underlying PD pathogenesis and how they connect to the clinical endpoints is essential to inform drug discovery and development. The models in this review represent a starting point in this sense. Being confined at the molecular level, they have to settle for surrogate endpoints such as ROS, aSyn aggregate, and LB levels to identify disease phenotypes. Moving to the cellular, tissue, and organ levels through network-based, epidemiological, and compartmental PK/PD approaches (Carbonell et al., 2018; Bloomingdale et al., 2021a; Bloomingdale et al., 2022a; Lin et al., 2022), these models can account for aSyn prion-like propagation between neurons and across multiple brain areas, using potential PD biomarkers such as aSyn concentrations in plasma and CSF (Parnetti et al., 2019) and magnetic resonance imaging measurements.

Overall, this review makes a case for using mechanistic models of aSyn homeostasis as valuable tools to uncover PD pathogenesis, suggesting their integration in a QSP framework to provide blueprints for therapeutic interventions.

A MECHANISTIC MODEL OF PURE AND LIPIDIC α -SYNUCLEIN AGGREGATION FOR ADVANCING PARKINSON'S THERAPIES

Chapter Abstract Alpha-synuclein (aSyn) plays a crucial role in Parkinson's disease, with various aggregates proposed as pathogenic triggers and therapeutic targets. However, anti-aSyn aggregation compounds often fail due to limited knowledge of the underlying molecular basis. In particular, interactions with lipid membranes are central to both physiological and pathological roles of aSyn, yet their underlying mechanisms remain unclear. Disrupting this balance may drive Parkinson's onset and progression, underscoring the need for a mechanistic understanding of pure and lipid-mediated aggregation. Building on well-established *in vitro* aggregation studies, we propose a mathematical model of aSyn accumulation incorporating both aggregation routes via a nucleation-conversion-polymerization process with self-amplifying loops and toxic oligomers. Model calibration uses data from *in vitro* assays mimicking physiologically relevant conditions, providing insights into transient and stable aSyn intermediates. Incorporating aSyn-lipid interactions enables *in silico* exploration of how lipid-to-aSyn ratio influences aggregation, with possible implications for neurodegeneration. Sensitivity analysis highlights secondary nucleation inhibition as a potential anti-aggregation strategy. Overall, our work contributes to a unified framework for investigating *in vitro* aSyn aggregation and evaluating Parkinson's therapies by building on existing models. It can serve as a stand-alone tool and a modular component in multiscale models, with potential applications in quantitative systems pharmacology.

This Chapter is based on the following work: Righetti et al., *A mechanistic model of pure and lipidic α -synuclein aggregation for advancing Parkinson's therapies* (Righetti et al., 2025). As first and co-corresponding author of this manuscript, I conceptualized the project design, developed the model, extracted and curated experimental data from the literature, performed model calibration and validation, conducted *in silico* analyses, and wrote the manuscript.

3.1 INTRODUCTION

The neuronal protein alpha-synuclein and its aggregation play a critical role in the onset and progression of Parkinson's disease. This close connection between aSyn and PD pathology is underscored by recent advances in aSyn-based biomarkers, which have defined a new biological framework for the disease (Simuni et al., 2024; Soto et al., 2025). Moreover, different aSyn aggregates have been suggested as crucial pathogenic triggers of PD neurodegeneration and considered potential therapeutic targets. Therefore, each new insight into aSyn molecular landscape, and ultimately its thorough understanding, could serve as a linchpin for the rational design of anti-aSyn aggregation therapies. Over the past few decades, significant progress has been made in characterizing aSyn aggregation. It is well-established that, upon misfolding due to post-translational modifications, increased oxidative stress, or other cellular stressors, aSyn monomers slowly aggregate into oligomeric species, thus creating nucleation seeds. In return, seeding-competent oligomers can rapidly accumulate, forming protofibrils and insoluble fibrils. Multiple aSyn species eventually gather into large cytoplasmic inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs), representing one of PD main molecular hallmarks. The transition from monomers to fibrils through multiple intermediate species relies on a complex molecular network. The resulting aggregates differ in size, conformational structure, and role in the aggregation pathway. The mechanisms involved can be divided into primary and secondary reactions, and their corresponding reverse reactions. Primary events include (i) homogeneous and heterogeneous (*i.e.*, surface-catalyzed) nucleation mechanisms combining monomers into nucleation seeds, *i.e.*, newly formed oligomers, as the first step of the aggregation cascade, (ii) conformational changes between intermediate aggregates, and (iii) fibril formation and elongation by monomer addition. On the other hand, nucleation catalyzed by the surface of existing fibrils and fibril fragmentation are labeled as secondary events, resulting in positive feedback loops.

Uncontrolled accumulation hinges on aSyn oligomers, which have been identified as crucial early steps of the aggregation pathway and suggested as key contributors to PD neurodegeneration. Two oligomeric species, *i.e.*, type-A and type-B oligomers, have been differentiated based on their structural conformation and degradation susceptibility (Cremades et al., 2012; Zunke et al., 2018). Type-A oligomers are intrinsically disordered and highly susceptible to proteinase-K treatment, whereas type-B oligomers have a partially formed fibrillar structure characterized by high β -sheet content and high resistance to proteinase-K degradation. This distinction arises not only from the different structural conformations of type-A and type-B oligomers but also from their related roles in the process. Experimental evidence points to oligomers as the major source of inherent toxicity within the aggregation pathway, compared to insoluble aggregates such as mature fibrils and Lewy bodies. Indeed, oligomer

formation results in higher levels of oxidative stress, increased cell membrane permeability, deregulated calcium and metal ion homeostasis, impaired mitochondrial function, and dysfunctional protein degradation (Gracia et al., 2020; Cascella et al., 2021). In this context, type-B oligomers have been suggested as the most cytotoxic aSyn species due to their specific conformation associated with high degrees of β -sheet content and hydrophobic exposure, in contrast to non-toxic type-A oligomers (Cremades et al., 2012; Gracia et al., 2020). For instance, cell-free *in vitro* and *in vivo* experiments have shown that these oligomers can rapidly and significantly increase cytoplasmic reactive oxygen species (ROS) production (Cremades et al., 2012; Zunke et al., 2018; Fusco et al., 2017). These oligomeric species are sequential intermediates in fibril formation, thus defined as on-pathway. Given the heterogeneous and elusive nature of oligomers, additional species may result from side reactions (*i.e.*, off-pathway), depending on experimental conditions and cellular co-factors, and their presence cannot be ruled out (Alam et al., 2019; Paslawski et al., 2014; Lorenzen et al., 2014; Gracia et al., 2020). However, we focus on type-A and type-B oligomers as they represent the primary path to fibrils in aSyn aggregation (Cremades et al., 2017; Dear et al., 2020a).

Strong evidence also supports co-aggregation between aSyn and lipid molecules, both *in vitro* and *in vivo*, in the pathological scenario. For instance, Lewy bodies have been observed as cytoplasmic deposits of aSyn filaments alongside crowded membranes from vesicles and fragmented organelles (Shahmoradian et al., 2019), potentially incorporated at every stage in the formation process (Hellstrand et al., 2013). In more controlled environments such as *in vitro* aggregation assays, lipid-protein co-assemblies can spontaneously form from mixtures of monomeric protein and negatively charged lipid membranes, typically introduced as small unilamellar vesicles (SUVs). Different factors, *e.g.*, vesicle composition and lipid-to-aSyn ratio, affect the co-aggregation process, thus modulating fibril morphology and aggregation kinetics (Hellstrand et al., 2013). Galvagnion et al. (2024) showed that lipid-aSyn binding can lead to the rapid but reversible breakup of vesicles into small disc-like lipid-protein structures that mediate favorable protein-protein interactions, inducing aggregation. As a result, lipidic fibrils emerge as lipid-aSyn co-aggregates, differing from pure protein fibrils in flexibility and thermodynamical stability. Nonetheless, these species share a connection. Despite these differences, lipid-decorated fibrils can undergo structural conversion upon heating or proteolytic protein removal, potentially transitioning into pure protein fibrils (Galvagnion et al., 2024). Pure protein and lipidic fibrils not only differ in structure but also in their modes of formation. Both fibrillar species result from monomer nucleation and oligomeric conversion but differ in the elongation mechanism. Indeed, new mechanistic insights suggest that lipids act as reactants consumed during aggregation, wherein the interaction of existing fibrils with monomer-coated lipid vesicles boosts fibril elongation (Galvagnion et al., 2024; Dear et al., 2024). These insights result from the extensive study of aSyn aggregation in test-tube,

in vitro, and *in vivo* experiments ([Knowles et al., 2009](#); [Meisl et al., 2022](#)). Of particular interest, due to their invaluable contribution to the field, are the chemical kinetic models, developed to complement test-tube experiments. Evolving alongside advancing methodologies for aggregate detection, these models aim to determine the microscopic events that govern aggregation and their kinetic rates under specific experimentally controlled scenarios ([Knowles et al., 2009](#); [Meisl et al., 2016](#); [Dear et al., 2024](#); [Rinauro et al., 2024](#)).

Notwithstanding ongoing progress in understanding the central role of aSyn aggregation in PD neurodegeneration, many unclear aspects still hamper the quest for effective disease-modifying therapies, which remain unavailable to date. Indeed, the biological complexity of the aggregation process comes with the limited availability of experimental measurements of aSyn intracellular dynamics, particularly in cell cultures and living systems. This data paucity often results from technical difficulties in tracking aggregate formation over time and addressing the heterogeneous and elusive nature of oligomers. As a result, a quantitative analysis of the aggregation kinetics in these settings requires continuous improvement. Moreover, different aspects of aSyn-lipid interplay are still elusive since this protein interacts with lipid membranes as part of both its physiological and pathological roles in the brain. Its unfolded form has essential functions mainly associated with the synaptic vesicles. The most agreed-upon yet elusive activity involves a lipid-bound helical form regulating synaptic vesicle recycling and neurotransmitter release through interactions with phospholipids of synaptic vesicle membranes ([Hallaceli et al., 2022](#); [Sharma et al., 2023](#)). On the other hand, aSyn-lipid co-aggregation can have pathological consequences: lipid membranes interfere with aggregation, and, in turn, various aSyn species can lead to alterations in membrane morphology and permeability, resulting in a vicious cycle. A slight perturbation of this finely-tuned balance between functional and deleterious interactions of aSyn and lipids may result in PD pathogenicity ([Killinger et al., 2019](#)). Therefore, understanding the mechanisms underlying aSyn-lipid interactions and the interplay between pure and lipidic aSyn aggregation can lead to crucial physiological implications.

These knowledge gaps would benefit from a unified mechanistic framework for *in vitro* aSyn aggregation that builds on existing insights to explore new scenarios *in silico* and support therapeutic development. Such a framework would also strengthen multiscale modeling approaches that connect molecular events to cellular, tissue, and clinical outcomes, particularly in QSP and PBPK models. While these models account for various biological processes (*e.g.*, degradation pathways and oxidative stress), they often simplify aSyn aggregation, reducing its complexity to coarse-grained variables that overlook the details of aggregation kinetics ([Righetti et al., 2022](#)). Conversely, when aggregation is explicitly modeled, kinetic parameters are often drawn from diverse literature sources ([Geerts et al., 2023a](#)), which can lead to inconsistencies due to differences in experimental conditions, some of which do not reflect phys-

iological environments. Integrating a unified molecular-level model of aSyn aggregation into these multiscale frameworks would provide a clearer link between microscopic aggregation events and system-level proteostasis failure, leading to a bridge between *in vitro* findings and *in vivo* disease mechanisms and deepening our understanding of PD neurodegeneration (Bloomington et al., 2022b; Cassotta et al., 2022; Menon et al., 2024; Denaro et al., 2024).

Chapter outline In this chapter, we propose such a model of *in vitro* aSyn aggregation. Section 3.2.1 describes the model structure in detail, comparing it to existing chemical kinetic models and outlining how pure and lipid-mediated pathways are represented and interact. Sections 3.2.2 and 3.2.3 present model calibration across multiple *in vitro* experimental scenarios and evaluate its ability to reproduce observed aggregation kinetics. Section 3.2.4 shows how the model can be used as a virtual lab, simulating perturbations such as changes in the lipid-to-aSyn ratio and modifications of specific rate constants to mimic potential drug effects. Finally, we critically assess the model limitations and suggest possible extensions to include degradation pathways, cell-level mechanisms, and integration into QSP/PBPK frameworks (see Section 3.3).

3.2 RESULTS

Chemical kinetic models have provided significant insights into *in vitro* aSyn aggregation (Knowles et al., 2009; Meisl et al., 2016; Dear et al., 2024; Rinauro et al., 2024). Tailored to *ad-hoc* test-tube experiments, they typically include a minimal set of reactions relevant to aggregation, following the principle of model parsimony. By leveraging their framework and mechanistic insights, we developed a mathematical model that integrates pure and lipid-mediated aggregation pathways into a unified framework grounded in *in vitro* knowledge. This approach allows to explore diverse experimental conditions and aggregation dynamics while aligning with established findings.

3.2.1 MODEL STRUCTURE

The system includes microscopic events involved in pure protein and lipidic aggregation and are specifically related to aSyn as reported in the literature. As shown in Figure 3.1, it represents a nucleation-conversion-polymerization process that describes fibril formation from monomers via oligomeric intermediates. It explicitly accounts for two oligomeric species and the corresponding monomer-independent conversion steps en route to fibrils, from type-A to type-B oligomers to fibrils. Type-A oligomers appear early in the process, resulting from different primary nucleation mechanisms. They can dissociate

back into monomers or undergo a structural conversion that results in type-B oligomers, which eventually form fibrils through another conformational change and do not spontaneously revert to type-A oligomers. Similarly, fibrils do not convert back to type-B oligomers. Structural transitions in amyloid aggregation pathways often result in more stable species and can therefore be effectively treated as irreversible (Iljina et al., 2016). Accordingly, we consider type-B oligomer formation as a unidirectional process, as these oligomers exhibit increased structural stability and resistance to reversal, making the relatively slow reverse reaction negligible. This assumption aligns with existing chemical kinetic models of aSyn aggregation that explicitly include type-A and type-B oligomers (Cremades et al., 2012; Iljina et al., 2016; Dear et al., 2020b). Additionally, the system accounts for pure protein and lipidic fibrils (Galvagnion et al., 2015; Galvagnion et al., 2024; Dear et al., 2024), thus reflecting another critical biological and mechanistic distinction between aSyn species.

The model describes the temporal evolution of protein species, including free and vesicle-bound monomers (m and m_b), type-A and type-B oligomers (A and B), newly formed fibrils (F^*), pure protein and lipidic fibrils (F_p and F_l), as well as free, monomer-coated, and fibril-bound vesicles (V_f , V_b , and $V_{F,b}$). In addition to monitoring the number concentration of each aSyn species, the model also incorporates mass concentration to keep track of the monomer molecule count within each aSyn species. Such a choice of variable follows the chemical kinetic framework proposed by Knowles and colleagues (Knowles et al., 2009) by leveraging a (stochastic) master equation approach (Oosawa et al., 1962), detailed in the Material and Methods section. As outlined in Table 3.1, M_A , M_B , M_{F^*} , $M_{F,p}$, and $M_{F,l}$ represent the concentration of monomers in oligomeric and fibrillar aggregates. In particular, we assumed that fibrils are the only species that can elongate by monomer addition at their ends, whereas oligomers, being nonfibrillar, are characterized by negligible elongation rate constant. This assumption results in the relationship $M_A = nA$ and $M_B = nB$. Such a coarse-grained representation of oligomer populations aligns with the kinetic modeling framework in Iljina et al. (2016) and Dear et al. (2020b). The limited contribution of oligomers to monomer consumption, as indicated by smFRET data (Iljina et al., 2016), supports the assumption of a negligible oligomeric elongation rate constant compared to the dominant/driving process of fibril elongation. In our notation, $M_{F,p}$ and $M_{F,l}$ both include the mass concentration of newly generated fibrils $M_{F,*}$, with the differences indicated by $\tilde{M}_{F,p}$ and $\tilde{M}_{F,l}$, respectively. Therefore, the total mass concentration of fibrils $M_{F,TOT}$ corresponds to the combined species concentration ($M_{F,p}$ and $M_{F,l}$) that accounts for the overlap ($M_{F,*}$), i.e., $M_{F,TOT} = \tilde{M}_{F,p} + \tilde{M}_{F,l} + M_{F,*} = M_{F,p} + M_{F,l} - M_{F,*}$. Accordingly, the concentration of both fibrillar species includes the amount of newly generated fibrils, with $F_p = \tilde{F}_p + F^*$ and $F_l = \tilde{F}_l + F^*$, where $\tilde{F}_p$ and $\tilde{F}_l$ refer to pure protein and lipidic fibrils originating through F^* elongation and vesicle- F^*

binding, respectively; and $F_{TOT} = \tilde{F}_p + \tilde{F}_l + F^* = F_p + F_l - F^*$. These variables are interconnected through primary and secondary nucleation, oligomer conversion and dissociation, fibril formation, pure and vesicle-mediated fibril elongation, and monomer-vesicle binding, listed in Table 3.1. Reactions such as monomer-independent unimolecular oligomer structural conversions, oligomer dissociation, fibril formation, reversible monomer-vesicle binding, and pure fibril elongation are regulated by first- or second-order mass action rate laws. In contrast to homogeneous primary nucleation that cannot display saturation effects, heterogeneous primary nucleation involves a two-step enzymatic reaction catalyzed by various surfaces within the reaction vessel (*e.g.*, the plate surface or the air-water interface concentration). To form oligomers A , monomers interact with a surface W according to the reaction:



The corresponding Hill function $H(K_{max,I}, K_P, n)$ is defined as $k_{max,I} \frac{m^n}{m^n + K_P^n}$, with half-saturation constant $K_P = (k_{-p} + k_{cat,I})/k_p$. The maximal production rate is given by $k_{max,I} = k_{cat,I}W_{TOT}$, where W_{TOT} , subsumed into the rate constant, represents the total concentration of surfaces catalyzing this reaction. Likewise, secondary nucleation involves a two-step catalytic reaction catalyzed by fibril surfaces S :



The corresponding Hill function $H(k_{max,II}, K_S, n)$ is given by $k_{max,II} \frac{m^n}{m^n + K_S^n} S$, where $K_S = (k_{-s} + k_{cat,II})/k_s$ represents the half-saturation constant, and $k_{max,II} = k_{cat,II}$ denotes the maximal production rate constant. Here, S is assumed to be proportional to fibril mass concentration. Specifically, in the model, secondary nucleation can occur on pure, lipidic, and newly generated fibril surfaces, which are assumed proportional to $\tilde{M}_{F,p}$, $\tilde{M}_{F,l}$, and $M_{F,*}$, respectively. The corresponding secondary nucleation rate constants are defined as $k_{max,II}^p$, $k_{max,II}^l$, and $k_{max,II}^*$. When only one secondary nucleation mechanism is considered, the rate constant is indicated as simply $k_{max,II}$. Note that assuming the same rate constant for different secondary nucleation mechanisms results in a corresponding flux proportional to the total fibril mass concentration, *i.e.*, $k_{max,II} \frac{m^n}{m^n + K_S^n} M_{F,TOT}$. In lipidic environments, monomers reversibly bind to and dissociate from free vesicles in solution, regulated by a dissociation constant $K_D = k^-/k^+$. This process contributes to the dynamic partitioning of monomers between the free and vesicle-bound states. The reversible binding is represented by a second-order mass action:



Here, $m_b = M_s/\gamma V_b$, with M_s and γ denoting the lipid count of a vesicle (estimated to approximately 6000 lipids per SUV) and the number of lipids within a vesicle involved in binding to a protein monomer (estimated to approximately 30 lipids per protein), respectively (Galvagnion et al., 2015). Therefore, although Eq. (3.3) is written as a 1:1 binding reaction, vesicles function as multivalent scaffolds with numerous lipid binding sites. This allows multiple monomers to bind to the same vesicle, up to a maximum capacity of M_s/γ monomers. The resulting monomer-coated vesicles V_b play a crucial role in fibril elongation by delivering both lipids and monomers to growing fibrils. According to Dear et al. (2024), lipid-mediated fibril elongation proceeds via a co-elongation mechanism: lipids are actively incorporated into the growing fibril structure during the elongation phase, rather than at the initial nucleation stages, and this process depends on the relative rates of protein and vesicle addition. Specifically, monomer-coated vesicles initially bind to fibril ends at rate constant k_{on} . As approximately y monomers are sequentially added to fibrils at rate constant k_{l2} , these monomers intermix with lipids provided by vesicles to form lipid-protein co-aggregates, which thus do not result from pre-bound “lipidic monomers”. While vesicle binding to fibril surfaces and monomer addition to fibril ends are reversible reactions, we assumed back reactions to be significantly slower than their corresponding forward reactions and thus negligible (Dear et al., 2024). In particular, vesicles likely do not remain permanently bound to the fibril surface but stay temporarily to enable elongation, facilitating the formation of lipid-protein co-aggregates without requiring vesicle engulfment or disassembly. Given that the model focuses on the overall aggregation kinetics—not on tracking the precise dynamics of individual vesicles, the vesicle dynamics is simplified to a “delivery” model: upon y monomers incorporation per binding event, a new vesicle needs to “dock” to the new growing end and “deliver” its cargo of lipids to further elongation, implying that the vesicle is no longer contributing to fibril growth. Therefore, the k_{on} constant effectively accounts for both vesicle binding and the delivery of lipids necessary for the process. This mechanism is represented by a function depending on the amount of free monomers and monomer-coated vesicles, defined as:

$$f(y, k_{on}, k_{l2}) = yk_{on}V_b \frac{k_{l2}m}{k_{l2}m + yk_{on}V_b} F_1 \quad (3.4)$$

where k_{on} and k_{l2} correspond to the rate constants of vesicle-fibril binding and lipidic fibril elongation by monomer addition, respectively. We assumed lipidic fibrils to elongate at the same rate constant as pure fibrils, *i.e.*, $k_{l1} = k_{l2}$. This function has been recently proposed by Dear et al. (2024), and a more detailed description of its derivation is provided in the Material and Methods section. The model accounts for pure and lipidic aggregation routes by assuming shared initial aggregation and distinct elongation processes for the two fibrillar species. Specifically, on-pathway oligomers are assumed to be pure in both

pathways, given the lack of detailed structural and mechanistic characterization of lipidic oligomers and the central role of lipid incorporation during the co-elongation phase of lipidic fibrils. While no mechanistic description of lipid-oligomer interactions is available in the literature to our knowledge, the existence of lipidic oligomers cannot be entirely excluded. According to [Dear et al. \(2024\)](#), various protein-only and protein-lipid clusters develop during nucleation in the lipid vesicle experimental setup. While these clusters could be intermediates, the authors identified the nucleus by its co-aggregate nature and ability to grow into fibrils—*i.e.*, as “the first species that structurally resembles the final aggregates and can rapidly grow through monomer addition”—rather than as an earlier-stage intermediate in the aggregation pathway. Therefore, early oligomerization may follow a protein-only pathway, with lipid incorporation becoming more prominent during fibril elongation. Building on these insights, our model focuses on monomer- and fibril-vesicle interactions as key reactions in lipidic fibril formation, reflecting the mechanistic emphasis on lipid incorporation at later stages rather than at early oligomerization or nucleation. Figure 3.1a provides a graphical representation of the molecular mechanisms underlying our model, formalized by the diagram in Figure 3.1b. Overall, the model of aSyn aggregation is based on a system of Ordinary Differential Equations (ODEs) describing the time evolution of monomer and aggregate levels. It consists of 12 ODEs, with 12 variables, 12 reactions, and up to 20 parameters (if we consider three distinct secondary nucleation mechanisms), and two algebraic equations corresponding to the conservation law of total monomer and vesicle concentrations, ensuring mass balance throughout the process. Table 3.1 lists the model variables and parameters; the highlighted variables appear in the model equations.

Integration of chemical kinetic model components A detailed comparison between our work and other chemical kinetic models of aSyn aggregation ([Iljina et al., 2016](#); [Dear et al., 2020b](#); [Gaspar et al., 2017](#); [Galvagnion et al., 2015](#); [Dear et al., 2024](#); [Xu et al., 2024](#)) can be found in Table A.1 of the Appendix A, highlighting differences in mechanisms, variables, and reaction kinetics (*e.g.*, mass action vs. catalytic processes). Chemical kinetic models often exclude intermediate species ([Meisl et al., 2016](#); [Gaspar et al., 2017](#)), focusing on nucleation-polymerization processes, or consider a single oligomeric state ([Galvagnion et al., 2015](#); [Xu et al., 2024](#)), facing limitations of data availability. Specific aSyn oligomers, however, play a crucial role in aggregation dynamics and cytotoxicity. Our model explicitly includes on-pathway oligomers undergoing structural conversion (from type-A to type-B), following the approach of [Iljina et al. \(2016\)](#). In addition, our model distinguishes between homogeneous and heterogeneous primary nucleation, in line with evidence suggesting distinct roles for these processes under specific experimental conditions.

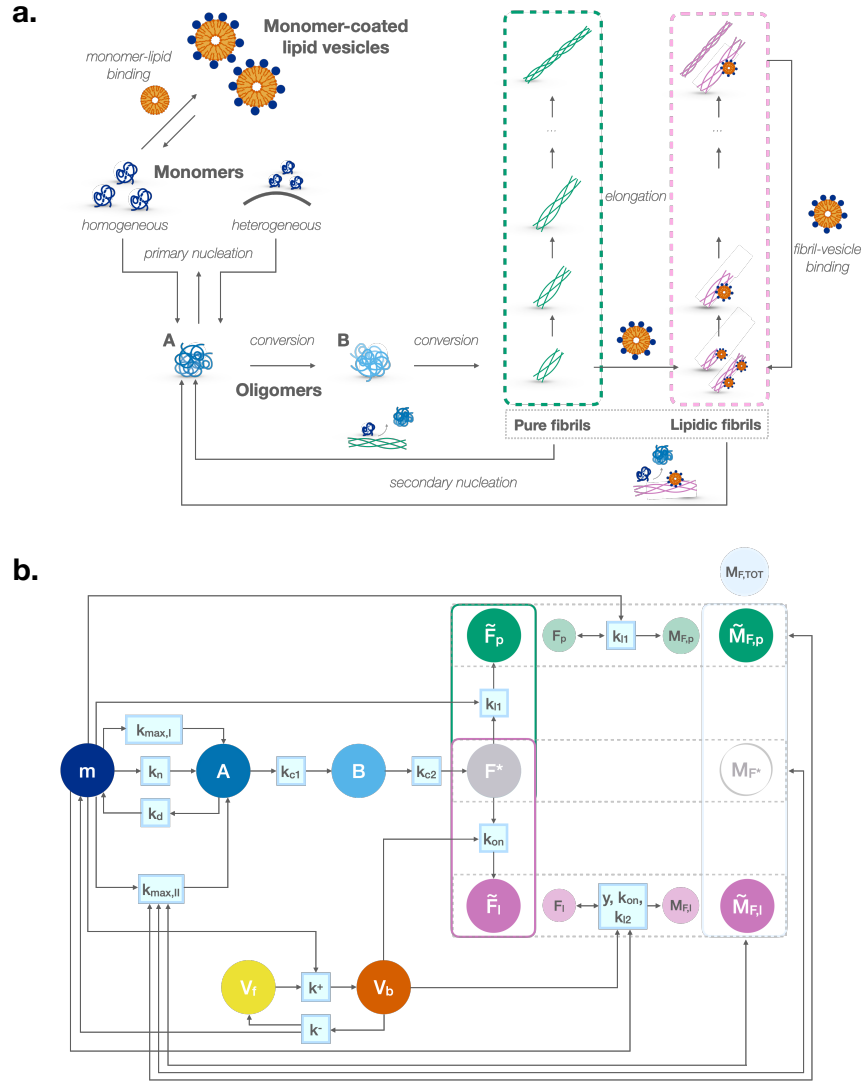


Figure 3.1: Graphical representation of aSyn aggregation. (a) A conceptual scheme of pure protein and lipidic aggregation pathways represented in our model, including all microscopic events involved in a nucleation-conversion-polymerization process and the interactions between aSyn and lipidic vesicles. Green and pink boxes represent pure protein and lipidic aggregation pathways, respectively. The two oligomeric species are explicitly represented due to their crucial role in aggregation-related cytotoxicity. **(b)** Model diagram. Circles correspond to the system variables, *i.e.*, monomers (m), type-A and type-B oligomers (A and B), newly generated fibrils (F^*), pure and lipidic fibrils in terms of both number concentration (F_p and F_l) and mass concentration ($M_{F,p}$ and $M_{F,l}$), and free and bound vesicles (V_f and V_b); with $\tilde{F}_p$ and $\tilde{F}_l$ as *auxiliary* variables indicating pure and lipidic fibrils that are not newly generated, *i.e.*, such that $F_p = \tilde{F}_p + F^*$ and $F_l = \tilde{F}_l + F^*$. Edged arrows represent the reactions governing the dynamics of the system variables, including nucleation, elongation, structural conversions, and binding events, with the corresponding parameters. This model diagram presents a variable choice that relies on the distinction between number and mass concentrations. Green and pink boxes, together with supplementary circles, highlight pure protein and lipidic fibril species, respectively, whereas the grey box represents $M_{F,TOT}$ as the union of $M_{F,p}$ and $M_{F,l}$. For simplicity of representation, we neglect the terms $k_{on} V_b M_{F^*}$ and $k_{11} m M_{F^*}$, involving the mass concentration of newly formed, pure protein, and lipidic fibrils. There is no arrow between F^* and M_{F^*} , given that newly formed fibrils F^* transition to $\tilde{F}_p$ upon elongation by monomer addition and do not grow in monomer count.

Table 3.1: **Model variables and parameters.** The variables highlighted in grey are those included in the model equations, as reported in the Material and Methods section.

Model variables	
Variable name	Symbol
Monomer concentration	m
Type-A oligomer concentration	A
Type-B oligomer concentration	B
Newly generated fibril concentration	F^*
Pure fibril concentration	F_p
Type-A oligomer mass concentration	M_A
Type-B oligomer mass concentration	M_B
Newly generated fibril mass concentration	M_{F^*}
Pure fibril mass concentration	$M_{F,p}$
Free vesicle concentration	V_f
Monomer-coated vesicle concentration	V_b
Fibril-bound monomer-coated vesicle concentration	$V_{F,b}$
Vesicle-bound monomer concentration	m_b
Fibril-bound vesicle-coating monomer concentration	$m_{F,b}$
Lipidic fibril concentration	F_l
Lipidic fibril mass concentration	$M_{F,l}$
Total vesicle concentration	V_{TOT}
Total monomer concentration	m_{TOT}
Total fibril mass concentration	$M_{F,TOT}$
Model parameters	
Parameter name	Symbol
Number of monomers involved in nucleation	n
Rate constant for homogeneous primary nucleation	k_n
Maximum rate for heterogeneous primary nucleation	$k_{max,I}$
Nucleation equilibrium constant (heterogeneous)	K_P
Maximum rate constant for secondary nucleation	$k_{max,II}$
Nucleation equilibrium constant (secondary)	K_S
Rate constant for oligomer conversion	k_{c1}
Rate constant for oligomer dissociation	k_d
Rate constant for fibril formation	k_{c2}
Rate constant for pure fibril elongation	k_{l1}
Rate constant for lipidic fibril elongation	k_{l2}
Coefficient for vesicle-mediated fibril elongation	y
Rate constant for vesicle-fibril binding	k_{on}
Dissociation constant for monomer-vesicle binding	K_D
Backward rate constant for monomer-vesicle binding	k^-
Forward rate constant for monomer-vesicle binding	k^+
Number of lipids binding a monomer	γ
Average lipid count in vesicles	M_s

While homogeneous nucleation is required for initiating any aggregation process, heterogeneous nucleation is the dominant primary nucleation pathway for aSyn in many cases (Dear et al., 2024). Although secondary nucleation is represented as a mass action process of order n , with $n < 1$ representing saturation, in many models (Gaspar et al., 2017; Galvagnion et al., 2015; Dear et al., 2020b; Xu et al., 2024), we explicitly represent it as a catalyzed reaction, where fibril surfaces—assumed to be proportional to fibril mass concentration—act as enzymes for type-A oligomer formation. For lipidic aggregation, our model incorporates the detailed aSyn-lipid interaction mechanisms described by Dear et al. (2024) and includes explicit monomer-vesicle binding, moving beyond pre-equilibrium assumptions. While chemical kinetic models often focus on either pure or lipid-mediated aggregation, our approach acknowledges that both likely occur simultaneously in physiological environments, where aSyn interacts with various cellular co-factors and undergoes dynamic changes in its surroundings (e.g., pH shifts, variations in local lipid concentration, etc). Overall, our model uniquely integrates reaction types relevant to aSyn aggregation, from oligomer conversions and secondary nucleation to lipid interactions.

3.2.2 CONTEXT-DEPENDENT MECHANISMS OF IN VITRO ASYN AGGREGATION: MODEL CALIBRATION AND VALIDATION

The mechanisms involved in the model can contribute to the whole aggregation process to a different extent depending on the pathophysiological conditions of the surrounding environment. In *in vitro* aggregation assays, aSyn monomers in solution are resistant to nucleation, and specific catalysts are required to trigger their aggregation. The process can be accelerated or slowed down by different reactions depending on the nature of the initiating agents, physicochemical factors (e.g., pH, ionic strength, water activity), and solution conditions. For instance, the presence of lipid vesicles significantly boosts fibril elongation (Dear et al., 2024), whereas secondary nucleation dominates the aggregation process as a result of pH variations and the addition of preformed fibril seeds (Gaspar et al., 2017). This information has emerged from the rigorous analysis of aSyn aggregation kinetics (Knowles et al., 2009; Buell et al., 2014; Galvagnion et al., 2015; Iljina et al., 2016; Meisl et al., 2016; Gaspar et al., 2017; Dear et al., 2024). To account for the significant variations in the contribution of each microscopic event, we calibrated and validated the model on different sets of published data from *in vitro* aggregation assays of recombinant purified aSyn (Iljina et al., 2016; Gaspar et al., 2017; Galvagnion et al., 2015). Specifically, we considered four aggregation scenarios, three shown in Figure 3.2 and the fourth in Figure 3.3:

1. constant agitation of aSyn monomers with oligomer data obtained using single-molecule Förster Resonance Energy Transfer (smFRET) measurements (Iljina et al., 2016);

2. pH variations from neutral to mildly acidic levels and inclusion of pre-formed fibrils seeds with Thioflavin-T (ThT) dye fluorescence data of pure aSyn fibrillation (Gaspar et al., 2017);
3. introduction of anionic phospholipidic vesicles, *i.e.*, DMPS SUVs, in solution with monomers with a ThT-derived fibril mass dataset (Galvagnion et al., 2015);
4. cytosolic ionic strength, neutral pH, and seeding with fibril and oligomer mass data obtained by ThT dye fluorescence and single-molecule fluorescence-free electrophoresis (FFE), respectively (Xu et al., 2024).

Although calibrating the model on smFRET-derived data obtained under mechanical stress provides valuable insights into aSyn oligomerization, there is a potential mismatch between constant agitation of monomers in solution and the initiating factors of aggregation *in vivo*. To address this limitation, we incorporated more physiologically relevant scenarios; aSyn is predominantly cytosolic, where the pH ranges from 7.2 to 7.4, but also interacts with mildly acidic cellular compartments, such as intracellular vesicles, endosomes, and lysosomes, where a slightly lower pH can influence its aggregation dynamics. In this context, a shift from neutral to mildly acidic pH, coupled with the strong seeding of preformed fibrils, represents a physiologically relevant scenario. Similarly, the presence of lipid vesicles at varying lipid-to-aSyn ratios provides an additional layer of biological relevance, given the well-established role of membrane interactions in aSyn aggregation. Finally, to further account for physiological conditions mimicking the cellular environment, we extended our analysis to a dataset reflecting aggregation under cytosolic ionic strength and neutral pH (Xu et al., 2024).

The model allows for calibration with pure protein or lipidic fibrillation datasets. Under the experimental conditions of the lipid vesicle setup, pure protein fibrils do not form under these conditions, and lipidic fibrils are the main aggregation product (Galvagnion et al., 2024; Dear et al., 2024). Accordingly, when calibrating on lipid-mediated aggregation data, we deactivated pure aggregation. For all scenarios, the model was calibrated on data associated with the highest and lowest initial monomer, seeding conditions, and lipid-to-aSyn levels, and validated on the remaining time series. For effective calibration across multiple datasets, we implemented a step-wise approach guided by data availability and biological knowledge, as detailed in the Material and Methods section. Parameter estimation followed two main principles: (i) for reactions consistently shared with existing models (such as standard and lipid-induced fibril elongation and oligomer dissociation), we maintained literature-derived values; (ii) the remaining parameters, including rate constants for oligomeric conformational changes and nucleation mechanisms, were estimated. To address data limitations, particularly the lack of oligomer measurements across different scenarios, we applied additional constraints such as maintaining fixed ratios between oligomer conversion rates, derived by calibration on available

oligomer data from shaking-induced aggregation (Iljina et al., 2016). The resulting parameter estimates are shown in Table 3.2 and Table 3.3. When the comparison is feasible in terms of unit of measure, our parameter estimates align with previously published values while extending the model applicability across diverse experimental conditions (see Table A.2). In addition, we performed uncertainty quantification on the parameter estimates related to the best fit to assess whether the model design is flexible enough to reflect the entire variability expressed by the data while retaining a meaningful and interpretable parametrization.

Table 3.2: Parameter estimates derived from a step-wise model calibration procedure on data from *in vitro* aggregation assays of recombinant purified aSyn under various conditions: (a) mechanical stress through constant agitation, (b) a shift in pH from neutral to mildly acidic & preformed fibril seeds, and (c) the presence of lipid vesicles, as detailed in the main text and shown in Figure 3.2. We assumed a fixed size for oligomers, setting the parameter n to 2 as in the literature (Thompson et al., 2021). Fitted parameters in each scenario are highlighted in light blue. Following a step-wise approach, the parameters highlighted in grey are carried over from the model calibration of the previous scenario (from left to right). The remaining parameters are fixed to literature values; those without a reference are derived from relationships defined in the literature, as detailed in the Material and Methods section. (*) Cremades et al. (2012) and Dear et al. (2020b), (**) Buell et al. (2014), (***) Galvagnion et al. (2015).

(a) Mechanical stress			(b) Mildly acidic pH & seeding			(c) Lipid vesicles		
Parameter	Estimates	Unit	Parameter	Estimates	Unit	Parameter	Estimates	Unit
n	2	unitless	n	2	unitless	n	2	unitless
k_n	1.21e-06	$\mu M^{-1} h^{-1}$	k_n	1.21e-06	$\mu M^{-1} h^{-1}$	$k_{\max, I}$	3.09e-03	$\mu M h^{-1}$
$k_{\max, I}$	6.32e-03	$\mu M h^{-1}$	$k_{\max, I}$	6.32e-03	$\mu M h^{-1}$	K_P	5.25	μM
K_P	5.25	μM	K_P	5.25	μM	k_d	2.4e-03 *	h^{-1}
k_d	2.4e-03 *	h^{-1}	$k_{\max, II}$	7.89e-03	h^{-1}	k_{c1}	5.91e-01	h^{-1}
k_{c1}	4.28e-02	h^{-1}	K_S	2.13	μM	k_{c2}	8.86e-02	h^{-1}
k_{c2}	7.33e-03	h^{-1}	k_d	2.4e-03 *	h^{-1}	(= 1.5e-01 k_{c1})		
k_{l1}	7.92 **	$\mu M^{-1} h^{-1}$	k_{c1}	2.76e-01	h^{-1}	k_{l2}	7.92 **	$\mu M^{-1} h^{-1}$
			k_{c2}	4.13e-02	h^{-1}	y	457.5	unitless
			(= 1.5e-01 k_{c1})			k_{on}	7.52e01	$\mu M^{-1} h^{-1}$
			k_{l1}	7.92 **	$\mu M^{-1} h^{-1}$	K_D	3.8e-01 ***	μM
						k^-	1.63e-03	h^{-1}
						k^+	4.30e-03	$\mu M^{-1} h^{-1}$
						γ	3e01 ***	unitless
						M_s	6e03 ***	unitless

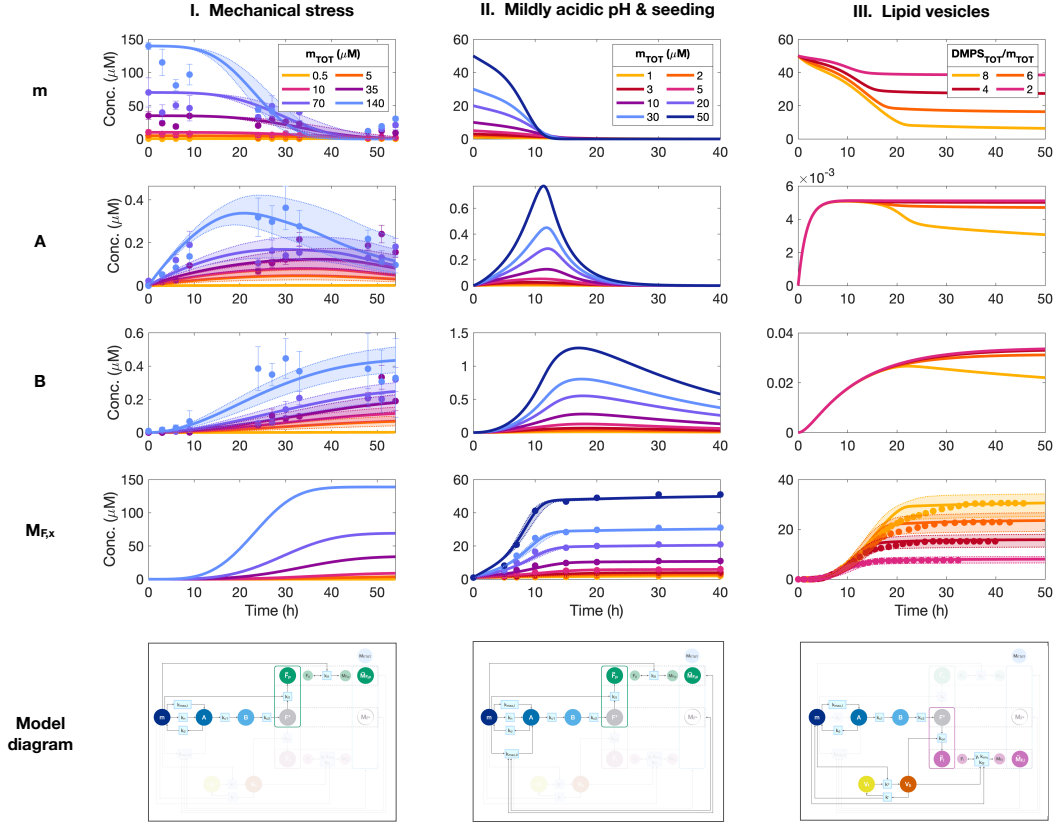


Figure 3.2: Calibration and validation of the model of pure and lipid-mediated aSyn aggregation on experimental data from *in vitro* aggregation assays of monomers in solution with different initiators. The three scenarios differ in terms of solution conditions and measurement techniques: **I.** Mechanical stress by constant agitation at 200 rpm, neutral pH, $T = 37^\circ$, smFRET (Iljina et al., 2016); **II.** Mildly acidic pH ($= 5.5$) and preformed fibril seeds ($M_{F,P} = 1 \mu M$), quiescent solution, $T = 37^\circ$, ThT dye fluorescence (Gaspar et al., 2017); **III.** DMPS SUVs, quiescent solution, neutral pH, $T = 30^\circ$, ThT dye fluorescence (Galvagnion et al., 2015). Here, ODE deterministic simulations (lines) represent the time evolution of monomer, oligomer, and fibril mass concentrations. Dots indicate the experimental data and bars the standard deviation, if available. Simulations are color-coded for different initial monomer levels, for the first two scenarios, and multiple lipid-to-aSyn ratios ($DMPS_{TOT}/m_{TOT}$), obtained by varying the lipid amount and keeping total monomer concentration fixed at $50 \mu M$. Uncertainty quantification on the parameter estimates related to the best-in-fit was performed to produce lower and upper bands corresponding to the 5th and 95th percentiles of the simulations for the fitted variables. The model diagrams in the bottom row represent activated reactions in each calibration scenario, depending on the aggregation nature (pure vs. lipidic).

3.2.3 THE MODEL RECAPITULATES CRITICAL ASPECTS OF ASYN AGGREGATION DYNAMICS ACROSS DIVERSE IN VITRO SCENARIOS

The resulting model simulations against experimental data are shown in Figure 3.2, with the corresponding parameter estimates in Table 3.2. Fibril mass concentration exhibits the characteristic sigmoidal profile, identified by an initial lag phase, followed by a growth phase due to increased monomer-to-fibril conversion until a plateau is reached, usually due to monomer depletion. This curve shows a propensity for faster monomer addition to existing aggregates rather than *de novo* oligomer formation. As shown in the second scenario, the presence of preformed fibrils can bypass primary nucleation. As the pH becomes acidic, the estimated rate constant for secondary nucleation exceeds the rate constants associated with primary nucleation reactions, underscoring the significance of secondary nucleation in driving the aggregation process (Gaspar et al., 2017). An increase in the preexisting fibril seed level or the total monomer level (while maintaining the same initial concentration of fibrils) leads to a faster overall aggregation reaction. Conversely, the initial lag phase is prolonged when aggregation is initiated not by the presence of aggregates but by other factors, such as mechanical stress or lipidic vesicles. Furthermore, our model reproduces fibrils with average lengths consistent with *in vitro* experimental data across different aggregation scenarios (see Figure A.1 in Appendix A).

The lipidic environment considered for model calibration involves intermediate lipid-to-protein ratios, corresponding to a surplus of free α Syn monomers over the amount that saturates the vesicle membranes. The relative proportion of lipids to monomers significantly affect the aggregation kinetics. These specific conditions promote the formation of amyloid fibrils, where the experimental plateau levels scale linearly with lipid concentration (Galvagnion et al., 2015). Our model captures this feature and other critical aspects of lipidic α Syn aggregation, owing to the specific formulation of the flux associated with vesicle-mediated fibril elongation (see eq. 3.4) (Dear et al., 2024). Indeed, the dual dependence of the rate of lipid-mediated fibril elongation on both the concentrations of monomer-coated vesicles and free monomers triggers a rapid transition in the rate of fibril formation within the specified time interval and across the majority of initial lipid-to- α Syn ratios. This transition results from a shift in the rate-limiting step of aggregation, moving from protein-dependent to lipid-dependent kinetics. In the following section, we provide the specifics of this case and discuss the broader implications.

In both pure protein and lipidic aggregation pathways, the structural conversion reactions between oligomeric and fibrillar species are crucial steps. The smFRET method predominantly detects stable oligomers, which persist throughout the aggregation pathway. Therefore, by fitting smFRET-derived oligomer data from a shaking-induced aggregation assay, our model effectively

captures the dynamics of these long-lived oligomers. In particular, model simulations can grasp the sequential nature of stable oligomers: type-A oligomer concentration reaches the peak before type-B oligomers, confirming that both oligomeric types are on-pathway. In the lipid-rich environment, the sequential order of oligomeric peaks is maintained, albeit with a delayed peak time. On the other hand, the oligomeric abundance decreases by two orders of magnitude compared to scenarios of pure protein aggregation. According to our model calibration on oligomer data (Iljina et al., 2016), the conformational change from type-B oligomers to fibrils is one order of magnitude slower (*i.e.*, $k_{c2}/k_{c1} = 0.15$) than fast conversion between the two oligomeric species. Assuming that the ratio k_{c2}/k_{c1} is maintained across all aggregation scenarios, our model implied that the rates of oligomer structural conversion increase substantially to capture the observed fibril growth profile in the second and third scenarios (see Table 3.2).

Providing mechanistic insights into secondary nucleation and oligomer nature in physiological conditions Recent work by Xu et al. (2024) introduced an *in vitro* aggregation assay that mimics the cellular environment by maintaining neutral pH and cytosolic ionic strength across various seeding scenarios. Our model, originally calibrated under mildly acidic conditions, successfully captured fibril mass concentration-time profiles for intermediate-to-high seeding, *i.e.*, 1% and 10% seeds, but showed limitations at low seeding, *i.e.*, 0% and 0.1% seeds (see Figure A.2). This validation suggests that cytosolic ionic strength likely governs aggregation initiation at low seed concentrations, whereas preformed fibrils are the primary catalysts of aggregation at higher seeding levels. Indeed, robust performance of the model in higher seeding scenarios stems from its calibration dataset, which spans seeding levels from 2% to 100% (1 μM preformed fibril mass, 1 – 50 μM initial monomer concentration). These results indicate that secondary nucleation parameters remain reasonably constrained across varying pH conditions when sufficient seeds are present. While pH strongly modulates secondary nucleation rates under low seeding conditions as previously shown (Buell et al., 2014), the effect of pH on this mechanism becomes less critical due to the high local concentration of catalytic surfaces. Oligomers reported in Xu et al. (2024), on the other hand, showed substantially higher abundance (45 to 65-fold increase) and faster dissociation rates (hours versus days) compared to those in Iljina et al. (2016), which provided the initial calibration data. This discrepancy stems from fundamental differences in detection methods rather than solution conditions (see Figure A.2 and Table A.3 in Appendix A). While offering detailed insights into stable oligomers, traditional smFRET is limited by sample dilution, which hampers the detection of transient species. In contrast, newer minimally perturbative techniques, such as single-molecule microfluidics (Xu et al., 2024), preserve oligomer equilibrium and capture fleeting, transient species. These methods may detect either transient species or the total oligomer population, of which

stable oligomers represent a small fraction. Since the model was initially calibrated on smFRET-derived data, it specifically captures the dynamics of long-lived oligomers. To account for short-lived oligomers observed under native-like conditions, we recalibrated the model using combined fibril and oligomer mass time-concentration profiles for 0% and 1% seeded aggregation (see the Materials and Methods section for further details). The model fitted both fibril and oligomer kinetics for 0% and 0.1% seeds and was able to reproduce the the fibril mass profile for 0.1% seeding conditions, as shown in Figure 3.3 and Figure A.3. Notably, the updated secondary nucleation and oligomer dissociation rate constants reached comparable magnitudes, indicating a balance between oligomer formation and dissociation into monomers under physiological conditions. This result confirmed secondary nucleation as the primary source of aSyn oligomer formation (Xu et al., 2024). Moreover, removing the prior constraint on oligomer conversion rates (*i.e.*, $k_{c2}/k_{c1} = 0.15$), previously established for long-lived species, revealed that fibril formation outpaces inter-oligomer conversion in short-lived oligomers (*i.e.*, $k_{c2}/k_{c1} \sim 2$).

Table 3.3: Parameter estimates derived from model calibration on aSyn aggregation data obtained under physiological solution conditions, as reported by Xu et al. (2024). Fitted parameters are highlighted in light blue. Corresponding model simulations are shown in Figure 3.3. (*) Xu et al. (2024).

Parameter	Estimates	Unit
n	2	unitless
k_n	1.21e-06	$\mu M^{-1} h^{-1}$
$k_{\max, I}$	6.32e-03	$\mu M h^{-1}$
K_P	5.25	μM
$k_{\max, II}$	6.67	h^{-1}
K_S	44.40	μM
k_d	1*	h^{-1}
k_{c1}	8.67e-03	h^{-1}
k_{c2}	1.71e-02	h^{-1}
k_{l1}	7.92**	$\mu M^{-1} h^{-1}$

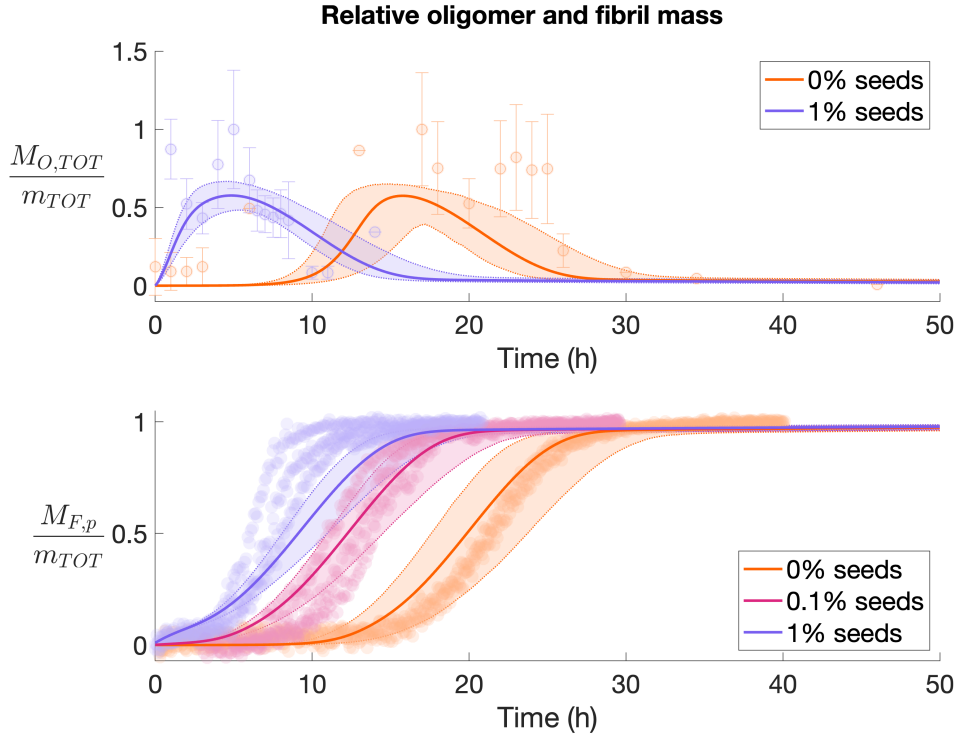


Figure 3.3: Quantitative analysis of aSyn aggregation kinetics under physiological solution conditions. Neutral pH ($= 7.4$), quiescent solution, $T = 37^\circ$, cytosolic ionic strength, and varying preformed fibril seeds. Model simulations (solid lines) compared with experimental measurements (dots), *i.e.*, total oligomer mass ($M_{O,TOT}$) and fibril mass ($M_{F,p}$), measured by single molecule microfluidics and ThT dye fluorescence, respectively, and normalized to total protein content $m_{TOT} = 100 \mu M$; low experimental accuracy of oligomer measurements indicated by the error bars (standard deviation) results from a yet-to-optimize protocol. Experimental data is obtained from [Xu et al. \(2024\)](#). Model calibration using combined fibril and oligomer mass measurements from 0% (orange) and 1% (purple) seeding conditions, showing oligomer dynamics with time-shifted peaks and corresponding fibril formation with distinct lag phases. Model validation with updated parameter estimates against fibril mass measurements for 0.1% (light pink) seeding conditions. Lower and upper bands constraining the shaded areas represent the 5th and 95th percentiles of the uncertainty quantification of the fitted variables. Updated parameter estimates are provided in Table 3.3.

3.2.4 A VIRTUAL LAB FOR IN SILICO EXPERIMENTS

Our model provides a computational environment for running a broad spectrum of *in silico* experiments, enabling the prediction and validation of hypotheses that underpin experimental investigations. In particular, it can provide insights into the interplay between pure protein and lipidic pathways and suggest target mechanisms to counteract aggregation.

Reproducing the impact of lipid-to-aSyn ratio on the dynamics of lipidic aggregation
As highlighted in the literature ([Galvagnion et al., 2015](#); [Dear et al., 2024](#)) and confirmed in the model calibration phase, the lipid-to-aSyn ratio R (*i.e.*, $DMPS_{TOT}/m_{TOT}$ here) is a crucial control parameter for *in vitro* lipidic aSyn aggregation. While the calibration step focused on intermediate ratios, the model can be used to expand the analysis to a wider range of initial conditions,

from surplus of monomers to excess of vesicles, thus providing predictions to be tested *in vitro*. As shown in Figure 3.4 (column 1 - pink/purple curves and points), our model faithfully reproduces the dynamics of lipidic aggregation, which was first experimentally observed by Galvagnion et al. (2015) and more recently analyzed in mechanistic terms by Dear et al. (2024). The system includes the reversible binding between lipidic vesicles and free monomers, which is faster than fibril formation (Galvagnion et al., 2015; Dear et al., 2024). Therefore, an excess of lipids in the solution at the beginning results in lipid-bound monomers and a minimal amount of free monomers left for aggregation. Vice versa, a significant excess of monomers over lipids underlies an insufficient amount of monomer-coated vesicles to enable the elongation of lipidic fibrils by monomer addition, thus inhibiting aggregation. Notably, in this case, oligomers persist longer and primarily give rise to newly generated fibrils, which become the dominant aggregate type as the scarcity of lipidic vesicles prevents their elongation into fibrils (Figure 3.4a, column 1). These behaviors arise from an unbalance between free and vesicle-bound monomers. Conversely, when the concentrations of the two species are balanced such that there is a surplus of monomers over the amount that saturates the lipidic vesicles, the system induces aggregation. Specifically, lipidic fibrils increase linearly in both number and mass concentrations with increasing total lipid concentrations, but only up to a certain lipid-to-protein ratio ($\text{DMPS}_{\text{TOT}}/m_{\text{TOT}} \sim 10$ in our simulations with $50 \mu\text{M}$ aSyn monomers and 100 to $5000 \mu\text{M}$ DMPS vesicles). Beyond this threshold, lipidic aggregation drops. The third scenario used for model calibration falls within this latter case of intermediate lipid-to-aSyn ratios.

While the lipidic aggregation rate is initially determined by the flux related to type-B oligomer conversion, it can be reasonably approximated by the flux term related to vesicle-mediated fibril elongation (Dear et al., 2024) at prolonged times. This reaction rate depends on the availability of free monomers and monomer-coated vesicles. Following the work of Dear et al. (2024), we can identify two regimes: (i) when bound vesicles are in excess over free monomers ($y k_{\text{on}} V_{\text{b}} \gg k_{12} m$), the lipidic aggregation rate is limited by free monomer concentration and, thus, the plateau level is reached upon monomer depletion. Conversely, (ii) in the case of free monomer excess over bound vesicles ($y k_{\text{on}} V_{\text{b}} \ll k_{12} m$), the lipidic aggregation rate is limited by the availability of monomer-coated vesicles, and the fibril mass concentration stabilizes at a plateau level upon vesicle depletion. Here, the plateau of lipidic fibril mass concentration $\tilde{M}_{F,1}$ scales linearly with total lipid concentration, being defined by the number of free monomers added to fibril ends upon vesicle binding y and the total vesicle concentration V_{TOT} , with no dependence on the initial monomer concentration, in agreement with experimental results (Galvagnion et al., 2015). Unlike pure protein aggregation, a certain amount of free monomers remains in solution instead of being incorporated into fibrils. Moreover, the monomer count of newly generated fibrils significantly contributes to the total fibril mass concentration in the lipid-limiting regime, whereas

it is nearly zero in the monomer-limiting regime. The switch from protein-dependent to lipid-dependent kinetics occurs for $V_b/m = y^{-1} k_{l2}/k_{on}$, when the concentration of bound lipids is approximately 1.4 times the free monomer level, given the parameter estimates in Table 3.2. Whether this transition in the rate of fibril formation takes place depends on the initial lipid-to-protein ratio. Since our model explicitly describes monomer-vesicle binding, without a pre-equilibrium assumption, a short initial time interval exists during which monomer-coated vesicles are not yet formed in the deterministic simulations, thus $k_{l2}m \gg yk_{on}V_b$. This condition persists throughout the experiment, resulting in a lipid-limiting regime for low lipid-to-protein ratios, corresponding to an excess of monomers over lipids. Conversely, high lipid-to-protein ratios trigger and sustain the reverse condition ($k_{l2}m \ll yk_{on}V_b$), as bound vesicles significantly outnumber free monomers. Finally, intermediate lipid-to-protein ratios lead to an initial transition from the lipid- to the protein-limiting regime, followed by a shift back to the lipid-limiting kinetics, all within the same experimental time frame.

Exploring the interplay of pure and lipidic aggregation in response to varying lipid-to-aSyn ratios Under physiological conditions, aSyn likely undergoes both aggregation routes simultaneously. The propensity for one pathway over the other depends on various cellular co-factors and conditions, with lipid-to-aSyn ratio possibly playing an important regulatory role. For example, in response to endosomal sorting, recycling pathways, and environmental factors, cellular environments such as synaptic vesicles or endosomal compartments may experience fluctuations in local vesicle and protein concentrations, which could disrupt the fine-tuned balance between beneficial and detrimental aSyn-lipid interactions. A mechanistic understanding of the response of aggregation system responds to these perturbations is therefore crucial. In this regard, our model provides an *in silico* framework to elucidate how varying lipid-to-aSyn ratios influence the interplay between pure and lipidic aggregation.

The previous scenario, based on the experimental setup with lipidic vesicles used for model calibration, represented an idealized case where pure protein fibrils do not form (Galvagnion et al., 2015; Dear et al., 2024) (see Figure 3.4c). Indeed, the pure aggregation pathway was entirely suppressed by imposing a strict constraint on fibril elongation ($k_{l1} = 0$), preventing newly generated and pure fibrils from elongating via monomer addition and ensuring that all fibrillar products contain lipids. This analysis underscored the critical role of the lipid-to-aSyn ratio in regulating lipidic aggregation and driving distinct kinetic regimes. To explore how these dynamical and steady-state features of lipidic aggregation translate into a more physiologically relevant scenario—where pure and lipidic aggregation occur simultaneously—we extended the previous scenario by relaxing the constraint on pure fibril elongation (*i.e.*, $k_{l1} = k_{l2}$). In addition to enabling pure fibril growth, this modification introduces new fibril surface areas that promote oligomer formation, triggering a positive feedback

loop via secondary nucleation with rate constant $k_{\text{max,II}}^{\text{P}}$: oligomers, in turn, convert into newly generated fibrils, amplifying both pure and lipidic fibril formation due to their shared aggregation route. Figure 3.4 shows the comparison between lipidic aggregation combined with pure protein aggregation (in green) and lipidic aggregation in isolation (in pink), in terms of dynamics and specific aggregation metrics (detailed in the Material and Methods section). In particular, Figure 3.4c shows the relative mass of aSyn species—and system variables—at the final simulation time ($t_f = 120$). We performed an additional analysis to disentangle the effects of pure and lipidic elongation within the full aggregation system by excluding secondary nucleation mechanisms from both pathways. As shown in Figure A.5, the fundamental insights not specifically related to secondary nucleation remain unchanged.

In the absence of lipidic vesicles ($R = 0$), pure fibrillation is the only viable pathway. Newly generated fibrils cannot transition into lipidic fibrillar species but can elongate via standard monomer addition, forming pure fibrils. Therefore, in contrast to lipidic aggregation alone, oligomers appear transiently even at low lipid-to-protein ratios. Moreover, the availability of new fibril surfaces triggers the secondary nucleation flux (proportional to $\tilde{M}_{F,p}$), significantly increasing oligomer abundance. Oligomer peak levels are highest in the absence of lipidic vesicles and decreases as the lipid-to-monomer ratio rises, eventually matching levels observed when only lipidic aggregation occurred, with no secondary nucleation assumed. Conversely, at high lipid-to-monomer ratios ($R > 50$), both pure and lipidic pathways are inhibited due to monomer sequestration by lipids. Most monomers bind to vesicles, leaving minimal free monomers for either pathway. The remaining free monomers are divided between pure and lipidic fibril formation as conditions allow, leading to similar system behavior regardless of aggregation mode (lipidic pathway alone or combined).

At intermediate R , the system exhibits a competitive environment where pure and lipidic pathways influence each other kinetics and steady-state behavior. As lipidic vesicles are introduced, monomers initially bind vesicles before being depleted via pure and lipidic elongation. Increasing R reduces pure fibril mass concentration ($\tilde{M}_{F,p}$) while favoring lipidic fibrils ($\tilde{M}_{F,l}$) and monomer-coated vesicles—free or bound to fibrils (m_b or $m_{F,b}$), as described by Figure 3.4c. Eventually, the system mirrors lipidic aggregation alone as previously discussed. Despite reduced mass concentration across all ratios, the qualitative behavior of lipidic fibrils ($\tilde{M}_{F,l}$ and $\tilde{F}_l$) remains consistent with previous findings. When lipidic aggregation occurs in isolation, lipidic fibril mass and number concentrations increase linearly with total lipid concentration until a threshold ($R \sim 10$), beyond which aggregation declines. When pure and lipidic aggregation coexist, a scaled yet non-linear increase in lipidic fibril mass and number concentrations was observed at plateau, peaking at $R \sim 30$ before declining due to monomer depletion caused by vesicle excess.

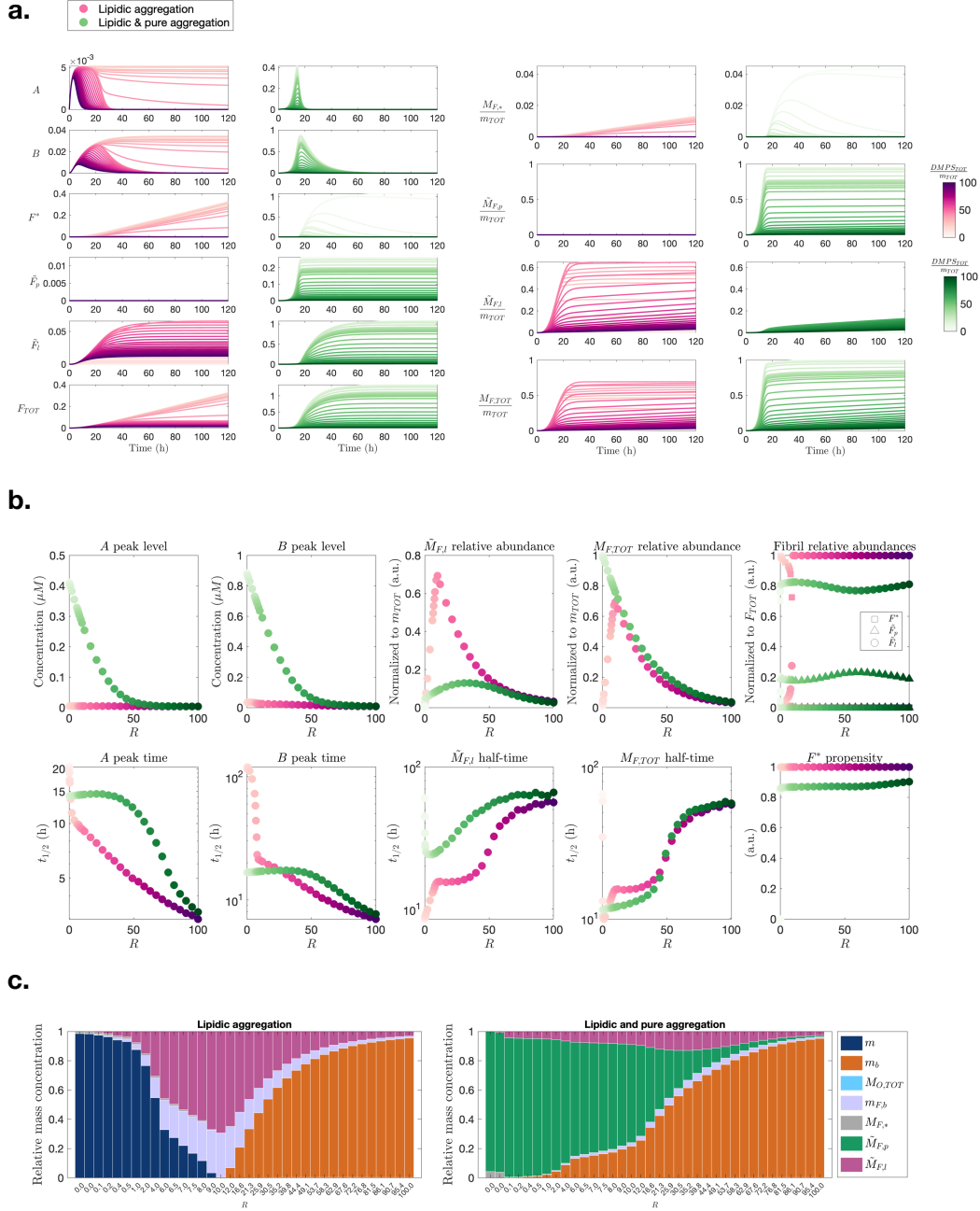


Figure 3.4: The impact of varying lipid-to-aSyn ratios on pure and lipidic aggregation. Pink and green shades denote lipid-mediated aggregation in isolation and in combination with pure protein aggregation, respectively. Concentrations (μM), time (hours). R represents the initial lipid-to-aSyn ratio (*i.e.*, $DMPSTOT/mTOT$). **(a)** Deterministic simulations of aSyn oligomer and fibril formation (number and mass concentrations) under varying lipid concentrations ($DMPSTOT$) with a fixed initial monomer concentration $mTOT = 50 \mu M$. Column one represents simulations with pure aSyn aggregation turned off, while column two depicts simulations with both pathways active, assuming equal rate constants for pure protein and lipidic fibril formation ($k_{11} = k_{12}$) and active secondary nucleation on pure fibrils ($k_{max,II}^p \neq 0$); **(b)** metrics including oligomeric peak time and level, lipidic and total fibril abundance (normalized to $mTOT$) and half-times, fibril number concentrations at $t_f = 120$ hours (normalized to F_{TOT}), and propensity for lipidic aggregation over pure protein aggregation; peak times and half-times are in log scale. Each point corresponds to a deterministic simulation colored according to the subplot above, with simulations ending at $t_f = 120$ hours; **(c)** relative mass concentration of aSyn species in the model, *i.e.*, (free and bound) monomer concentration and oligomer and fibril mass concentrations (normalized to $mTOT$).

Figure 3.4c (right) shows the remaining monomers distribute between pure fibrils and vesicle-bound monomers, with proportions varying by lipid-to-aSyn level. Overall, incorporating pure aggregation into the system shifts lipidic aggregation thresholds and breaks robustness of the plateau of lipidic fibril mass $\tilde{M}_{F,l}$ to variations in initial monomer levels (see Figure A.4) but does not blur the distinction between lipid- and protein-limiting kinetic regimes.

As shown by Figure 3.4b, total fibril mass ($M_{F,TOT}$) decreases monotonically from m_{TOT} (pure protein aggregation) as lipid concentration increases. This reflects an initial dominance of pure fibrils, followed by a balance between pure and lipidic fibrils at higher lipid concentrations, with unincorporated monomers accumulating due to vesicle surplus. Notably, newly generated fibrils strongly favor lipidic aggregation, even at low lipid-to-monomer ratios, reaching nearly 90% propensity for the lipidic pathway at high R . This trend is observed in fibril number concentrations (see Figure 3.4b). In particular, the switch from pure to lipidic dominance at very low R results from secondary nucleation, which enhances pure as well as lipidic aggregation due to their shared early-stage processes. As detailed in the Material and Methods section, we derived measures that quantify Lewy body composition. These indexes—albeit artificial *in vitro* conditions—can provide a system measure of the interplay of aggregation pathways. In combined aggregation (see Figure A.6), the system consistently forms heterogeneous Lewy body-like inclusions across most lipid-to-aSyn ratios (except at very low ones), characterized by limited pure fibril derivation (I^P up to 6%) and up to 66% lipidic fibril derivation (I^l), alongside significant cross index contribution (I^{cross}).

In silico-aided therapeutic strategies informed by local sensitivity analysis Mechanistic insights into aSyn aggregation can be crucial in guiding the development of Parkinson’s disease therapies. What is the ideal mechanism of action for small molecules targeting aSyn aggregation? Which reactions should be perturbed by candidate compounds? Our model of pure and lipid-mediated aSyn fibrillation allows to investigate the effect of therapeutic strategies that affect both aggregation routes, tuning their effect to maximize their efficacy potential. As previously discussed, our model assumes the overlap between pure and lipid-induced pathways at the initial steps of aggregation, until elongation mechanisms differentiate between pure and lipidic fibrils. Another assumption involved secondary nucleation mechanisms. Evidence for secondary nucleation in lipid-induced aggregation is significantly weaker than in pure protein aggregation (Galvagnion et al., 2015; Dear et al., 2024). Accordingly, we suppressed it when calibrating the model for lipidic aggregation at intermediate lipid-to-aSyn ratios. However, given the limited fibril surface exposure due to bound vesicles, secondary nucleation rate is likely reduced—not negligible—in the lipidic pathway. To analyze the impact of secondary nucleation on the interplay between pure and lipidic aggregation, we differentiated between two

secondary nucleation rate constants for pure and lipidic aggregation ($k_{\max,II}^P$ and $k_{\max,II}^L$, respectively). We assumed $k_{\max,II}^L$ to be one order of magnitude slower than $k_{\max,II}^P$ and newly generated fibrils, shared between the two pathways, to be too short to provide sufficient surface area for oligomer formation (*i.e.*, $k_{\max,II}^* = 0$). A more refined approach could scale the lipidic contribution to the secondary nucleation flux according to the reduced accessible surface area dictated by vesicle binding ($< \tilde{M}_{F,I}$). To analyze the impact of secondary nucleation and the other aggregation mechanisms on the cross-talk between pure and lipidic pathways, we relied on the second parameter combination for pure protein aggregation (see Table 3.2), reflecting a mildly acidic pH scenario where all nucleation reactions contribute to the process to some extent, and on the third set of parameter estimates for lipidic aggregation.

As shown by Figure 3.5, we performed a local sensitivity analysis (LSA) to quantify the impact of model parameters on relevant aSyn species through the AUC-based sensitivity index (SI) defined in the Material and Methods section. The sensitivity of each parameter is indicated by the distance of its corresponding point from the center of the figure. Here, we also explored the impact of initial conditions, *i.e.*, the presence or absence of aggregates, on parameter sensitivity. For most parameters, such variations either have a minimal effect on the outcome or corroborate the trend observed in the sensitivity ranking from the alternative scenario. As expected, the rate constants of oligomer conversions k_{c1} and k_{c2} mainly affect oligomer and pure fibril kinetics. Moreover, when considering type-A and type-B oligomers, as well as lipidic fibrils in terms of number and mass concentration, our model identifies the rate constant of secondary nucleation on pure fibrils $k_{\max,II}^P$ as one of the most sensitive parameters. Interestingly, variations in this parameter significantly influence lipidic fibrils but do not affect pure fibril levels. In general, alterations of other parameters only marginally affect the monomer count of pure fibrils. Conversely, no variable seems to be affected by perturbation of secondary nucleation lipidic rate constant $k_{\max,II}^L$. Another relevant parameter is the rate constant associated with pure fibril elongation k_{11} , which consistently ranks among the top three sensitive parameters across relevant aSyn species, such as pure and lipidic fibrils and oligomers in terms of number concentration. In contrast, lipid-mediated fibril elongation rate constant k_{12} counts among the insensitive parameters, even for the lipidic species.

Relying on these results, we simulated the impact of potential compounds targeting the most sensitive reactions by varying the corresponding model parameter. The LSA-informed model can virtually explore how significant variations of the most sensitive parameters (up to 50%) affect the dynamics and features of aSyn oligomers and fibrils throughout the aggregation process. Metrics such as type-B oligomer peak level and time, as well as pure protein and lipidic fibril abundances and half-times, can be seen as gauges of the toxicity level of the aggregation process. Even though the specific mechanisms

of toxicity associated with aSyn aggregation are not fully understood, experimental evidence pointed to the inherent toxicity as well as seeding and spreading abilities of the aggregates as structural and functional drivers of the overall toxicity (Froula et al., 2019; Gracia et al., 2020). In particular, small fibril fragments have been suggested as primary spreading agents in PD because of their high seeding efficiency and internalization ability (Iljina et al., 2016; Hijaz et al., 2020). On the other hand, type-B oligomers have been identified as the most cytotoxic species due to their high β -sheet content (Cremades et al., 2012; Gracia et al., 2020).

Parameters such as k_{c1} , k_{c2} , and $k_{\max,II}^P$ are crucial as they directly control oligomer concentrations. In particular, as suggested by LSA and shown in Figures 3.5b and 3.5c, a 50% inhibition of secondary nucleation catalyzed by pure fibrils leads to significant changes in oligomer and fibril dynamics. Specifically, this perturbation reduces the oligomeric peak time while lowering the peak level of type-A and type-B oligomers by 40% and 20%, respectively. Given the high toxicity of type-B oligomers, *e.g.*, linked to increased levels of reactive oxygen species, this intervention could mitigate the harmful impact of aSyn aggregation. The effect of this inhibition on fibrils is less straightforward. As shown in Figure 3.5c, under nominal parameter conditions, the system is characterized by a highly lipidic fibrillar content ($\sim 80\%$ of total fibril concentration) and a strong propensity for lipidic over pure protein aggregation. Conversely, perturbing secondary nucleation only marginally affects these metrics. In particular, halving the rate constant $k_{\max,II}^P$ decreases both lipidic and pure fibril concentrations ($\tilde{F}_l$ and $\tilde{F}_p$). This results in a slight reduction in lipidic aggregation propensity while promoting a significant increase in pure fibrillar content—though not enough to overturn lipidic dominance. In terms of monomer counts, total fibril mass concentration shows a minimal overall loss (under 0.03% to 50% inhibition), driven by a small increase in pure fibrils counterbalanced by a significant decrease in lipidic fibrils. In summary, while strong inhibition of secondary nucleation favors pure fibril formation, it is insufficient to substantially reduce overall lipidic contributions, as also reflected in Lewy body composition indexes.

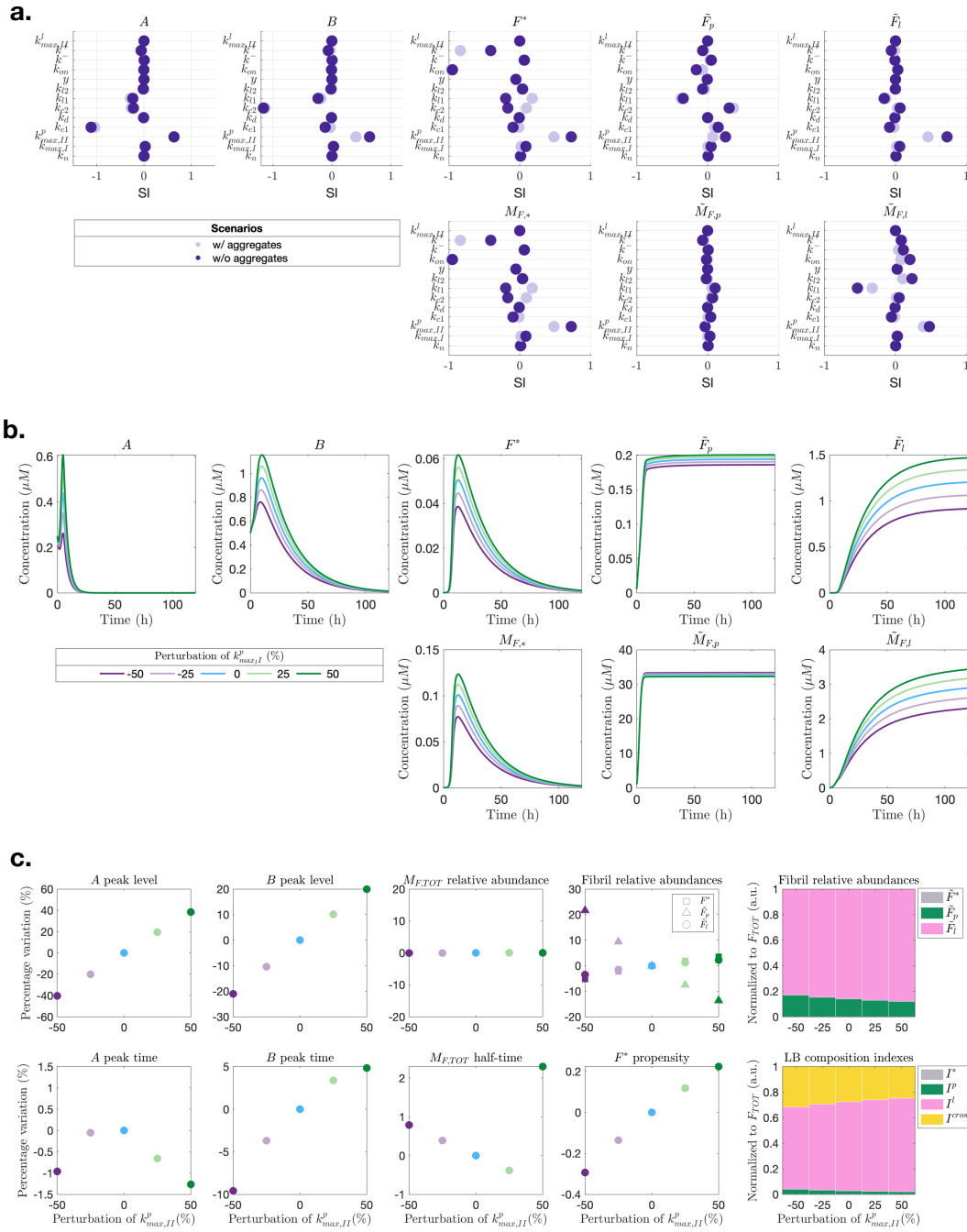


Figure 3.5: Local sensitivity analysis-informed modeling of the effect of anti-aSyn aggregation drugs. (a) Relative AUC-based sensitivity index corresponding to the 1% perturbation of each rate constant. Different colors correspond to different initial concentrations: absence (dark) and presence (light) of aggregates at $t = 0$, i.e., $m = 35 \mu M$, $\tilde{M}_{F,p} = 1 \mu M$, and $M_A + M_B = 4\% m_{TOT}$. (b) Deterministic simulations representing the variations in the model dynamics in response to perturbations of the secondary nucleation rate constant $k_{max,II}^p$, up to 50% of the nominal level; (c) Percentage variation of specific metrics describing the dynamics and features of oligomers and fibrils throughout the aggregation process. Each point is associated with the deterministic simulation of the corresponding color in the subplot above, with final time $t_f = 120$ hours.

3.3 DISCUSSION

By elucidating the dynamics of pure and lipid-mediated aSyn aggregation in diverse environments, our model offers a unified representation of specific mechanisms underlying PD pathogenesis. Our work is grounded in insights mainly provided by the chemical kinetic models (Knowles et al., 2009; Meisl et al., 2016; Dear et al., 2024; Rinauro et al., 2024). Tailored on *ad-hoc* test-tube experiments, these models include a minimal set of reactions relevant to protein aggregation in a specific scenario. For instance, they are typically coarse-grained to the extent that they either entirely exclude intermediate species or consider a single oligomeric state (e.g., see Galvagnion et al. (2015) and Cremades et al. (2012)), due to the principle of model parsimony and limitations in data availability. Since we aimed at a unified description of aggregation *in vitro*, we propose a mathematical model that accounts for microscopic events physiologically related to uncontrolled aSyn accumulation in the literature, exploiting prior biological and mechanistic knowledge and a step-wise calibration on various test-tube aggregation datasets. A detailed comparison between our work and other models of aSyn aggregation, in terms of structure and parameter estimates, is available at Table A.1 and Table A.2.

The mathematical description of aggregation presented here incorporates competing nucleation reactions, suggesting a dominant role for secondary nucleation in aSyn aggregation and thus in anti-aSyn aggregation therapies. This result aligns with recent findings that highlight the role of this reaction in oligomer and fibril formation under physiological conditions, either with acidic (Buell et al., 2014; Gaspar et al., 2017) or neutral pH (Horne et al., 2023; Xu et al., 2024). Parameters estimated at acidic pH successfully described fibril formation driven by secondary nucleation at neutral pH for medium-high seeding conditions. However, this result may rise some doubts given previous findings on pH-dependence of secondary nucleation. Specifically, Buell et al. (2014) reported that secondary nucleation flux increases dramatically at acidic compared to neutral pH. However, the experimental setup required to reveal this pH dependence relied on very low seed levels (nM seeds vs. μM monomers). Therefore, our model suggests that seeding conditions can modulate the impact of pH on secondary nucleation-dependent processes. Yet, the specific mechanisms by which pH affects secondary nucleation rates require further investigation.

In line with previous work focused on stable oligomers (Iljina et al., 2016; Dear et al., 2020b; Dear et al., 2020a), our model confirms the critical role of the kinetics of oligomer structural conversion in determining the overall fibril growth dynamics in the given aggregation scenarios. We considered two sequential oligomeric types connected by conformational changes with distinct rate constants, comparable to estimates of oligomer conversion rates from the literature (Iljina et al., 2016; Cremades et al., 2012; Dear et al., 2020b; Galvagnion et

al., 2015). The ratio $k_{c2}/k_{c1} = 0.15$ suggests that type-B oligomers predominate during aggregation, indicating higher stability than type-A oligomers. Given the higher toxicity of type-B oligomers, the conversion rate may be a crucial factor in controlling neuronal damage. Targeting this reaction may represent an effective approach adopted by intracellular defense mechanisms (Cremades et al., 2012) and holds promise as a strategy for therapeutic intervention by molecular chaperones and small molecules. These results on oligomer conversion derive from model calibration using smFRET data and, thus, refer to long-lived oligomers. Given their prolonged presence and association with increased ROS production, long-lived oligomers represent a toxicologically relevant yet small fraction of the total oligomeric population. Indeed, recent methodologies indicate that short-lived oligomers are substantially more abundant. By investigating their kinetics under physiological neutral conditions (as described in Xu et al. (2024)), our model confirmed previous results on short-lived oligomer formation driven by secondary nucleation and mainly balanced by oligomer dissociation, and suggests a higher stability of type-A compared to type-B species ($k_{c2}/k_{c1} \sim 2$). Such a kinetic dichotomy between transient and stable oligomeric species further points to fundamental differences in their biological roles. For instance, since their formation is catalyzed by fibrils, short-lived oligomers may act not only as bulk nucleation products influencing aggregation kinetics but also as sources of toxicity and aggregate spreading, through mechanisms that remains to be fully elucidated.

Xu et al. (2024) modeled transient oligomers kinetics by integrating two processes: a complete nucleation-polymerization pathway—encompassing primary nucleation, fibril elongation, and secondary nucleation—and an oligomer formation/dissociation cycle catalyzed by fibrils. Focusing on the dominant mechanisms of oligomer formation and depletion, this approach suggests an off-pathway nature for short-lived oligomers while not excluding the presence of distinct on-pathway species. The model was calibrated in two steps: first to fibril mass data across seeding scenarios (1% – 10% seeds, Amylofit, Xu et al. (2024)) and then to oligomer data (0% and 1% seeds). Our work raises mechanistic questions about these oligomers. For example, do short-lived oligomers undergo structural conversion as their long-lived counterparts? Are both transient and stable oligomers on-pathway and governed by the same formation and depletion mechanisms? Our model assumes that both transient and stable oligomers are on-pathway to fibril formation and undergo sequential conformational changes (from type-A to type-B). Upon calibration on combined fibril and oligomer mass data, the model successfully reproduce fibril and oligomer profiles for various seeding conditions. This good performance, comparable to that in Xu et al. (2024), challenges the strictly off-pathway nature of microfluidic-detected oligomers. They could represent either early unstable intermediates or aggregation products generated alongside fibril formation. Nonetheless, their fibril-catalyzed formation makes them an integral part of the aggregation landscape. Further single molecule microflu-

idic data on transient species would be needed to address these mechanistic knowledge gaps. Overall, our quantitative analysis requires caution due to the sparsity and noisiness of these measurements compared to smFRET data. Expanding datasets obtained by minimally perturbative techniques, particularly time series under varying monomer concentrations, will be essential to better constrain model parameters and refine models that capture both short- and long-lived oligomers, thereby advancing our understanding of oligomer dynamics.

Building on previous mechanistic insights into aSyn-lipid interactions (Galvagnion et al., 2015; Dear et al., 2024), we included aSyn monomer-lipid binding and vesicle-boosted fibril elongation in our model. These mechanisms capture the linear scaling of lipidic fibril concentration at plateau. However, the model does not fully account for the fibril growth phase in this aggregation scenario, suggesting hidden non-linear interactions between aSyn fibril and lipids. Future modeling efforts should explore this mechanism in collaboration with experimentalists. Additionally, further refinement should focus on the role of lipids in heterogeneous primary nucleation, a controversial aspect in lipidic aggregation research (Galvagnion et al., 2015; Galvagnion et al., 2024; Dear et al., 2024). We assumed that heterogeneous primary nucleation occurs at air-water interfaces rather than on lipidic vesicles, as suggested by Dear et al. (2024). While lipid incorporation was modeled at the fibril elongation stage, we cannot exclude the possibility that some lipids integrate into intermediate species forming at the air-water interface during nucleation. A lipid monolayer at this interface could provide a ready supply of lipids, influencing the nucleation process. Since the concentration of heterogeneous nucleation sites is incorporated into the rate constant ($k_{\max, I}$) in the model, any lipid-induced effect at this stage would be reflected in its value. Another refinement could involve monomer-vesicle binding. While our model assumes a fixed monomer count per vesicle, an alternative approach could account for vesicles with varying bound monomer counts, though this would require additional experimental data to characterize their distribution and impact on fibril elongation as well as on aSyn aggregation dynamics.

Exploring the interplay between pure and lipidic aggregation and the impact of lipid-to-aSyn ratio can offer insights into pathological mechanisms and can suggest potential therapeutic strategies. To this end, our model provides a virtual lab for investigating how the availability of free monomers and lipidic vesicles affects the dynamics of *in vitro* aSyn aggregation via pure and lipidic routes and how this may relate to PD onset and development. Integrating pure and lipidic aggregation preserved the qualitative behavior of lipidic fibrils across lipid-to-aSyn ratios: monomer excess inhibits lipidic fibril formation; vesicle surplus suppresses all aggregation by sequestering monomers; a balance between monomers and lipidic vesicles results in a scaled yet non-linear increase of lipidic fibril mass and number concentrations with lost robustness of the plateau level of $\tilde{M}_{F, I}$ to variations in initial monomer concentration. As a

result, total fibril mass concentration decreases monotonically with increasing lipid-to-aSyn ratios, reflecting the competitive interplay between the two pathways. Therefore, lipid and protein availability can reshape fibril composition and kinetics in this crosstalk, with relevant physiological and pathological implications. For example, the model can be used for testing different hypotheses on the *in vitro* formation and crowding of vesicular structures interspersed with aSyn filaments, hinting at the role of aSyn-lipid interactions in modulating Lewy body composition (Hellstrand et al., 2013; Shahmoradian et al., 2019). However, these insights, along with the Lewy body composition indexes introduced in this work, should be seen as indicative rather than directly informative of *in vivo* scenarios, given that the model does not account for aSyn production and degradation, as discussed below.

Given its confirmed crucial role in *in vitro* aSyn aggregation, the lipid-to-aSyn ratio could serve as a proxy for specific risk factors for Parkinson's disease, such as genetic alterations and aging. Specifically, investigating how variations of this control parameter affect aSyn dynamics can be relevant for understanding disease-associated mutations leading to aSyn overexpression, *e.g.*, via gene duplication and triplication. Even small perturbations of free monomer concentrations can disrupt the physiological balance between free vesicles and monomeric aSyn and, thus, substantially affect aggregation. On the other hand, aging can be associated with lipid vesicle concentration alterations. As discussed in Kiechle et al. (2020), age-related factors often lead to decreased local lipid-to-protein ratio. To showcase the model potential, we simulated an *in silico* scenario mimicking the conditions of a damaged dopaminergic neuron flooded with aSyn aggregates and the impact of oscillations in the DMPS to free aSyn monomer ratio (see Figure A.5). Our model suggests that such imbalances between available free vesicles and monomers can enhance aSyn aggregation. Overall, perturbations of the lipid-to-aSyn ratio may disrupt the balance between benign and harmful effects of aSyn-lipid interactions, leading to PD pathogenicity. Notably, the lipid-to-aSyn ratio can also serve as a proxy for the mechanism of action of candidate drugs targeting aSyn-lipid interactions or modulating vesicle concentrations, such as genipin. This iridoid glucoside is currently under investigation for its direct interaction with aSyn monomers, which alters the nucleation step, and its putative effect on lipid storage, which leads to reduced lipid-to-aSyn ratio (Rosado-Ramos et al., 2023). Both modes of action and their impact on aSyn aggregation can be investigated through our model.

In these critical times, when clinical trials for PD continue to fail, modeling can play a crucial role in supporting the design of new therapeutic compounds and drugs currently under investigation in various ways. Besides affecting the local lipid concentration, a candidate compound can directly bind aSyn in one of its various states, *e.g.*, monomers, oligomers, or fibrils, perturbing one or more mechanisms within the aggregation process. In this context, our model can facilitate the analysis of anti-aSyn aggregation compounds (Fields et al.,

2019), such as those that have reached the clinical state of development, *i.e.*, Anle-138b (Antonschmidt et al., 2022) and UCB-0599 (Schwarz et al., 2023), other small molecules, *e.g.*, EGCG and SynuClean-D, and nanobodies, *e.g.*, NbSyn2 and NbSyn87 (Iljina et al., 2017). Moreover, it can be used to investigate therapeutic strategies involving a combination of small molecules with different modes of action. As suggested by the results of the LSA analysis, it can also support the identification of potential pharmacological targets. In particular, our model suggests that inhibiting secondary nucleation may be an effective therapeutic strategy to reduce harmful oligomeric species, thus supporting the idea that developing compounds targeting fibril surfaces could be crucial for lowering aggregation inherent toxicity (Dear et al., 2024). Indeed, fibrils are critical sites where oligomeric species form and lipids interact with growing fibrils, acting as a common element in both aggregation pathways. Such an effect is associated with various inhibitors of secondary nucleation, for example, small molecules binding the catalytic sites on fibril surfaces (Staats et al., 2020; Horne et al., 2024) and aminosterol compounds with high blood-brain barrier penetrability, *e.g.*, trodusquemine (Perni et al., 2018; Limbocker et al., 2020). Our model also predicts that inhibiting secondary nucleation can have a mixed impact on aSyn aggregation: while it reduces toxic type-B oligomers, its effect on fibril composition remains ambiguous. This intervention favors pure fibril formation but does not significantly reduce overall fibril mass concentration and lipidic fibril dominance. Given the uncertainty regarding whether lipidic or pure fibrils are more detrimental to neurons, the overall impact of this strategy remains unclear. Therefore, understanding the implications of lipidic dominance and clarifying the pathological roles of different fibril types is crucial to determining whether this intervention could effectively counteract aSyn aggregation. To complement our LSA analysis and resolve ambiguities, global sensitivity analysis (GSA)—for example, Latin hypercube sampling combined with logarithmic sensitivity coefficients (Simoni et al., 2021)—can systematically explore parameter interactions across the full uncertainty space and test robustness over physiologically relevant ranges. While more computationally demanding than LSA, GSA is expected to confirm key drivers of aSyn aggregation identified previously, such as secondary nucleation on pure fibrils and fibril elongation, and to reveal additional interactions and nonlinear effects that may be critical for the design of effective therapeutic interventions. When exploring mechanistic effects in test-tube experiments using chemical kinetics models (Meisl et al., 2022), inherent challenges of translating results from *in vitro* to *in vivo* settings must be accounted. Indeed, there is a tight link between the contribution of each microscopic event to the overall aggregation and the solution conditions. For example, secondary nucleation has been proven to contribute to a different extent depending on the pH of the surrounding environment and seeding levels (Buell et al., 2014; Gaspar et al., 2017). Moreover, in the presence of lipids, their identity strongly affects the aggregation kinetics (Galvagnion et al., 2015; Dear et al., 2024), and DMPS

SUVs have a different composition from vesicles found in a synaptic bouton or functional synaptosome (Galvagnion et al., 2015). On the other hand, we expect several aspects of aSyn aggregation highlighted by our model to translate into *in vivo* scenarios. Indeed, the monomer concentrations associated with the *in vitro* aggregation assays used for model calibration fall within the physiologically relevant range (Rabe et al., 2013), given that aSyn reaches micromolar concentrations in synaptic boutons (Wilhelm et al., 2014). Moreover, as highlighted by recent studies (Dear et al., 2024; Xu et al., 2024; Horne et al., 2023), a wide range of lipids results in aSyn-lipid co-assemblies as the main species in the aggregation pathway, and secondary nucleation has a central role in aSyn aggregation also in neutral pH solutions under quiescent conditions. More generally, despite potential variations in rate constants and rate-limiting steps, the fundamental mechanisms of aSyn aggregation remain consistent. Therefore, although our model is calibrated for specific experimental conditions, it hinges on processes that retain *in vivo* translatability, such as oligomeric conversion, secondary nucleation, and fibril elongation via the incorporation of monomers and lipids. This consistency aligns with the holistic approach of QSP and PBPK frameworks, which integrate system-level dynamics to model complex biological processes.

To capture the impact of aSyn aggregation on neuronal function *in vivo*, future work should extend the model presented here to encompass additional mechanisms, particularly those involving fibril breakdown mechanisms. For example, based on its negligible impact on aggregation dynamics, we have chosen to disregard fibril depolymerization, *i.e.*, the reverse of fibril elongation by monomer addition, as a contributing factor in our model. This process is significantly slower than other dominant aggregation events and becomes especially insignificant as monomer concentrations are depleted at equilibrium (Dear et al., 2020b). Moreover, our model does not include fibril disaggregation into oligomers. This modeling choice aligns with existing chemical kinetic models of aSyn aggregation, which typically exclude this reaction due to its negligible impact under standard experimental setups and its limited mechanistic role under physiological conditions (Iljina et al., 2016; Cremades et al., 2012; Dear et al., 2020b; Xu et al., 2024). Indeed, fibril disaggregation into oligomers has been observed primarily under destabilizing conditions, such as monomer-depleted environments or altered pH (Cremades et al., 2012; Picotti et al., 2007). Moreover, this simplification supports the broader goal of integrating the model into a QSP framework, where excluding processes with minimal impact simplifies the model while still capturing the key features of aSyn aggregation relevant for drug development. Nevertheless, fibril disaggregation could play a more significant role in specific physiological contexts or under therapeutic interventions aimed at destabilizing fibrils. For example, fibril disaggregation can release cytotoxic oligomers that may accelerate neurodegeneration (Cremades et al., 2012), and intracellular processes such as chaperone-mediated disaggregation could influence this pathway (Bigi et al., 2022). Future iterations

of this model could incorporate fibril disaggregation to explore its potential contributions to disease progression and therapeutic outcomes, particularly in scenarios involving environmental or cellular stressors and enzymatic activity that promote fibril destabilization.

Current research highlights secondary nucleation as the candidate secondary mechanism governing aSyn aggregation, whereas fibril fragmentation is typically excluded from models of test-tube fibril formation due to its minimal impact on early-time dynamics (hours or days). Indeed, under physiological conditions, experimental measurements suggest that fragmentation rates are low compared to secondary nucleation (Xu et al., 2024). Nevertheless, this mechanism may become more relevant over longer timescales associated with disease progression (days to years), particularly in fibril length redistribution and potential prion-like spread. Fragmentation indeed generates shorter seeds ($< 50\text{ nm}$) with enhanced cell penetration and high cytotoxicity (Xue et al., 2009), potentially accelerating neuropathological spread in later disease stages. Further extensions of this model could incorporate fragmentation delayed pathological role for multi-timescale simulations of Parkinson's disease progression. This would be particularly relevant in scenarios where monomer depletion approaches critical concentrations or where mechanical stresses become more pronounced (e.g., trauma, shear forces in CSF, or cellular motility). However, while fragmentation becomes the main reason for fibril number increase at full monomer depletion, *in vivo* systems rarely reach full depletion due to continuous protein production.

While total protein mass concentration remains constant in test-tube experiments, aggregation dynamics is regulated by monomer production and protein clearance *in vivo*. Therefore, another model extension would consist in adopting a modular and incremental strategy to integrate the standalone aggregation module with a minimal reaction set, accounting for protein synthesis, misfolding, and generic degradation mechanisms (e.g., Thompson et al. (2021)). Such a model would provide insights into the interplay of aSyn aggregation and dysfunctional degradation and, thus, into PD pathogenesis. Indeed, while PD has commonly been identified as a proteinopathy, it is not merely a consequence of aSyn aggregation but manifests as a multifactorial disorder wherein various pathological mechanisms converge. Disrupted aSyn homeostasis, controlled by uncontrolled protein accumulation and age-related impairments of the degradation machinery, stands out among these processes. Therefore, targeting aSyn aggregation or degradation processes within the deregulated proteostasis network can be a promising strategy to slow down or halt PD progression. In this context, a mechanistic understanding of the role of aSyn aggregation in this intricate molecular landscape is critical for developing effective therapeutic interventions.

Although our model is rooted in *in vitro* findings, by offering a unified description of *in vitro* aggregation, it can serve both as a stand-alone tool for

advancing therapeutic strategy design through molecular-level mechanistic insights and as a modular component in multiscale models. Aligned with recent work in modeling neurodegenerative diseases (Ramakrishnan et al., 2023; Geerts et al., 2023b; Geerts et al., 2023a), this model is well-suited for integration into a mechanistic framework that includes a brain compartment along with compartments representing aSyn detection sites, such as cerebrospinal fluid and blood. This approach would leverage data from ongoing clinical trials on various disease-modifying therapies (Fields et al., 2019; Pagano et al., 2022; Lang et al., 2022). More specifically, our work provides a ready-to-go model grounded in prior *in vitro* mechanistic knowledge that can be seamlessly integrated into larger modeling frameworks. Overall, within a QSP framework, our model might serve as a key building block for an intraneuronal model of disrupted aSyn homeostasis in Parkinson's disease. Our work contributes to a coherent mechanistic understanding of aSyn aggregation by combining key findings and published *in vitro* data, acquired from various experimental conditions and through different techniques. The model corroborates mechanistic findings across diverse aggregation scenarios and provides insights into *in vitro* experimental outcomes. Therefore, it serves as a virtual lab for *in silico* experiments that, while impractical *in vitro*, may be relevant to *in vivo* biology, making it well-suited for integration into QSP and PBPK frameworks.

3.4 MATERIAL AND METHODS

ODE SYSTEM AND DETERMINISTIC IMPLEMENTATION

The ODE system of the model of pure and lipidic aSyn aggregation consists of 12 variables, 12 reactions, and 18 parameters, listed in Table 3.1. Refer to Figure 3.1 for a graphical representation of this model.

$$\begin{aligned}
 \frac{dm}{dt} = & \underbrace{\underbrace{-nk_n m^n}_{\text{homogeneous primary nucl.}}}_{\text{homogeneous primary nucl.}} + \underbrace{\underbrace{-nk_{\max, I} \frac{m^n}{m^n + K_P^n}}_{\text{heterogeneous primary nucl.}}}_{\text{heterogeneous primary nucl.}} + \underbrace{+nk_d A}_{\text{oligomer dissociation}} \\
 & \underbrace{\underbrace{-nk_{\max, II} \frac{m^n}{m^n + K_S^n} \tilde{M}_{F, P}}_{\text{pure fibril secondary nucl.}}}_{\text{pure fibril secondary nucl.}} + \underbrace{\underbrace{-nk_{\max, II} \frac{m^n}{m^n + K_S^n} \tilde{M}_{F, L}}_{\text{lipidic fibril secondary nucl.}}}_{\text{lipidic fibril secondary nucl.}} + \underbrace{\underbrace{-nk_{\max, II}^* \frac{m^n}{m^n + K_S^n} M_{F, *}}_{\text{newly generated fibril secondary nucl.}}}_{\text{newly generated fibril secondary nucl.}} \\
 & \underbrace{\underbrace{-k_{I1} m F_P}_{\text{pure fibril elongation}}}_{\text{pure fibril elongation}} + \underbrace{\underbrace{-yk_{\text{on}} V_b \frac{k_{I2} m F_L}{k_{I2} m + yk_{\text{on}} V_b}}_{\text{vesicle-mediated (lipidic) fibril elongation}}}_{\text{vesicle-mediated (lipidic) fibril elongation}} + \underbrace{\underbrace{+\frac{M_s}{\gamma} (-k^+ m V_f + k^- V_b)}_{\text{monomer-vesicle binding}}}_{\text{monomer-vesicle binding}} \\
 \frac{dA}{dt} = & +k_n m^n + k_{\max, I} \frac{m^n}{m^n + K_P^n} - (k_d + k_{c1}) A \\
 & + k_{\max, II}^P \frac{m^n}{m^n + K_S^n} \tilde{M}_{F, P} + k_{\max, II}^L \frac{m^n}{m^n + K_S^n} \tilde{M}_{F, L} + k_{\max, II}^* \frac{m^n}{m^n + K_S^n} M_{F, *}
 \end{aligned}$$

$$\begin{aligned}
\frac{dB}{dt} &= \underbrace{+k_{c1}A}_{\text{oligomer conversion}} - \underbrace{k_{c2}B}_{\text{fibril formation}} \\
\frac{dF^*}{dt} &= +k_{c2}B - \underbrace{k_{l1}mF^*}_{F^* \text{ elongation}} - \underbrace{k_{on}V_bF^*}_{\text{vesicle-}F^* \text{ binding}} \\
\frac{dM_{F^*}}{dt} &= +nk_{c2}B - k_{l1}mM_{F^*} - k_{on}V_bM_{F^*} \\
\frac{dF_p}{dt} &= +k_{c2}B - k_{on}V_bF^* \\
\frac{dM_{F,p}}{dt} &= +nk_{c2}B + k_{l1}mF_p - k_{on}V_bM_{F^*} \\
\frac{dF_l}{dt} &= +k_{c2}B - k_{l1}mF^* \\
\frac{dM_{F,l}}{dt} &= +nk_{c2}B - k_{l1}mM_{F^*} + yk_{on}V_b \frac{k_{l2}mF_l}{k_{l2}m + yk_{on}V_b} \\
\frac{dV_{f,b}}{dt} &= +k_{on}V_b \frac{k_{l2}mF_l}{k_{l2}m + yk_{on}V_b} \\
\frac{dV_f}{dt} &= -k^+mV_f + k^-V_b \\
\frac{dV_b}{dt} &= k^+mV_f - k^-V_b - k_{on}V_b \frac{k_{l2}mF_l}{k_{l2}m + yk_{on}V_b}
\end{aligned}$$

$$m_{TOT} = m + m_b + m_{F,b} + n(A + B) + M_{F,TOT} \quad (\text{monomer conservation law})$$

$$V_{TOT} = V_f + V_b + V_{F,b} \quad (\text{vesicle conservation law})$$

where $\tilde{M}_{F,p} = M_{F,p} - M_{F,*}$, $\tilde{M}_{F,l} = M_{F,l} - M_{F,*}$, and $M_{F,TOT} = M_{F,p} + M_{F,l} - M_{F^*} = \tilde{M}_{F,p} + \tilde{M}_{F,l} + M_{F,*}$. Also, $m_b = \frac{M_s}{\gamma} V_b$ and $m_{F,b} = \frac{M_s}{\gamma} V_{F,b}$. Given an initial lipid amount $DMPS_{TOT}$, the total vesicle concentration $V_{TOT} = DMPS_{TOT}/M_s$. Type-A and type-B oligomer mass concentrations correspond to $M_A = nA$ and $M_B = nB$, respectively.

We implemented the ODE system in MATLAB 2024a, using the built-in ODE solver function `ode23s` for simulations.

MASTER EQUATION APPROACH

Within the modeling framework proposed by Knowles and colleagues (Oosawa et al., 1962; Knowles et al., 2009), the deterministic model proposed here results from a moment-based approximation of a master equation, relying on the chemical kinetic representation of protein aggregation (Knowles et al., 2009). The master equation approach describes in probabilistic terms the time course of the species involved in the system and identified by their monomer count. Specifically, this stochastic representation presents the evolution of the molecule number of filaments of length j at time t , i.e, $f(j, t)$, using the concentration of monomers at time t . The resulting master equation summarizes

an infinite system of non-linear differential equations. By extracting the zero-th and first moments, *e.g.*, $F_X(t) = \sum_{j>n} f(j, t)$ and $M_{F,X} = \sum_{j>n} jf(j, t)$, we obtain the corresponding ODE deterministic system describing the dynamics of aggregate number and mass concentrations, respectively. Note that the reaction flow of lipid-mediated fibril elongation has been taken from [Dear et al. \(2024\)](#). This term relies on the assumption that newly generated fibrils F^* and fibrils with vesicles bound y monomers away from the fibril ends $F_{l,y}$ can bind a new monomer-coated vesicle, whereas the other fibrillar species $F_{l,i}$ ($0 \leq i < y$) can only elongate with rate constant k_{l2} . By adopting a mean field approach as in [Dear et al. \(2024\)](#), we derived the differential equations:

$$\begin{aligned}\frac{dF^*}{dt} &= k_n m^n + k_{\max, I} \frac{m^n}{m^n + K_p^n} + k_{\max, II} \frac{m^n}{m^n + K_s^n} M_{F,l} - k_{\text{on}} V_b (F^* + F_{l,y}) \\ \frac{dF_{l,0}}{dt} &= k_{\text{on}} V_b (F_{l,y} + F^*) - k_{l2} m F_{l,0} \\ \frac{dF_{l,i}}{dt} &= k_{l2} m F_{l,i-1} - k_{l2} m F_{l,i} \\ \frac{dF_{l,y}}{dt} &= k_{\text{on}} V_b (F_{l,y} + F^*) - k_{l2} m F_{l,0}\end{aligned}$$

Assuming that the nucleation rates are negligible compared to the elongation rates and approximating to a pre-equilibrium between the different species ([Dear et al., 2024](#)) result into

$$\begin{aligned}F_{l,0} &= \frac{k_{\text{on}} V_b}{k_{l2} m} (F_{l,y} + F^*) \\ F_{l,i} &= F_{l,i-1} \quad (0 < i < y) \\ F^* + F_{l,y} &= \frac{k_{l2} m}{k_{\text{on}} V_b} F_{l,y-1} \\ F_l &= \sum_{i=0}^y F_{l,i} + F^* = y F_{l,0} + F^* = \left(1 + y \frac{k_{\text{on}} V_b}{k_{l2} m}\right) (F_{l,y} + F^*)\end{aligned}$$

leading to the lipid-mediated elongation term

$$\begin{aligned}\frac{dM_{F,l}}{dt} &= \sum_{i=0}^{y-1} k_{l2} m F_{l,i} + \varepsilon = y k_{l2} m F_{l,0} + \varepsilon = \\ &= \frac{k_{\text{on}} V_b}{k_{l2} m} (F_{l,y} + F^*) + \varepsilon = y \frac{k_{\text{on}} V_b k_{l2} m F_l}{y k_{\text{on}} V_b + k_{l2} m} + \varepsilon\end{aligned}$$

where ε represents the additional terms governing $M_{F,l}$ time derivative.

MODEL CALIBRATION AND VALIDATION

Calibration of pure protein aggregation Given the crucial role of oligomers in the aggregation pathway, we first focused on available data of aSyn oligomerization in solution, corresponding to the first pure protein aggregation scenario (Iljina et al., 2016). Retrieving time-series data on oligomer levels proves challenging due to their diverse and transient nature; smFRET stands out among the few experimental techniques that can monitor oligomers' time evolution (e.g., quantitative size exclusion chromatography, multispectral microchip free-flow electrophoresis) as it can differentiate between type-A and type-B oligomers linked to low and high FRET levels. Therefore, we used the available smFRET measurements of monomers and oligomers to calibrate oligomeric conversion and fibril formation, in addition to primary nucleation reactions. Conversely, rate constants associated with oligomer dissociation and fibril elongation were set to fixed values derived from the literature (Buell et al., 2014; Dear et al., 2020b). Drawing on existing biological insights into the role of secondary events in this scenario, we switched off secondary nucleation and ruled out fibril fragmentation in our model. Indeed, fibril-catalyzed nucleation plays a marginal role compared to homogeneous and heterogeneous primary nucleation, especially at early reaction times, when high fibril levels are not reached yet. Moreover, even though constant agitation is known to promote fibril fragmentation, we excluded this reaction from the model diagram upon assessment of its negligible impact on early-time dynamics. At the same time, constant agitation promotes heterogeneous primary nucleation by introducing shearing forces or increasing turnover at the air–water interface (Campioni et al., 2014; Gaspar et al., 2017). Therefore, while mechanical stress is not explicitly modeled, its effects are implicitly captured through the calibrated parameters, particularly by the heterogeneous nucleation rate constant.

Approaching a more physiologically relevant scenario as compared to shaking-induced aggregation, we calibrated the model to reproduce the dynamics of pure aSyn aggregation triggered with preformed fibril seeds and exacerbated by a pH shift from neutral to mildly acidic. Secondary nucleation plays a predominant role in this setting compared to primary nucleation (Gaspar et al., 2017). Parameters associated with this mechanism were fitted while keeping those related to primary nucleation mechanisms fixed, by leveraging the results from the initial calibration step. Note that we estimated the rate constant of secondary nucleation on pure and newly generated fibrils as a single parameter $k_{\max,II}$ (i.e., $k_{\max,II}^P = k_{\max,II}^*$). Simultaneously, we adjusted the rate constants governing oligomer conformational changes, maintaining a fixed ratio k_{c2}/k_{c1} . In contrast to the first calibration step, the model was calibrated on measurements of fibril mass concentration by Thioflavin-T (ThT) dye fluorescence. Although more precise than smFRET, this standard technique cannot provide oligomer data but only detect fibril levels.

In addition to smFRET, single-molecule microfluidics can also detect oligomer mass data (Xu et al., 2024). Here, we focused on ThT-derived fibril mass and single molecule FFE-derived oligomer mass data obtained under solution conditions that mimic the cytosolic environment (neutral pH and physiological ionic strength). To recalibrate the model for 0% and 1% seeded aggregation, we (i) retained primary nucleation and fibril elongation estimates from the second calibration scenario, (ii) increased the oligomer dissociation rate constant ($k_d = 1 \text{ h}^{-1}$ vs. $k_d = 2.4 \times 10^{-3} \text{ h}^{-1}$ in previous calibrations), (iii) removed constraints on oligomeric conversion rate constants for long-lived oligomeric species (*i.e.*, $k_{c2} = 0.15k_{c1}$). As for the previous scenario, we estimated the rate constant of secondary nucleation on pure and newly generated fibrils as a single parameter $k_{\max, \Pi}$ (*i.e.*, $k_{\max, \Pi}^p = k_{\max, \Pi}^*$).

Calibration of lipid-mediated aggregation We considered another biologically meaningful scenario related to aSyn normal functions in the synapses. DMPS vesicles are introduced in neutral pH solutions with monomers at different lipid-to-aSyn ratios, obtained by adjusting the initial lipid amount while maintaining a constant total monomer concentration. DMPS phospholipids, commonly used in lipid-mixing vesicle preparation to form thin membranes, promote significant lipid uptake in aSyn co-aggregates (Hellstrand et al., 2013). This *in vitro* aggregation assay has proven valuable for analyzing lipidic aSyn aggregation. By leveraging this experimental setup, Dear et al. (2024) challenged the traditional perspective that lipid bilayers act as catalysts for primary nucleation, increasing the local protein concentration on their surface to facilitate aggregation (Galvagnion et al., 2015). While lipids may have a role in primary nucleation, their primary function is actively boosting fibril elongation, which governs the aggregation process (Galvagnion et al., 2024; Dear et al., 2024). We integrated these recent mechanistic findings into the model structure and set some of the parameters associated with lipidic interactions ($K_D = 3.8 \times 10^{-1} \mu\text{M}$, $\gamma \sim 30$, $M_s = 6000$, and $7.5 \leq k_{\text{on}}/k_{12} \leq 16$) to values provided by the existing literature (Galvagnion et al., 2015; Dear et al., 2024).

We relaxed the pre-equilibrium assumption on monomer-vesicle binding set by Dear et al. (2024). As a result, model calibration required assessing the rate constant of monomer-vesicle unbinding k_- , such that $K_D = k_-/k_+$, and fitting the coefficient of vesicle-mediated fibril elongation y as a free parameter instead of deriving it directly from stoichiometric relationships. To obtain this parameter value, Dear et al. (2024) used the formula $y = M_s/\gamma - M_s/\chi$, where the stoichiometry of lipid-to-protein in lipidic fibrils χ was experimentally estimated at approximately 15 lipids per protein monomer under optimal conditions (Galvagnion et al., 2015; Dear et al., 2024). Without pre-equilibrium, the assumption of a fixed stoichiometry no longer strictly applies, and the explicit relationship between y , χ , and other parameters becomes dynamic/-less deterministic, with deviations in stoichiometry arising under sub-optimal conditions. In our model, y represents no longer a fixed number but an em-

pirical average number of monomers added per vesicle binding event; thus, it reflects an average behavior influenced by experimental conditions such as lipid-to-protein ratio, vesicle size (M_s), and binding kinetics (k_+ and k_-). The estimated value still aligns with the values obtained in the literature. Nevertheless, treating y as a fitted parameter can provide flexibility in modeling variability across experimental conditions while capturing essential features of lipid-mediated co-elongation and maintaining consistency with experimental observations. Furthermore, we assumed that lipidic fibrils elongate at the same rate constant as pure fibrils, *i.e.*, $k_{11} = k_{12}$, with the parameter value derived from the literature (Buell et al., 2014). Based on parameter estimates from prior calibrations, we adjusted the rate constants related to oligomer structural conversions while keeping their ratio fixed. Moreover, we switched off all nucleation reactions except for heterogeneous primary nucleation (estimated), which has been identified as the predominant nucleation reaction in this experimental setting (Dear et al., 2024). In line with this, the fitting procedure suggested a negligible impact of secondary nucleation in this experimental scenario ($k_{\max,II}^1 = k_{\max,II}^* = 0$).

Optimization algorithm and uncertainty quantification The parameter estimation procedure relied on a global stochastic optimization method computing the covariance matrix of the search distribution to estimate unknown model parameters, namely, the covariance-based evolutionary optimization (CMA-ES) algorithm (Real et al., 2017). The optimization problem corresponded to minimizing a weighted least squares objective function tailored for each calibration scenario. To avoid model overfitting and take into account the differences in initial monomer and lipid concentrations, we calibrated the aggregation module on multiple time-series for the considered species in the system corresponding to the highest and lowest initial monomer concentrations/lipid-to-monomer ratios. Complementarily, we validated the model on the time series related to intermediate levels of initial monomer concentrations and lipid-to-monomer ratios. To perform uncertainty quantification, we randomly sampled 1000 parameter sets from a standard normal distribution centered in the parameter estimates with coefficient of variation of 20% (10% for y). We then run model simulations for each parameter set and computed the median and the 5th and 95th percentiles of the time series distribution for each model species.

On the optimization strategy choice. Our calibration problem involved a low-dimensional parameter space (3–5 parameters per scenario) with reasonable initial estimates, making gradient-based methods initially appealing (Villaverde et al., 2019). However, multi-start gradient approaches did not yield satisfactory fits. We therefore selected CMA-ES: its population-based strategy, requiring approximately 10 function evaluations per iteration, provides robust global search while remaining computationally efficient. Its invariance properties and adaptive covariance matrix updating further help overcome local optima

and parameter scaling issues, which are common in biochemical kinetic models. Bayesian optimization could have been considered as an alternative, as it is well-suited for low-dimensional problems, naturally incorporates prior knowledge, and provides uncertainty estimates. However, its implementation requires careful kernel and acquisition function specification, and probabilistic surrogate model (*e.g.*, Gaussian process) inference adds computational overhead that grows with the number of function evaluations. For our case, CMA-ES offered an effective solution enabling a straightforward sequential calibration strategy without the need for additional prior specification.

AGGREGATION METRICS

For comparison across *in silico* experiments, we computed different metrics that directly relate to aggregation:

- the oligomer peak level and time;
- the final level of pure, lipidic, and newly generated fibril mass concentration normalized to the total monomer concentration (*i.e.*, $\tilde{M}_{F,p}$, $\tilde{M}_{F,l}$, and $M_{F,*}$ relative abundance), along with the corresponding half-time.
- the final level of pure, lipidic, and newly generated fibril number concentration normalized to the total fibril concentration (*i.e.*, $\tilde{F}_p$, $\tilde{F}_l$, and F^* relative abundance), serving as a measure of the composition of the fibrillar population.
- the propensity of newly generated fibrils to undergo lipidic over pure protein aggregation normalized for the time range, identified by

$$\int_0^{t_f} \frac{k_{on} V_b(t)}{k_{li} m(t) + k_{on} V_b(t)} dt \quad ,$$

serving as a measure of the relative occurrence of the two reactions and, thus, determining the overall dominance of lipidic fibril formation over pure protein fibril formation.

- Lewy body composition indexes measuring the composition of Lewy body-like structures that result from our *in vitro* model system. These inclusions are not direct analogs of the cytoplasmic Lewy bodies in PD patients. Instead, they emerge as a natural outcome of *in vitro* aggregation, where “LB” seeds form through fibril-fibril interactions, and growth occurs through further fibril incorporation. The indexes introduced below track contributions of different fibril types. Given $AUC(\cdot)$ as the area under the curve over a specified time interval (*i.e.*, $AUC(x(t)) = \int_0^T x(t) dt$), we define:

- self-contribution indexes for pure, lipidic, and newly generated fibrils:

$$I^p = \frac{AUC(\tilde{F}_p^2)}{AUC(\tilde{F}_{TOT}^2)} \quad , \quad I^l = \frac{AUC(\tilde{F}_l^2)}{AUC(\tilde{F}_{TOT}^2)} \quad , \quad I^* = \frac{AUC(\tilde{F}_*^2)}{AUC(\tilde{F}_{TOT}^2)} \quad , \quad (3.5)$$

which sum to $I^{\text{self}} = I^{\text{p}} + I^{\text{l}} + I^* = \frac{\sum_{i \in \{\text{p}, \text{l}, *\}} \text{AUC}(\tilde{F}_i^2)}{\text{AUC}(\tilde{F}_{\text{TOT}}^2)}$.

– a cross-contribution index for interactions between different fibril types:

$$I^{\text{cross}} = \frac{2 \sum_{i,j \in \{\text{p}, \text{l}, *\}} \text{AUC}(\tilde{F}_i \tilde{F}_j)}{\text{AUC}(\tilde{F}_{\text{TOT}}^2)} \quad (3.6)$$

By definition, $I^{\text{self}} + I^{\text{cross}} = 1$, ensuring a complete decomposition. A detailed derivation of these indexes is provided in Appendix A.

LOCAL SENSITIVITY ANALYSIS

We carried out a local sensitivity analysis (LSA) to quantify the impact of the model parameters on the system dynamics (Simoni et al., 2020). For each parameter in $\mathbf{p} = (p_1, \dots, p_j, \dots, p_m)$, we computed a logarithmic sensitivity index (SI) based on the area-under-the-curve (AUC) of each model variables in $\mathbf{x} = (x_1, \dots, x_i, \dots, x_n)$ within the experimental time interval $[0, t_f]$. Relying on a numerical approximation given by the second order central finite difference formula, the sensitivity index $S_{i,j}$ associated with the model variable x_i and the $\delta = \pm 1\%$ perturbation of the parameter p_j from its nominal value was obtained as

$$\begin{aligned} \text{SI}_{i,j} &= \frac{\partial \log \text{AUC}(x_i(p_j))}{\partial \log p_j} = \frac{\partial \text{AUC}(x_i(p_j))}{\partial p_j} \frac{p_j}{x_i(p_j)} = \\ &= \frac{\text{AUC}(x_i(p_j + \delta p_j)) - \text{AUC}(x_i(p_j - \delta p_j))}{2\delta p_j} \frac{p_j}{\text{AUC}(x_i(p_j))} \end{aligned}$$

where $\text{AUC}(x_i(p_j)) = \int_0^{t_f} x_i(p_j, t) dt$, with $x_i(p_j, t)$ denoting the i -th model variable at time t considering the nominal value of the j -th parameter p_j .

ACCOUNTING FOR EXPERIMENTAL BIAS IN MECHANISTIC PREDICTIONS OF UNBOUND DRUG FRACTION IN BRAIN HOMOGENATES

Chapter abstract Unbound drug exposure in the brain is a critical factor in CNS drug development. Among the unbound concentrations definable in brain tissue, we focus on the interstitial unbound concentration C_{iw} . It depends on total brain concentrations and the fraction unbound in brain tissue (fuT), defined as the ratio of interstitial water to total tissue drug concentration. While fuT is often estimated using equilibrium dialysis of brain homogenates, this experimental method disrupts cell membranes and pH compartmentalization, introducing experimental artifacts. Mechanistic models, such as the widely used Rodgers & Rowland (R&R) methods, enable *a priori* predictions of fuT . However, model predictions are typically evaluated against homogenate data, leading to a mismatch between model assumptions and experimental endpoints. To address this, we derived a homogenate-specific metric, fuH_{tis} , building on the R&R framework to account for the experimental bias. We compared fuH_{tis} and fuT across a dataset of over 50 CNS-active small molecules, analyzing the influence of ionization state and tissue composition—especially brain lipid levels. Our results support fuH_{tis} as a mechanistically appropriate endpoint for homogenate assays, provided brain lipid composition is well characterized. Overall, this chapter underscores the need to reconcile experimental artifacts with mechanistic models and supports improving *a priori* predictions of unbound drug exposure in the brain within PBPK frameworks.

This chapter is based on the following work: Righetti et al., *Evaluating a priori fraction unbound predictions against brain homogenate data: Accounting for the experimental bias*, presented as a conference poster at Population Approach Group Europe (PAGE) 2025, and *Lost in homogenization: Reconciling a priori mechanistic predictions with experimental estimates of brain fraction unbound*, manuscript in preparation. This project is part of an ongoing collaboration with Prof. Huisinga's lab at the University of Potsdam. I co-designed the study, curated the data, performed all the analyses, created the figures, and wrote all related content included in this chapter.

4.1 INTRODUCTION

Assessing CNS drug distribution is a critical step in developing brain-active therapeutics. According to the free drug hypothesis (or theory [Summerfield et al. \(2022\)](#)), only the fraction unbound of a drug is pharmacologically active, as it is the only form available for interaction with molecular targets. Therefore, the unbound drug concentration in brain tissue is a key determinant of both efficacy and safety. Unlike most peripheral organs, the brain is protected by specialized anatomical and physiological barriers, such as the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB), and the barriers separating the cerebrospinal fluid from the brain interstitial space (*e.g.*, ependyma, pia mater). These barriers restrict passive diffusion and also involve active transport mechanisms that significantly affect CNS drug kinetics. The extent of brain penetration has traditionally been described by the total brain tissue-to-plasma concentration ratio, $K_{p_{bra}}$, or its unbound counterpart, $K_{pu_{bra}}$ ([Reichel, 2015](#)). These parameters are widely used in PBPK models for peripheral organs, where the total tissue-to-unbound plasma partition coefficient $K_{pu_{tis}}$ is typically calculated as $1/fu_P \cdot K_{p_{tis}}$, with fu_P being the fraction unbound in plasma. This approach assumes fast exchange across the vascular endothelium, so that the unbound plasma concentration reflects the unbound interstitial concentration within the tissue. This assumption, however, does not hold in the brain. The restrictive nature of the BBB often leads to unbound plasma concentrations equal to or higher than those in the brain interstitial fluid ([Liu et al., 2009](#)). As a result, $K_{p_{bra}}$ and $K_{pu_{bra}}$ do not fully capture the complexity of CNS-specific distribution. To address this, a more mechanistically relevant metric has been proposed ([Hammarlund-Udenaes et al., 2008](#); [Loryan et al., 2022](#)), which corrects for nonspecific drug binding to both brain tissue and plasma. The unbound brain-to-unbound plasma partition coefficient, $K_{puu_{bra}}$, is defined as

$$K_{puu_{bra}} := \frac{C_{iw}}{C_{pw}}, \quad (4.1)$$

where C_{iw} and C_{pw} represent unbound drug concentrations in the brain interstitial fluid and plasma, respectively. This quantity is more directly linked to pharmacological activity than conventional $K_{pu_{tis}}$. It also more accurately reflects transport equilibrium across the BBB, ranging from 0.01 to 5, with $K_{puu_{bra}} \sim 1$ suggesting passive diffusion; values substantially different from 1 indicate active efflux or uptake. The definition of fraction unbound in brain tissue has evolved alongside this conceptual shift from $K_{pu_{bra}}$ to the more mechanistically relevant $K_{puu_{bra}}$. In earlier literature, fu_T was obtained as $1/K_{pu_{tis}} = C_{pw}/C_{tis}$ ([Rodgers et al., 2005](#); [Rodgers et al., 2006](#); [Maurer et al., 2005](#); [Summerfield et al., 2006](#)), while more recent work adopts the mechanistic definition based on interstitial water ([Kalvass et al., 2007](#); [Fridén et al., 2009](#); [Fridén et al., 2011](#); [Spreafico et al., 2013](#)). Specifically, fractions unbound in

plasma and interstitial fluid do not correlate, as plasma and brain differ substantially in protein and lipid content (Reichel, 2015). This distinction is important when comparing fuT values across studies or data sources, as inconsistent definitions may lead to misinterpretation. The definition of fuT adopted in this work is the most recent one, related to interstitial water:

$$\text{fuT} := \frac{C_{\text{iw}}}{C_{\text{tis}}} , \quad (4.2)$$

i.e., the ratio of unbound interstitial water concentration to total tissue concentration. Reliable estimation of fuT—whether through prediction or measurement—can be crucial for assessing the unbound brain-to-unbound plasma partition coefficient, $K_{\text{puu}_{\text{bra}}}$, which is rarely measured directly in practice. Instead, it is typically estimated by leveraging the following relationship between $K_{\text{puu}_{\text{bra}}}$ and fuT:

$$K_{\text{puu}_{\text{bra}}} = K_{\text{p}_{\text{bra}}} \cdot \frac{\text{fuT}}{\text{fuP}} . \quad (4.3)$$

Therefore, despite being mechanistically independent of $K_{\text{p}_{\text{bra}}}$, fuT, and fuP, $K_{\text{puu}_{\text{bra}}}$ can be derived from these more accessible experimentally or predictable quantities (Liu et al., 2018). As such, accurate fuT estimation is fundamental in CNS drug development and in PBPK modeling to evaluate brain exposure and support translational predictions.

The fraction unbound fuT can be estimated either experimentally or through *a priori* mechanistic prediction.

Experimental methods. Measuring fuT in intact brain tissue remains technically challenging. Several experimental techniques exist, each with distinct strengths and limitations (Chowdhury et al., 2021).

- *In vivo* microdialysis provides direct, real-time sampling of unbound drug concentrations in the brain interstitial fluid of awake animals, in specific brain regions. It is considered the most accurate reference method (Darvesh et al., 2011); it is, however, invasive, technically demanding, time-consuming, and low-throughput, limiting its applicability in drug discovery, especially for highly lipophilic compounds (Reichel, 2015; Loryan et al., 2014; Hammarlund-Udenaes et al., 2009).
- Brain slice methods offer a valid *in vitro* alternative, enabling measurement in a physiologically relevant tissue environment that preserves cellular structures (Kakee et al., 1996; Fridén et al., 2010; Loryan et al., 2013; Fridén et al., 2009). The output, *i.e.*, the volume of distribution in the brain, aligns well with values from *in vivo* microdialysis (within a 3-fold deviation). These methods offer higher throughput than *in vivo* microdialysis but remain limited by tissue degradation and operational complexity, and are generally less scalable than other approaches such as brain homogenate methods (Chowdhury et al., 2021).

- Equilibrium dialysis of brain homogenates is currently the standard *in vitro* experimental method in drug discovery due to its high throughput, cost-effectiveness, and compatibility with early screening workflows (Reichel, 2015; Summerfield et al., 2008). Nevertheless, homogenization disrupts cellular membranes and physiological compartmentalization, introducing experimental artifacts. This can affect drug binding, particularly for compounds whose distribution is sensitive to pH-dependent partitioning (Fridén et al., 2011). Despite their pitfalls, brain homogenate assays remain useful for rapid comparative assessments, with rodent brain homogenate measurements being used as a reference in CNS drug discovery and development (Read et al., 2010; Reichel, 2015; Loryan et al., 2022). Extensive data on rat brain homogenate binding are available across multiple compound classes (e.g., Culot et al. (2013), Fridén et al. (2011), Kalvass et al. (2007), and Longhi et al. (2011)). These measurements are routinely used in toxicology, pharmacodynamics, and human pharmacokinetic predictions. This practice stems from the widely accepted assumption of species independence in brain tissue binding, underpinning the extrapolation of rodent data to other species, including humans (Di et al., 2011). As a result, homogenate-derived values of the fraction unbound are often used as surrogates for f_{uT} in estimating $K_{puu_{bra}}$.

Mechanistic approaches and the mismatch with homogenate experimental data. Models such as the well-established Rodgers & Rowland (R&R) framework enable *in silico* prediction of f_{uT} based on physicochemical properties and tissue-specific parameters. These tissue composition-based models (TCMs, Poulin et al. (2024)) predict drug tissue partitioning (*i.e.*, the distribution of a drug within the tissue spaces) *a priori* by relying on three core ideas: (i) representing a tissue as a composition of its most relevant tissue constituents and barriers; (ii) approximating drug interactions with these constituents using *in vitro* assay readouts; (iii) finally, calculating drug partitioning into total tissue by summing the contributions from each constituent, weighted by their respective volumes. The predictive performance of these methods, however, is typically evaluated against homogenate data, which include experimental biases not accounted for in the mechanistic assumptions of the model. This mismatch can reduce confidence in f_{uT} predictions when they are validated against biased measurements in tissue homogenates, commonly used in drug discovery. In other words, while the mechanistic assumptions of the model remain sound, the inherent limitations of homogenate assays introduce uncertainty in evaluating model performance, calling for cautious interpretation of validation outcomes. This gap poses several key questions from a mechanistic modeling perspective:

1. Should *a priori* predictions of f_{uT} be evaluated against homogenate-derived values?

2. How can mechanistic models be adapted to reflect homogenate-specific alterations in tissue composition?
3. Does accounting for these experimental biases improve predictive accuracy?

Addressing these questions requires understanding how homogenization alters drug binding and how such changes can be mechanistically incorporated in current *a priori* methods. Bridging the gap between intact tissue predictions and homogenate-based measurements is essential for more reliable evaluation of fuT. This would improve the interpretation of fraction unbound data and the predictive utility of *in vitro* assays in CNS drug discovery and development.

Chapter outline In this chapter, we first introduce the relevant definitions, notation, and mechanistic framework. Specifically, in Section 4.2, we define the mechanistic basis of brain tissue binding and clarify the key quantities used to characterize CNS drug distribution. We outline the core concepts of mechanistic modeling for *a priori* predictions of fractions unbound, with a focus on the R&R methodology, with its assumptions and approximations (Rodgers et al., 2005; Rodgers et al., 2006). In Section 4.3, we derive the standard equation for predicting the fraction unbound in intact brain tissue and, as our main contribution, we propose a homogenate-specific expression for the fraction unbound in homogenized brain tissue (fuH_{tis}), building on R&R equations to reflect experimental artifacts. Our goal is to mechanistically explain the relationship between fuT and fuH_{tis} , and understand whether accounting for experimental bias improves the alignment between predicted and experimental values. Therefore, we assess the impact of homogenization on fraction unbound predictions by comparing fuT and fuH_{tis} against each other (Section 4.3.1) and against experimental brain homogenate data (Section 4.3.2), after characterizing compound-dependent homogenization biases. As discussed in Section 4.4, by clarifying the limitations of using homogenate-derived values as proxies for the fraction unbound in intact brain tissue, this work ultimately aims at enhancing the reliability of *a priori* predictions for CNS-active small molecules. Section 4.5 (Materials and Methods) includes detailed derivations of fractions unbound in intact and homogenate brain tissue, as well as the formula for the experimentally determined fraction unbound in undiluted brain homogenate. Here, we also provide details on compound selection, and show the experimental data used for evaluation of fractions unbound in intact and homogenate brain tissue. For a detailed picture of the R&R equations underlying drug partitioning into brain tissue components, interstitial and cellular compartments, plasma and red blood cells (and whole blood), we refer the reader to Appendix B.

4.2 MATHEMATICAL FRAMEWORK FOR A PRIORI PREDICTION OF BRAIN FRACTION UNBOUND

For a long time, a major bottleneck in early drug development has been estimating tissue distribution from *in vitro* and *in silico* data, without relying on costly and compound-intensive *in vivo* experiments. In the context of PBPK modeling, accurate prediction of drug tissue distribution and, thus, drug fractions unbound remains a crucial challenge—especially for compounds exhibiting high lipophilicity and extensive tissue distribution. In their seminal work, Poulin & Theil ([Poulin et al., 2002](#)) developed a set of mechanistic equations to *a priori* predict tissue-to-plasma and tissue-to-unbound plasma concentration ratios (or partition coefficients), K_p and K_{pu} , for diverse drug classes based on physiological and physicochemical properties, towards overcoming the limitations of empirical pharmacokinetic models. The key idea underlying this approach, and all the subsequent *a priori* methods predicting the unbound drug concentration in a specific subspace (be it the cellular and interstitial spaces, plasma, or red blood cells), consists in approximating drug distribution into tissue constituents (lipids, water, and binding proteins) and relying on readily available *in vitro* assay readouts. As a result, this approach requires physiological and drug-specific input parameters.

Rodgers & Rowland approach While the Poulin & Theil method ([Poulin et al., 2002](#)) accounted for drug distribution based on tissue composition (lipids, water, and proteins), it tended to underpredict the tissue-to-plasma partition coefficients for highly basic drugs. To address this limitation and improve prediction accuracy, by relying on the Poulin & Theil model, Rodgers & Rowland introduced a mechanistic framework that explicitly considers unique ionization-dependent electrostatic interactions between moderate-to-strong basic drugs and tissue acidic phospholipids, as well as binding to extracellular proteins for acids, neutral compounds, and weak bases ([Rodgers et al., 2005](#); [Rodgers et al., 2006](#)). This extended approach provides a more physiologically realistic estimate of tissue partition coefficients and is widely adopted in PBPK model development as the most recognized TCM. Indeed, it offers a detailed yet tractable framework for *a priori* prediction of tissue-to-plasma partition coefficients, based on physiological tissue composition and compound-specific physicochemical properties. Various versions have been proposed over time and their prediction accuracy has been thoroughly examined, *e.g.*, [Schmitt \(2008\)](#), [Lukacova et al. \(2008\)](#), [Poulin et al. \(2012\)](#), [Schmitt et al. \(2021\)](#), and [Poulin et al. \(2024\)](#). However, no current available version consistently yielded the most accurate model predictions for all drugs tested in the preclinical species and humans. We adopt the Rodgers & Rowland framework, *i.e.*, its theoretical basis and core assumptions, to *a priori* predict the fraction unbound in intact and homogenate brain tissues (see Section 4.5 for specific details on R&R assumptions and approximations).

TISSUE CONSTITUENTS AND SUBSPACES

To model drug distribution in the brain, we define biologically relevant subspaces and their associated constituents. The primary subspaces include:

- **interstitial space** (int): the extracellular matrix and the interstitial fluid (ISF), containing interstitial water (iw) and binding proteins (pr(i)).
- **cellular space** (cel): the cellular membrane and the intracellular environment, which includes cellular water (cw), binding proteins (pr(c)), neutral lipids (nl(c)), neutral phospholipids (np(c)), and acidic phospholipids (ap(c)).
- **peripheral blood space** (blo): a lumped compartment including plasma (pla) and red blood cells (RBCs), with respective water phases (pw and rw) and binding constituents.

Among the distinct tissue constituents influencing drug distribution, there are:

- **water phases** (wa), serving as the medium for the distribution of unbound drug molecules in cellular and interstitial spaces, plasma, and RBCs;
- **binding proteins** (pr), such as albumin or α_1 -acid glycoprotein, representing reversible interaction sites with affinity depending on the drug type (acid, base, neutral);
- **acidic phospholipids** (ap) in the cellular space, being a major site of accumulation for ionized bases in the cellular space due to strong electrostatic interactions with positively charged (moderate to strong) basic drugs;
- **neutral lipids** (nl) in cellular, RBC, and plasma spaces, preferentially sequestering highly lipophilic (non-polar) compounds through nonspecific partitioning;
- **neutral phospholipids** (np) in cellular, RBC, and plasma spaces, interacting with both hydrophilic and lipophilic drugs, thus supporting moderate binding through hydrophobic interactions.

The concentration of unbound drug in any compartment refers specifically to the drug dissolved in the water phase (for example, C_{iw} for interstitial water), and the fraction unbound of a drug in a tissue subspace is the ratio of the drug concentration in the water phase to the total concentration in that subspace (*e.g.*, fu_P , fu_C , and fu_I for plasma, cellular, and interstitial spaces, respectively). Given a tissue constituent (sub), we define the tissue constituent-to-water distribution coefficient $K_{sub:wa}$ as C_{sub}/C_{wa} , with $C_{sub} = A_{sub}/V_{sub}$ as the drug concentration bound to or in the tissue constituent and C_{wa} as the free drug concentration in water/buffer characterized by pH_{wa} .

INPUT PARAMETERS

A priori prediction methods for fraction unbound in brain tissue used in this work rely on both physiological and drug-specific parameters.

Drug-specific input parameters Key drug-specific parameters influencing brain tissue binding include:

- the acid dissociation constant K_A , often expressed in logarithmic form as pKa. It determines the ionization state of a drug in physiological fluids or buffered solutions. Specifically, the pH of the solution and the drug pKa values define the fraction of the drug in its neutral, cationic, anionic, and zwitterionic forms — denoted as fn_{wa} , $fc_{at_{wa}}$, $f_{an_{wa}}$, and $f_{z_{wi_{wa}}}$, respectively — via the Henderson–Hasselbalch equation (Henderson, 1908; Hasselbalch, 1916; Rodgers et al., 2005). These ionization fractions, in turn, affect both membrane permeability and nonspecific binding;
- the *n*-octanol:water partition coefficient $P_{O:W}$, commonly reported in logarithmic form as logP, which reflects compound lipophilicity and influences nonspecific tissue binding;
- the blood-to-plasma ratio (B:P) and the fraction unbound in plasma (fu_P), which are species-dependent parameters describing blood partitioning and plasma protein binding.

These input parameters can be either measured experimentally as *in vitro* readouts or predicted *in silico* using software tools (e.g., ADMET Predictor, GastroPlusTM). Section B.1 provides a more detailed explanation of these parameters and how they are typically computed or estimated within the R&R framework.

Brain physiological parameters Physiological and anatomical parameters describing brain structure and composition—whether species-specific or species-independent—include brain weight, interstitial and cellular volume fractions (and their associated water compartments), brain lipid composition, and pH values across different brain regions. Typical values, drawn from well-established literature sources (e.g., Valentin (2002), Brown et al. (1997), Poulin et al. (2002), and Rodgers et al. (2006)), are summarized in Table 4.2 and Table 4.1. Among these, fractional volumes of specific brain lipid classes are key determinants of drug partitioning into brain tissue. There is, however, limited consensus in the literature regarding the composition and quantitative distribution of brain lipids, even within the same species. For example, Rodgers & Rowland (Rodgers et al., 2005; Rodgers et al., 2006) relied on brain lipid volume fractions for rats based on Poulin et al. (2002) data and physiological assumptions about brain cell composition and lipid distribution. In contrast, Xu et al. (2020) provided updated estimates derived from direct lipid quantification using modern analytical techniques (e.g., high resolution mass

spectrometry). As shown in Table 4.1, these two sources report markedly different values, particularly for acidic phospholipids and neutral lipids, which can substantially affect predicted binding. For humans, direct measurements are even more scarce, leading to reliance on parameter sets such as those in Poulin et al. (2002) and extrapolation of acidic phospholipid values from rat data. As a result, for humans, the acidic phospholipid fractional volume ($V_{ap(c)}/V_{tis}$), defined as percentage of total phospholipid fraction volume $V_{p(c)}/V_{tis}$, was varied over a plausible range informed by both rat data and classical biochemical analyses.

This lack of consistency across sources reflects methodological differences, differences in sampled brain regions, and variable assumptions about brain structure and composition. In addition, brain lipid composition is known to vary with age, brain region, and disease state (Osetrova et al., 2024; Mota-Martorell et al., 2022; Fitzner et al., 2020; Naudí et al., 2015).

The range spanned the R&R baseline up to low-teen percentages, consistent with phosphatidylserine (PS) constituting roughly 13-15% of total brain phospholipids in the human cortex (Svennerholm, 1968; Kim et al., 2014). Indeed, PS was taken as a proxy for the total acidic-phospholipid pool, as it is the predominant acidic class and the binding characteristics of other acidic lipids (phosphatidylinositol, phosphatidylglycerol, and phosphatidic acid) are comparable for cationic drugs.

Table 4.1: **Brain tissue lipid composition.** (*) Corrected for residual blood using equation A1 in Rodgers & Rowland (Rodgers et al., 2006) and assuming residual blood fractional volume of 0.04 (Brown et al., 1997); (**) plausible range of acidic-phospholipid fractional volumes based on rat data (Rodgers et al., 2005; Xu et al., 2020) and classical biochemical analyses in human cortex (Svennerholm, 1968; Kim et al., 2014); (***) derived by sum of acidic and neutral phospholipids—not explicitly stated in (Rodgers et al., 2005). When unavailable, neutral phospholipid levels are calculated by difference between total and acidic phospholipid fractional volumes.

Parameter	Brain lipid content			
	Rat			Human
	Poulin et al. (2002)	Rodgers et al. (2005), Rodgers et al. (2006)	Xu et al. (2020)	Poulin et al. (2002)
$V_{nl(c)}/V_{tis}$	0.0391	0.0391	0.0001831	0.0509*
$V_{p(c)}/V_{tis}$	0.0533	0.0019 (***) by sum	0.0205754 (by sum)	0.0564*
$V_{ap(c)}/V_{tis}$	0.0004	0.0004	0.0022441	[0.7, 15]% $V_{p(c)}/V_{tis}$ **
$V_{np(c)}/V_{tis}$	0.0529 (by diff.)	0.0015	0.0183313	by diff.

Table 4.2: General physiological parameters of rat and human brains. Most parameters are assumed to be species-independent. Species-specific values (highlighted in yellow) include brain weight—based on a male F344 rat (250 g) and a male human (70 kg)—as well as interstitial and cellular water volume fractions. (*) Corrected for residual blood using Eq. A2 from Rodgers & Rowland (Rodgers et al., 2006), assuming a residual blood volume fraction of 0.04 (Brown et al., 1997); (**) calculated as $V_{iw}/V_{tis} = (V_{iw}/V_{wtot})|_{\text{rat}} \cdot V_{wtot}/V_{tis}$; (***) derived from the ratio V_{iw}/V_{tis} to V_{wtot}/V_{tis} , and assumed to be conserved across species.

Parameter	Physiological input parameters			
	Rat		Human	
	Value	Reference	Value	Reference
Brain weight (g)	0.1425	Brown et al. (1997)	1450	Brown et al. (1997)
Brain density (g/mL)	1	Brown et al. (1997)	1	Brown et al. (1997)
$V_{\text{cel}}/V_{\text{tis}}$	0.75	Reichel (2015)	0.75	Reichel (2015)
$V_{\text{int}}/V_{\text{tis}}$	0.25	Reichel (2015)	0.25	Reichel (2015)
$V_{\text{wtot}}/V_{\text{tis}}$	0.753	Poulin et al. (2002)	0.7457*	Poulin et al. (2002)
$V_{\text{iw}}/V_{\text{tis}}$	0.162	Rodgers et al. (2005)	0.1603**	derived (Poulin et al., 2002; Rodgers et al., 2005)
$V_{\text{iw}}/V_{\text{wtot}}$	0.215***	Rodgers et al. (2006)	0.215***	assump. (Rodgers et al., 2006)
$V_{\text{cw}}/V_{\text{tis}}$	0.5910	by diff. (Rodgers et al., 2006)	0.5854	by diff. (Poulin et al., 2002)
$V_{\text{iw}}/V_{\text{isf}}$	1	R&R assump.	1	R&R assump.
pH _{iw}	7.3	Saleh et al. (2021), Fridén et al. (2011), Davson et al. (1996)	7.3	Saleh et al. (2021), Fridén et al. (2011), Davson et al. (1996)
pH _{cw}	7.0	Saleh et al. (2021), Fridén et al. (2011), Davson et al. (1996), Rodgers et al. (2005)	7.0	Saleh et al. (2021), Fridén et al. (2011), Davson et al. (1996), Rodgers et al. (2005)
pH _{rw}	7.22	Rodgers et al. (2005)	7.22	Rodgers et al. (2005)
pH _{pw}	7.4	Rodgers et al. (2005)	7.4	Rodgers et al. (2005)
PR _{tis} /PR _{pla}	0.048 (albumin)	Rodgers et al. (2006)	0.048 (albumin)	Rodgers et al. (2006)
	0.041 (lipoproteins)	Rodgers et al. (2006)	0.041 (lipoproteins)	Rodgers et al. (2006)

INTACT VS. HOMOGENATE BRAIN TISSUE

In intact brain tissue, the interstitial (V_{int}) and cellular (V_{cel}) spaces—whose sum defines the total tissue volume $V_{\text{tis}} = V_{\text{int}} + V_{\text{cel}}$ —are separated by cell membranes, maintaining physiological pH compartmentalization with distinct interstitial and cellular pH levels ($\text{pH}_{\text{iw}} = 7.3$ and $\text{pH}_{\text{cw}} = 7.0$, respectively, as reported in [Saleh et al. \(2021\)](#), [Fridén et al. \(2011\)](#), and [Davson et al. \(1996\)](#)). Importantly, while intact cell membranes serve as barriers that maintain concentration differences for drugs transported actively (via uptake or efflux), they do not act as barriers for passively diffusing compounds, which eventually reach equilibrium across compartments. This setup mirrors the conditions found in brain slice experiments, where the structural integrity of the tissue is almost preserved.

In contrast, homogenate brain tissue refers to a uniform mixture of all components of the brain tissue (*e.g.*, cells, extracellular matrix, interstitial fluid), where cell membranes are disrupted. In this uniform environment, all water phases are mixed, and tissue constituents have full access to the whole tissue. Homogenization eliminates the compartmentalization of aqueous environments that normally maintain distinct pH values. As a result, there is a single, homogeneous water phase (hw) with a uniform pH (pH_{hw}), given by the volume-weighted average of hydrogen ion concentrations across interstitial, cellular, and buffer spaces (pH_{iw} , pH_{cw} , $\text{pH}_{\text{buffer}}$), also accounting for the dilution factor DF used in the experimental setup, as described in Section 4.5. The present framework does not include intracellular sub-compartmentalization for either intact or homogenized brain tissue; hence, pH contributions from disrupted organelles (*e.g.*, lysosomes, mitochondria, endosomes, etc.) are not explicitly accounted for in the homogenate pH calculations. As a result, pH_{cw} provides an approximation that reflects the different pH values of all intracellular subcompartments, such as organelles and cytosol.

While the homogenized tissue no longer contains distinct interstitial and cellular spaces, the R&R approach—by approximating drug distribution in tissue based on distribution among tissue constituents—allows us to conceptually assign these constituents to fictitious interstitial and cellular spaces (see Figure 4.1). Therefore, in our theoretical derivation, we consider the undiluted homogenate brain tissue that is assumed to have the same total volume as the original intact tissue. This volume corresponds to the sum of the fictitious interstitial and cellular space volumes, expressed as:

$$V_{\text{hom}} = V_{\text{int}} + V_{\text{cel}} , \quad (4.4)$$

and the volume of homogenate water is defined as the sum of the interstitial and cellular water volumes included in the initially intact brain tissue:

$$V_{\text{hw}} = V_{\text{iw}} + V_{\text{cw}} . \quad (4.5)$$

Such a representation of the undiluted homogenate brain tissue allows for the estimation of drug partitioning in a disrupted, but compositionally representative, tissue matrix. Experimentally, brain homogenate assays typically involve performing equilibrium dialysis on the homogenate brain tissue by adding buffer to the homogenized tissue, thereby diluting the sample and determining the final pH. The buffer composition and the dilution factor (DF) influence the measured concentration of unbound drug. Operationally, the unbound drug concentration in the homogenate C_{hw} is measured in the diluted homogenate and, then, a correction is applied using a standard formula to predict it in undiluted homogenate tissue (see Section 4.5). The output is the fraction unbound in the undiluted homogenate brain tissue ($fuH_{exp(u)}$). Figure 4.1 summarizes the key differences between the compartmentalized structure of intact brain tissue and the unified environment of the homogenate. Tables 4.3 and 4.4 list the relevant abbreviations, variables, and parameters introduced in this section and in the chapters hereafter.

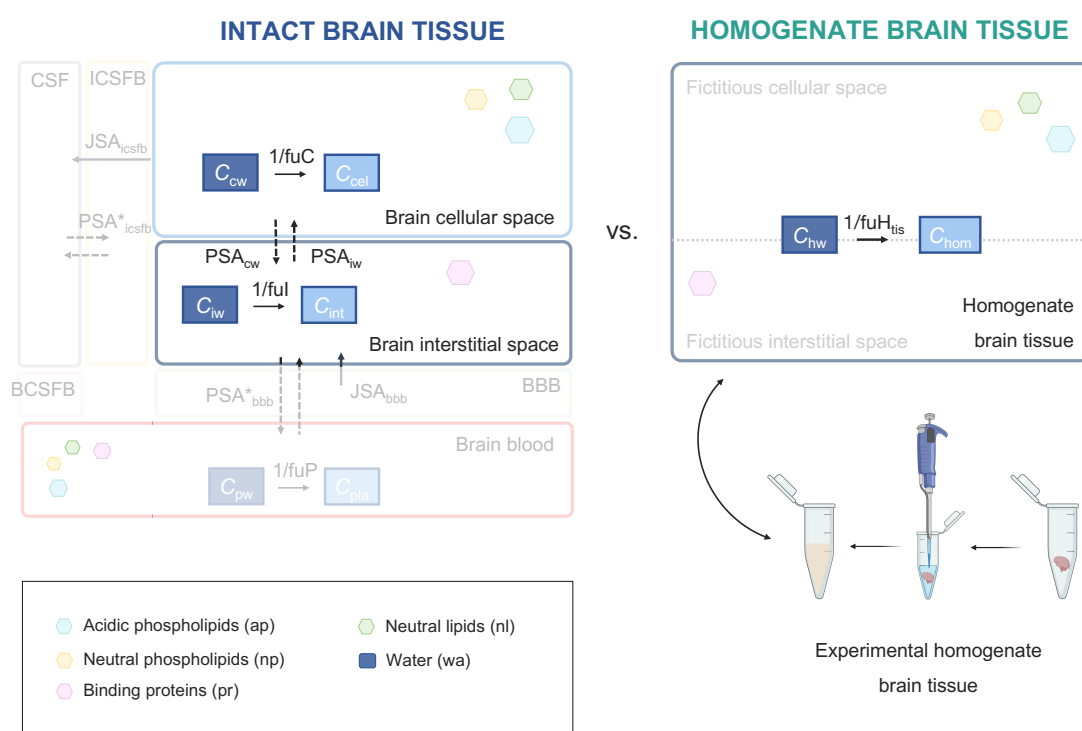


Figure 4.1: Conceptual representation of drug distribution in intact versus homogenate brain tissue. The left box illustrates an intact brain tissue model, in which drug distribution is governed by extravasation from a lumped blood compartment (plasma and RBCs), followed by permeation into interstitial and cellular water subspaces. Drug concentrations are tracked in water subspaces (C_{pw} , C_{iw} , C_{cw}) and total subspaces (C_{pla} , C_{int} , C_{cel}), linked via fractions unbound fuP , fuI , and fuC . Color-coded boxes indicate tissue constituents. The right box panel depicts the homogenate brain tissue, derived from brain homogenate assays (bottom right inset). Homogenization results in a single homogenate water phase with a unified pH (pH_{hw}), shaped by subspace pH values, buffer pH, and dilution factor. Drug concentrations in the homogenate are described by total (C_{hom}) and unbound (C_{hw}) levels, linked via the fraction unbound in homogenate brain tissue (fuH_{tis}).

Table 4.3: **Abbreviations in brain tissue partitioning.** Mathematical notation for tissue constituents, subspaces, water phases, and homogenate-related parameters.

Name	Symbol	
Tissue constituents		
Water	wa	
Neutral lipids	nl	
Neutral phospholipids	np	
Acidic phospholipids	ap	
Binding proteins	pr	
Tissue subspaces & spaces		
Plasma	pla	
Red blood cells	rbc or RBC	
Peripheral blood	blo	
Interstitial space	int	
Interstitial space fluid	isf	
Cellular space	cel	
Tissue (or parenchyma for brain)	tis	
Homogenate tissue	hom	
Homogenate tissue fluid	htf	
Water spaces		Associated pH
Extracellular water	ew	pH_{ew}
Interstitial water	iw	pH_{iw}
Cellular water	cw	pH_{cw}
Plasma water	pw	pH_{pw}
RBC water	rw	pH_{rw}
Homogenate tissue water	hw	pH_{hw}
Homogenate pH		
Dilution factor	DF	
Buffer pH	buffer	$\text{pH}_{\text{buffer}}$

Table 4.4: Summary of parameters and symbols used in brain tissue partitioning. This table presents notation for volumes, concentrations, surface areas, permeability coefficients, and the fractions of neutral and ionized drug species across various water spaces. It also details fractions unbound and their relationships to drug concentrations within these water subspaces and drug-input parameters. This notation underpins the quantitative description of drug distribution in both intact and homogenate brain tissues, as well as blood.

Parameter name	Symbol
Volumes	V_{sub}
Concentrations	C_{sub}
Fractions neutral, cationic, anionic	$f_n, f_{\text{cat}}, f_{\text{ani}}$
Fraction neutral/cationic/anionic in plasma water	$f_{n_{\text{pw}}}, f_{\text{cat}_{\text{pw}}}, f_{\text{ani}_{\text{pw}}}$
Fraction neutral/cationic/anionic in interstitial water	$f_{n_{\text{iw}}}, f_{\text{cat}_{\text{iw}}}, f_{\text{ani}_{\text{iw}}}$
Fraction neutral/cationic/anionic in cellular water	$f_{n_{\text{cw}}}, f_{\text{cat}_{\text{cw}}}, f_{\text{ani}_{\text{cw}}}$
Fraction neutral/cationic/anionic in homogenate tissue water	$f_{n_{\text{hw}}}, f_{\text{cat}_{\text{hw}}}, f_{\text{ani}_{\text{hw}}}$
Fractions unbound	f_u
Ratio of plasma water-to-plasma concentration	f_{uP}
Ratio of interstitial water-to-(brain) tissue concentration	f_{uT}
Ratio of homogenate water-to-homogenate tissue concentration	$f_{uH_{\text{tis}}}$
Experimental fraction unbound in diluted homogenate tissue	$f_{uH_{\text{exp(d)}}}$
Experimental fraction unbound in undiluted homogenate tissue	$f_{uH_{\text{exp(u)}}}$
Relationship between fraction unbound and concentrations in water spaces	
Drug concentration in plasma water	$C_{\text{pw}} = f_{uP} C_{\text{pla}}$
Drug concentration in interstitial water	$C_{\text{iw}} = f_{uT} C_{\text{tis}}$
Drug concentration in homogenate tissue water	$C_{\text{hw}} = f_{uH_{\text{tis}}} C_{\text{hom}}$
Relevant brain-specific partition coefficients	
Brain tissue-to-plasma concentration ratio	$K_{p_{\text{tis}}} = C_{\text{tis}}/C_{\text{pla}}$
Brain tissue-to-extracellular water partition coefficient	$K_{pu_{\text{tis}}} = C_{\text{tis}}/C_{\text{ew}} = C_{\text{tis}}/C_{\text{iw}}$
Brain interstitial water-to-plasma water concentration ratio	$K_{puu_{\text{bra}}} = C_{\text{iw}}/C_{\text{pw}}$
Drug-specific input parameters	
Acid dissociation constant (logarithmic form)	pK_a
<i>n</i> -octanol:water partition coefficient (logarithmic form)	$\log P$
Blood-to-plasma ratio	$B:P$

4.3 RESULTS

4.3.1 ACCOUNTING FOR THE EXPERIMENTAL BIAS: TISSUE HOMOGENIZATION IMPACT ON FRACTION UNBOUND PREDICTIONS

Baseline differences between fractions unbound equations for intact and homogenate brain tissue Fractions unbound fuT and fuH_{tis} are fundamentally different quantities, as shown by their mechanistic equations derived below. In the intact brain tissue, building on the R&R framework, the fraction unbound can be expressed as a volume-weighted sum of the distribution coefficients of tissue constituents:

$$\frac{1}{fuT} = \frac{V_{iw}}{V_{tis}} + \frac{V_{pr(i)}}{V_{tis}} K_{pr(i):iw} + \left(\frac{V_{cw}}{V_{tis}} + \frac{V_{nl(c)}}{V_{tis}} K_{nl(c):cw} + \frac{V_{np(c)}}{V_{tis}} K_{np(c):cw} + \frac{V_{ap(c)}}{V_{tis}} K_{ap(c):cw} \right) K_{cw:iw} \cdot \quad (4.6)$$

To derive tractable expressions for these distribution coefficients, various parameterization strategies can be used, including protein-binding kinetics (charged or noncharged), partition coefficient-based models, membrane affinity regressions, or neutral and charged logP-based models ([Poulin et al., 2002](#); [Rodgers et al., 2005](#); [Schmitt, 2008](#)). Within the R&R framework:

- The cellular water-to-interstitial water distribution coefficient $K_{cw:iw}$, which characterizes the exchange across the cell membrane, is approximated as fn_{iw}/fn_{cw} , under the assumption that only neutral species cross membranes via passive diffusion.
- The cellular neutral lipid and neutral phospholipid partition coefficients, $K_{nl(c):cw}$ and $K_{np(c):cw}$, are estimated via logP-based parameterizations. Here, it is assumed that only the neutral species partitions into neutral lipids and phospholipids.
- The cellular acidic phospholipid-to-water ($K_{ap(c):cw}$) and interstitial protein-to-water ($K_{pr(i):iw}$) distribution coefficients rely on charged protein-binding parameterizations. Specifically, only the non-cationic species—namely, neutral and anionic forms of the drug—are assumed to interact with binding proteins in the interstitial fluid. In contrast, acidic phospholipids bind exclusively to cationic drug forms.

Therefore, by applying these approximations that use accessible *in vitro* read-outs and specific parameterizations (Rodgers et al., 2005; Rodgers et al., 2006), eq. 4.6 becomes:

$$\begin{aligned} \frac{1}{fuT} = & \frac{V_{iw}}{V_{tis}} + \frac{V_{iw}}{V_{isf}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{1 - fcat_{iw}}{1 - fcat_{pw}} \frac{PR_{tis}}{PR_{pla}} \\ & + \frac{V_{cw}}{V_{tis}} \frac{fn_{iw}}{fn_{cw}} + \frac{V_{nl(c)}}{V_{tis}} P_{o:w} fn_{iw} + \frac{V_{np(c)}}{V_{tis}} (0.3 P_{o:w} + 0.7) fn_{iw} \\ & + K_{A,ap(r)^-} AP_{tis}^- fcat_{cw} \frac{fn_{iw}}{fn_{cw}}. \end{aligned} \quad (4.7)$$

Here, $P_{o:w}$ is the *n*-octanol:water partition coefficient, while fn_{wa} and $fcat_{wa}$ refer to the neutral and cationic fractions in a given water phase (interstitial, cellular, or plasma). The interstitial water-to-interstitial fluid space fractional volume is assumed equal to 1. $K_{A,ap(r)^-}$ is the affinity constant for acidic phospholipids, and $K_{A,pr(p)}$ that for plasma binding proteins. The acidic phospholipid concentration in the tissue is defined as $AP_{tis}^- := A_{AP}^- / V_{tis}$, and the concentrations of binding proteins in tissue and plasma are PR_{tis} and PR_{pla} , respectively. Their ratio PR_{tis} / PR_{pla} depends on drug type: interstitial proteins correspond to lipoproteins for neutral compounds, and albumin for acids and weak bases.

For the homogenate brain tissue, we directly account for the experimental bias introduced by tissue homogenization. Using the same R&R principles, the fraction unbound in homogenate brain is written as the volume-weighted sum of tissue constituent distribution coefficients with respect to homogenate water:

$$\begin{aligned} \frac{1}{fuH_{tis}} = & \frac{V_{iw}}{V_{hom}} + \frac{V_{pr(i)}}{V_{hom}} K_{pr(i):hw} \\ & + \frac{V_{cw}}{V_{hom}} + \frac{V_{nl(c)}}{V_{hom}} K_{nl(c):hw} + \frac{V_{np(c)}}{V_{hom}} K_{np(c):hw} + \frac{V_{ap(c)}}{V_{hom}} K_{ap(c):hw}. \end{aligned} \quad (4.8)$$

Applying the same (previously mentioned) R&R approximations to the homogenate-specific distribution coefficients $K_{sub:hw}$ leads to:

$$\begin{aligned} \frac{1}{fuH_{tis}} = & \frac{V_{iw}}{V_{hom}} + \frac{V_{hw}}{V_{htf}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{1 - fcat_{hw}}{1 - fcat_{pw}} \frac{PR_{hom}}{PR_{pla}} \\ & + \frac{V_{cw}}{V_{hom}} + \frac{V_{nl(c)}}{V_{hom}} P_{o:w} fn_{hw} + \frac{V_{np(c)}}{V_{hom}} (0.3 P_{o:w} + 0.7) fn_{hw} \\ & + K_{A,ap(r)^-} AP_{hom}^- fcat_{hw}. \end{aligned} \quad (4.9)$$

Here, fn_{hw} and $fcat_{hw}$ are the neutral and cationic fractions in homogenate water. The homogenate water-to-total homogenate volume ratio is assumed

to be 1, as the interstitial water-to-fluid ratio assumed in the original R&R model. Acidic phospholipid concentration in the homogenate is expressed as $AP_{\text{hom}}^- := A_{\text{AP}}^-/V_{\text{hom}}$, and PR_{hom} is the total binding protein concentration in homogenized brain tissue. As with intact tissue, the ratio $PR_{\text{hom}}/PR_{\text{pla}}$ reflects the nature of the binding proteins relevant to the drug type. A detailed derivation of eqs. 4.6 and 4.7 for intact brain tissue, and eqs. 4.8 and 4.9 for homogenized brain tissue, is provided in Materials and Methods (Section 4.5), along with the specific assumptions and approximations involved.

The intrinsic difference between fuT and fuH_{tis} becomes clear when comparing their respective equations (eqs. 4.7 and 4.9). The key distinction lies in how pH compartmentalization is handled. In the intact brain, the drug distribution across compartments depends on the pH difference between the interstitial and cellular spaces. This is captured mechanistically in fuT via the cellular-to-interstitial water distribution coefficient, approximated as the ratio of the fractions neutral of the drug in the two spaces. As a result, fuT is a function of both interstitial and cellular pH:

$$fuT = fuT(pH_{\text{iw}}, pH_{\text{cw}}),$$

In contrast, tissue homogenization disrupts cellular membranes and eliminates this physiological compartmentalization, creating a single, homogeneous pH that governs drug ionization, as shown in eq 4.9. Thus, we can explicitly define fuH_{tis} as a function of the homogenate pH:

$$fuH_{\text{tis}} = fuH_{\text{tis}}(pH_{\text{hw}}).$$

While fuT and fuH_{tis} are not interchangeable, fuH_{tis} can be derived from fuT by adjusting pH_{iw} and pH_{cw} to pH_{hw} :

$$fuH_{\text{tis}} = fuT(pH_{\text{hw}}).$$

Since the standard R&R expression for fuT is readily available, this result provides a useful operational shortcut when an explicit equation for fuH_{tis} is lacking.

This approximation, however, does not imply that fuH_{tis} and fuT become equivalent under certain conditions. One might hypothesize that selecting a buffer and dilution factor yielding the homogenate pH close to the physiological interstitial value ~ 7.3 (Fridén et al., 2011; Saleh et al., 2021) could bring fuH_{tis} closer to fuT . Obtaining such a homogenate pH level requires adjusting the buffer pH (typically around 7.4) and, more significantly, the dilution factor of the experimental assay—which can vary considerably across studies (ranging from 2 to 10, as shown in Table 4.7). Lower dilution shifts the pH toward the more acidic intracellular pH value, whereas higher dilution levels move it closer to the buffer pH, which resembles interstitial or plasma

pH. However, this reasoning overlooks a critical point: homogenization irreversibly disrupts brain tissue structure. Even if pH_{hw} approximates interstitial pH, the tissue no longer maintains the pH compartmentalization that governs drug distribution *in vivo*. Some micro-compartmentalization (*e.g.*, vesicles or microsomes) may exist, but it is insufficient to recreate the native structural and functional barriers of intact brain tissue. This distinction is reflected in the mechanistic equations: fuT and fuH_{tis} are derived from fundamentally different conditions and assumptions. No careful choice of dilution factor or buffer composition can fully reconcile the two.

COMPOUND-TYPE DEPENDENT EFFECTS OF TISSUE HOMOGENIZATION

In the R&R mechanistic framework, binding interactions with interstitial proteins and cellular acidic phospholipids are modeled based on compound ionization characteristics, under the following assumptions

- Compounds with at least one cationic (basic) $\text{pK}_a \geq 7$, *i.e.*, moderate-to-strong bases and type I (ordinary or zwitterionic) ampholytes, are assumed to bind predominantly to acidic phospholipids in their cationic form. This assumption, introduced by the R&R model ([Rodgers et al., 2005](#)), reflects their expected electrostatic interactions within the cellular space. For these compounds, binding to interstitial proteins is generally considered negligible.
- Compounds with no cationic (basic) $\text{pK}_a \geq 7$, *i.e.*, acids, neutral compounds, weak bases, and type II ampholytes, are assumed to primarily interact with interstitial proteins. In particular, neutral compounds are modeled to bind to lipoproteins, while the others bind to albumin. For this group, distribution into cellular acidic phospholipids is typically not considered relevant.

These unique ionization-dependent electrostatic interactions between moderate-to-strong basic drugs and tissue acidic phospholipids, as well as binding to interstitial proteins for acids, neutral compounds, and weak bases, are central to the R&R work. Therefore, compounds with and without a cationic $\text{pK}_a \geq 7$ exhibit different binding behaviors, and, consequently, different fractions unbound in intact and homogenate brain tissues. Table 4.5 summarizes the corresponding equations obtained relying on eqs. 4.7 and 4.9. Building on these mechanistic differences, we evaluate how homogenization differentially affects fuH_{tis} —and its relation to fuT —across compound ionization classes.

Fraction unbound in intact and homogenate brain tissues

Compounds with at least one cationic $pK_a \geq 7$

$$\begin{aligned}
 1/fuT & \quad \frac{V_{iw}}{V_{tis}} + \frac{V_{cw}}{V_{tis}} + \frac{V_{nl(c)}}{V_{tis}} K_{nl(c):cw} + \frac{V_{np(c)}}{V_{tis}} K_{np(c):cw} + \frac{V_{ap(c)}}{V_{tis}} K_{ap(c):cw} \\
 & \quad \frac{V_{iw}}{V_{tis}} + \frac{V_{cw}}{V_{tis}} \frac{fn_{iw}}{fn_{cw}} + \frac{V_{nl(c)}}{V_{tis}} P_{o:w} fn_{iw} + \frac{V_{np(c)}}{V_{tis}} (0.3P_{o:w} + 0.7) fn_{iw} + \\
 & \quad K_{A,ap(r)}^- AP_{tis}^- fcat_{cw} \frac{fn_{iw}}{fn_{cw}} \\
 1/fuH_{tis} & \quad \frac{V_{iw}}{V_{hom}} + \frac{V_{cw}}{V_{hom}} + \frac{V_{nl(c)}}{V_{hom}} K_{nl(c):hw} + \frac{V_{np(c)}}{V_{hom}} K_{np(c):hw} + \frac{V_{ap(c)}}{V_{hom}} K_{ap(c):hw} \\
 & \quad \frac{V_{iw}}{V_{hom}} + \frac{V_{cw}}{V_{hom}} + \frac{V_{nl(c)}}{V_{hom}} P_{o:w} fn_{hw} + \frac{V_{np(c)}}{V_{hom}} (0.3P_{o:w} + 0.7) fn_{hw} + \\
 & \quad K_{A,ap(r)}^- AP_{hom}^- fcat_{hw}
 \end{aligned}$$

Compounds with no cationic $pK_a \geq 7$

$$\begin{aligned}
 1/fuT & \quad \frac{V_{iw}}{V_{tis}} + \frac{V_{pr(i)}}{V_{tis}} K_{pr(i):iw} + \frac{V_{cw}}{V_{tis}} + \frac{V_{nl(c)}}{V_{tis}} K_{nl(c):cw} + \frac{V_{np(c)}}{V_{tis}} K_{np(c):cw} \\
 & \quad \frac{V_{iw}}{V_{tis}} + \frac{V_{iw}}{V_{isf}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{(1-fcat_{iw})}{(1-fcat_{pw})} \frac{PR_{tis}}{PR_{pla}} + \frac{V_{cw}}{V_{tis}} \frac{fn_{iw}}{fn_{cw}} + \\
 & \quad \frac{V_{nl(c)}}{V_{tis}} P_{o:w} fn_{iw} + \frac{V_{np(c)}}{V_{tis}} (0.3P_{o:w} + 0.7) fn_{iw} \\
 1/fuH_{tis} & \quad \frac{V_{iw}}{V_{hom}} + \frac{V_{pr(i)}}{V_{hom}} K_{pr(i):hw} + \frac{V_{cw}}{V_{hom}} + \frac{V_{nl(c)}}{V_{hom}} K_{nl(c):hw} + \frac{V_{np(c)}}{V_{hom}} K_{np(c):hw} \\
 & \quad \frac{V_{iw}}{V_{hom}} + \frac{V_{hw}}{V_{htf}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{(1-fcat_{hw})}{(1-fcat_{pw})} \frac{PR_{hom}}{PR_{pla}} + \frac{V_{cw}}{V_{hom}} + \\
 & \quad \frac{V_{nl(c)}}{V_{hom}} P_{o:w} fn_{hw} + \frac{V_{np(c)}}{V_{hom}} (0.3P_{o:w} + 0.7) fn_{hw}
 \end{aligned}$$

Table 4.5: Equations of fractions unbound in intact and homogenate brain tissues for compounds from different ionization classes.

Compounds with at least one cationic $pK_a \geq 7$ For moderate-to-strong bases, and type I ordinary or zwitterionic ampholytes, interstitial protein binding is assumed negligible in the R&R framework. As such, the contribution of interstitial constituents in both fuT and fuH_{tis} is represented by the interstitial space-to-water volume ratio, V_{int}/V_{iw} , and the observed differences between intact and homogenate brain unbound fractions stem solely from cellular contributions. Therefore, the discrepancies arise primarily from two sources. First, variation in neutral and cationic fractions between intact and homogenate environments—ending down to ionization and pH differences—*i.e.*, fn_{hw} vs. fn_{iw} , and $fcat_{hw}$ vs. $fcat_{cw} \cdot fn_{iw}/fn_{cw}$, drives differences between fuT and fuH_{tis} . Since pH_{hw} typically falls in the 7.2 – 7.4 range (see Table 4.7), close to pH_{iw} , larger differences are expected to result not from fraction neutral terms—linked to distribution into neutral lipids and phospholipids—but from

fraction cationic terms that characterize binding to acidic phospholipids. Second, homogenization-induced disruption of the cellular membrane sets the cellular-to-interstitial water distribution ratio to unity, altering the contribution of the cellular water term and of the acidic phospholipid binding term. This effect is especially relevant for strong bases, where the pH shift can cause up to a 100% decrease in the cellular fraction neutral relative to the interstitial one. In contrast, compounds with cationic pK_a values near the homogenate pH range exhibit only a moderate reduction.

Compounds with no cationic $pK_a \geq 7$ For weak bases, acids, neutral compounds, and type II ordinary and zwitterionic ampholytes, fuH_{tis} is influenced by the neutral and cationic fractions at pH_{hw} —as in the previous case—as well as by the homogenate water-to-tissue fluid volume ratio, V_{hw}/V_{htf} . In contrast, fuT depends on neutral and cationic fractions calculated at physiological interstitial and cellular pH, and on the interstitial fluid-to-interstitial space volume ratio, V_{iw}/V_{isf} . In contrast to highly basic compounds, these drugs are characterized by relevant interstitial protein binding. Thus, both interstitial protein interactions and water term contributes to the fractions unbound in intact and homogenate brain tissue. If $V_{iw}/V_{isf} = 1$ (R&R assumption), the difference in interstitial terms is due to fn_{iw} vs. fn_{hw} . Additionally, binding to acidic phospholipids is negligible in this compound class, so cellular contributions are primarily affected by changes in fn between interstitial and homogenate water spaces. However, given that pH_{hw} and pH_{iw} are typically very similar, discrepancies due to differences between fn_{hw} vs. fn_{iw} and $fc_{at_{hw}}$ vs. $fc_{at_{iw}}$ are expected to be small. Finally, there is a small-to-moderate homogenization-induced change in the contribution of the cellular water term, due to the cellular-to-interstitial water distribution ratio being set to unity. For strong acids, this contribution can increase by up to 50%, with a smaller increase observed for weaker acids. For weak bases, the contribution decreases by approximately 30% (assuming a homogenate $pH \sim 7.3$). Neutral compounds remain unaffected, as their cellular-to-interstitial water distribution coefficient is already 1.

Overall, for both drug categories, homogenization shifts all pH-dependent terms to the homogenate value by removing pH compartmentalization. The impact, however, is expected to be greatest for highly basic compounds due to their unique electrostatic interactions with cellular acidic phospholipids. For other compounds, such as acids, neutrals, and weak bases, differences are expected to be generally smaller and dominated by minor shifts in neutral and cationic fractions (see Table 4.6 for a summary).

Table 4.6: Key differences between fuT and fuH_{tis} for different compound classes. Color-coded section headers indicate the two main ionization categories based on Rodgers & Rowland classification.

fuT and fuH _{tis} differences by compound class - Parameter changes		
Homogenization-induced change from intact to homogenate tissue	Expected magnitude of change (assuming pH _{hw} ~ pH _{iw})	Overall change
Compounds with at least one cationic pKa ≥ 7		
fn _{iw} → fn _{hw}	Small	Moderate to large (mainly cellular)
fcat _{cw} · $\frac{fn_{iw}}{fn_{cw}}$ → fcat _{hw}	Moderate to large	
$\frac{fn_{iw}}{fn_{cw}}$ → 1	Moderate to large	
Compounds with no cationic pKa ≥ 7		
fn _{iw} → fn _{hw}	Small	Small to moderate
fcat _{iw} → fcat _{hw}	Small	
$\frac{fn_{iw}}{fn_{cw}}$ → 1	Small to moderate	

4.3.2 BENCHMARKING A PRIORI PREDICTIONS AGAINST HOMOGENATE DATA

By capturing physiological compartmentalization, fuT is more appropriate for intact-tissue or brain slice experiments. In contrast, fuH_{tis} is designed to reflect homogenate conditions, where membrane structures are disrupted and pH levels equilibrate. As such, we expect fuH_{tis} to align more closely with experimental $fuH_{exp(u)}$ values from brain homogenate assays. To test this hypothesis, we evaluated predictions for the small molecule dataset in Table 4.5 by calculating the proportion of compounds falling within 2-, 3-, and 4-fold deviation from the observed $fuH_{exp(u)}$ listed in Table 4.10; 2- and 3-fold error thresholds are standard in pharmacokinetic modeling to assess predictive performance. The 2-fold criterion reflects the typical variability observed in experimental measurements (Poulin et al., 2002). The 3-fold threshold is widely accepted for *a priori* predictions of unbound fractions in tissues or plasma (Rodgers et al., 2005; Rodgers et al., 2006; Schmitt, 2008; Fridén et al.,

2009). To ensure that predictions of fuH_{tis} approach as much as possible the experimental context and improve the interpretability of model derivations, they were adapted to the specific assay conditions by adjusting the homogenate pH according to the dilution factor and buffer pH used in each experimental setup. It is indeed essential to match model inputs to the actual assay conditions as closely as possible, rather than relying on default or assumed values, as they can affect significantly the accuracy of fuH_{tis} predictions. As detailed in Table 4.7, most protocols use phosphate-based buffers at $\text{pH}_{\text{buffer}}$ around 7.4 (Di et al., 2011; Summerfield et al., 2008; Becker et al., 2006; Fridén et al., 2011; Kalvass et al., 2002; Kalvass et al., 2007; Maurer et al., 2005; Culot et al., 2013), although exceptions exist (*e.g.*, CSF surrogate buffer as in Longhi et al. (2011)), whereas reported DF can vary significantly across studies (from 2 to 10). Based on eq. 4.42, homogenate pH values (pH_{hw}) were estimated using literature-based reference pH values for the brain interstitial and intracellular compartments (Saleh et al., 2021; Fridén et al., 2011). Physiological input parameters, including protein and lipid content and compartmental volumes, followed literature standards from Rodgers & Rowland (Rodgers et al., 2005; Rodgers et al., 2006) for rats and Poulin et al. (2002) for humans. The acidic phospholipid fractional volume was assumed to be $\sim 0.7\%$ of the total phospholipid volume, consistent with values reported in Rodgers & Rowland (Rodgers et al., 2005) for rats (Table 4.1). Relying on these physiological assumptions, Figure 4.2 shows that fuT outperformed fuH_{tis} in both rats and humans. Specifically, within 3-fold deviation from $\text{fuH}_{\text{exp(u)}}$, fuT showed 65.4% accuracy in humans versus 59.6% for fuH_{tis} , and 69.6% versus 66.1% in rats, respectively. Yet, as expected, the greatest differences between the two metrics were observed for moderate-to-strong bases and, more generally, for compounds with at least one cationic $\text{pK}_a \geq 7$. Why did fuH_{tis} not outperform fuT as expected? To address this discrepancy, we explored the robustness of fuT better performance over fuH_{tis} by extending our analysis in a specific direction.

Table 4.7: Brain homogenate assay settings and buffer compositions used in selected studies. The table reports whether a brain homogenate assay was performed, the buffer solution used, the dilution factor (DF), the buffer pH ($\text{pH}_{\text{buffer}}$), and the estimated homogenate water phase pH (pH_{hw}), calculated by eq. 4.5 and assuming $\text{pH}_{\text{cw}} = 7.0$ (cellular water) and $\text{pH}_{\text{iw}} = 7.3$ (interstitial water), *i.e.*, reference pH values reported in [Saleh et al. \(2021\)](#), [Fridén et al. \(2011\)](#), and [Davson et al. \(1996\)](#). PBS: standard phosphate-buffered saline; DPBS: Dulbecco's PBS; [Fridén et al. \(2011\)](#) employed a more concentrated phosphate buffer (180 mM, pH 7.4), while [Culot et al. \(2013\)](#) used a custom buffer with increased phosphate (0.122 M phosphate buffer with 75 mM NaCl, pH 7.4), maintaining physiological ionic strength. (*) indicate pH values inferred from the solution composition rather than explicitly reported.

Reference	Brain ho- mogenate assay	Solution	DF	$\text{pH}_{\text{buffer}}$	pH_{hw}
Di et al. (2011)	✓	DPBS	5	7.4*	7.3067
Summerfield et al. (2008)	✓	PBS	3	7.4	7.2540
Fridén et al. (2011)	✓	PBS	4	7.4	7.2862
Longhi et al. (2011)	✓	CSF surrogate	2	7.4	7.1961
Culot et al. (2013)	✓	PBS	10	7.4*	7.3508

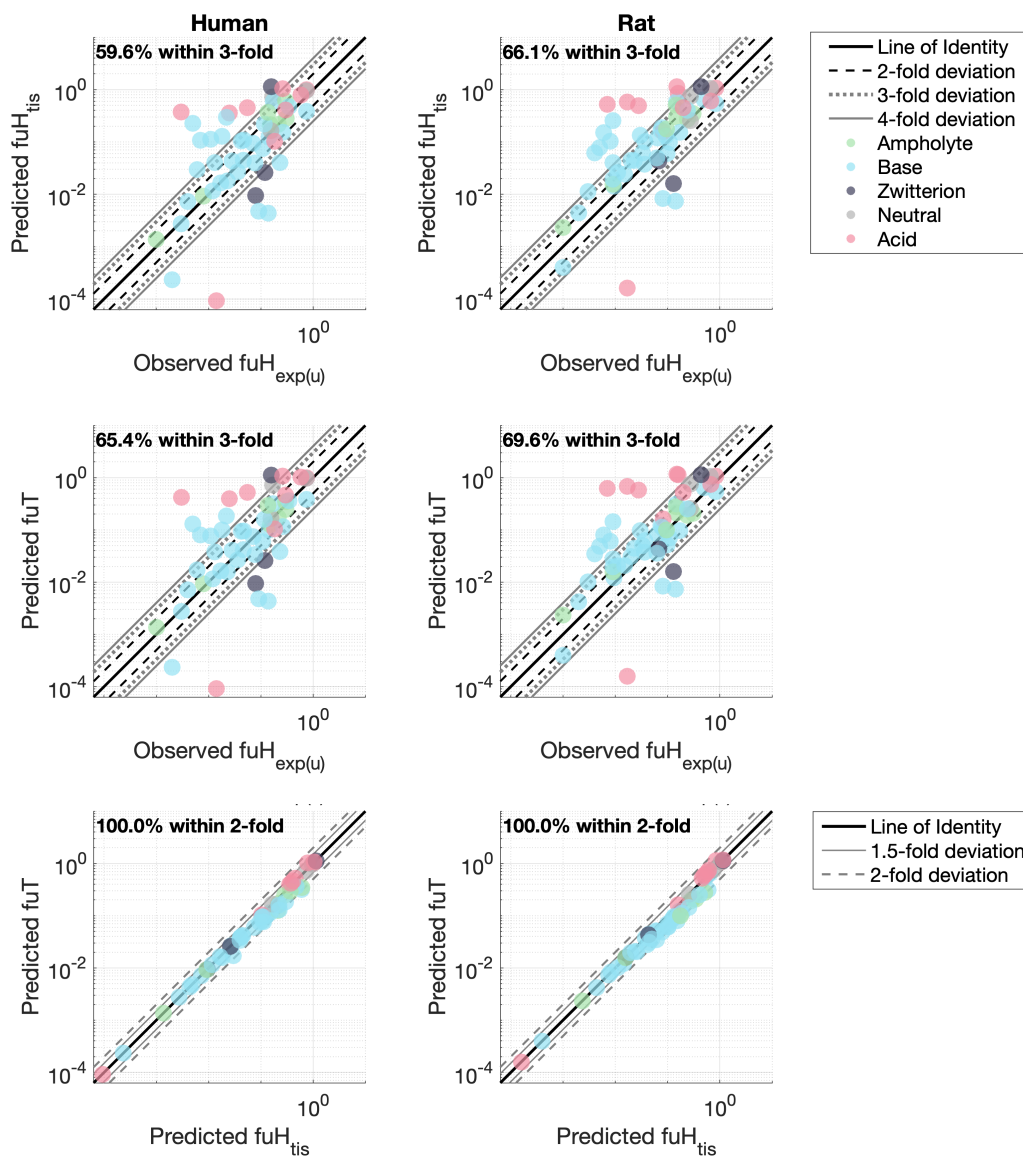


Figure 4.2: Predicted fuH_{tis} and fuT vs. experimental homogenate data $\text{fuH}_{\text{exp(u)}}$ ($n = 56$ and 52 compounds for rats and humans, respectively). The first two rows compare predicted fuH_{tis} and fuT with experimental $\text{fuH}_{\text{exp(u)}}$, with points color-coded by drug type. Experimental data: Table 4.10; physiological parameters: Table 4.1; brain lipid composition: Rodgers & Rowland (2005, 2006) (Rodgers et al., 2005; Rodgers et al., 2006) for rats and Poulin et al. (2002) for humans, see Table 4.1; same acidic phospholipid volume from Rodgers & Rowland (2005) (Rodgers et al., 2005) for both species. Axes are in log scale.

The role of brain lipid composition We tested three plausible rat brain lipid compositions reported in the literature (Rodgers et al., 2005; Poulin et al., 2002; Xu et al., 2020) (see Table 4.1). For each profile, both fuT and fuH_{tis} were recalculated and compared with experimental values. As shown by Figure 4.3, while fuT consistently showed better alignment with $\text{fuH}_{\text{exp(u)}}$ in rats, a clear trend emerged: both metrics became less accurate as the ratio of acidic phospholipid volume to total tissue volume ($V_{\text{ap(c)}}/V_{\text{tis}}$) increased. Among the three brain lipid profiles, the Poulin & Theil composition yielded the highest overall accuracy. In humans, we adopted the Poulin & Theil brain lipid profile (the only one available, to our knowledge), and assumed the same three values of $V_{\text{ap(c)}}/V_{\text{tis}}$ used for rats. The acidic-to-neutral phospholipid ratio varied accordingly, since the total phospholipid volume remains constant. Notably, under the highest $V_{\text{ap(c)}}/V_{\text{tis}}$ condition, fuH_{tis} surpassed fuT in accuracy, reaching a maximum of $\sim 60\%$ within 3-fold deviation and $\sim 70\%$ within 4-fold deviation. However, both methods—especially fuT —showed a marked drop in absolute performance at high $V_{\text{ap(c)}}/V_{\text{tis}}$ values. Expanding on previous results, Figure 4.4 shows compound-level differences in prediction performance between fuH_{tis} and fuT , based on the log2-difference in relative error relative to experimental $\text{fuH}_{\text{exp(u)}}$. Even when accounting for different brain lipid compositions, the differences between fuH_{tis} and fuT predictions remain generally minimal for acids, neutrals, weak bases, and type II ampholytes-compound classes that appear largely unaffected by homogenization bias. In contrast, the largest discrepancies are consistently observed for moderate-to-strong bases and compounds with at least one cationic $\text{pK}_a \geq 7$. As expected, these compounds are the most sensitive to homogenization-induced disruption of cell membranes and pH compartmentalization, with several showing notable improvements (e.g., amantadine) or deteriorations (e.g., citalopram) in prediction accuracy when using fuH_{tis} instead of fuT . While the overall dataset supports the species-independence of experimental brain fraction unbound (see Figure 4.7), a few compounds—such as nortriptyline—exhibit divergent behavior across species, with improved predictions in humans but not in rats. These results raised the hypothesis that sensitivity to brain lipid composition could play a significant role in shaping prediction accuracy. Therefore, the expected performance of fuH_{tis} may be obscured by parameter uncertainty rather than a flaw in the model itself. A key physiological uncertainty lies in the brain acidic phospholipid content. Reported values vary widely, from $\sim 0.7\%$ up to 15% of human total phospholipids level (Rodgers et al., 2005; Xu et al., 2020; Svennerholm, 1968; Kim et al., 2014). Since protonated bases bind strongly to acidic lipid headgroups, we hypothesized that this parameter could critically affect model performance—particularly for basic compounds.

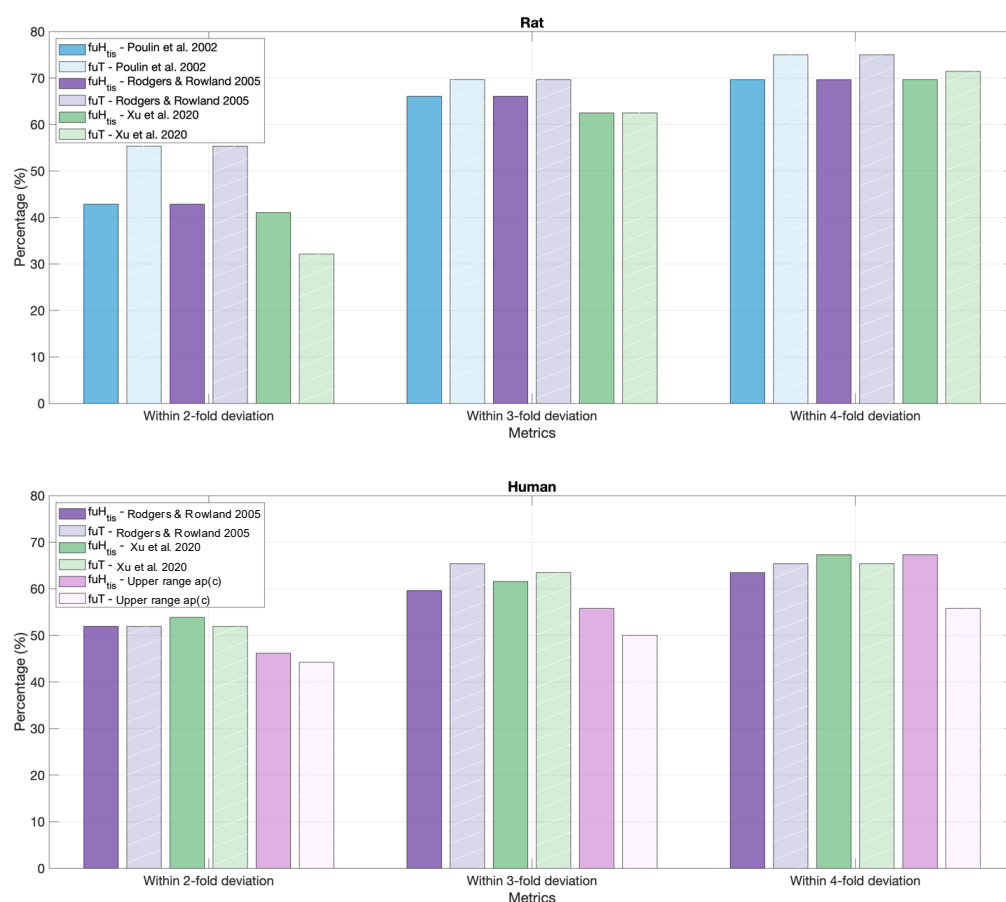


Figure 4.3: Prediction accuracy of fuH_{tis} and fuT in rats ($n = 56$) and humans ($n = 52$) with varying brain lipid compositions. Accuracy is evaluated across fold deviation thresholds from observed $fuH_{exp(u)}$ (Table 4.10). Physiological parameters are listed in Table 4.1. For rats, lipid volumes were taken from Poulin et al. (2002), Rodgers et al. (2005), and Xu et al. (2020). For humans, the baseline composition follows Poulin et al. (2002), combined with three increasing acidic phospholipid fractions from Rodgers et al. (2005) and Xu et al. (2020) and a representative level near the upper end of the plausible physiological range (corresponding to 12.5% of total phospholipids). Here, the bars are color-coded based on their acidic phospholipid source.

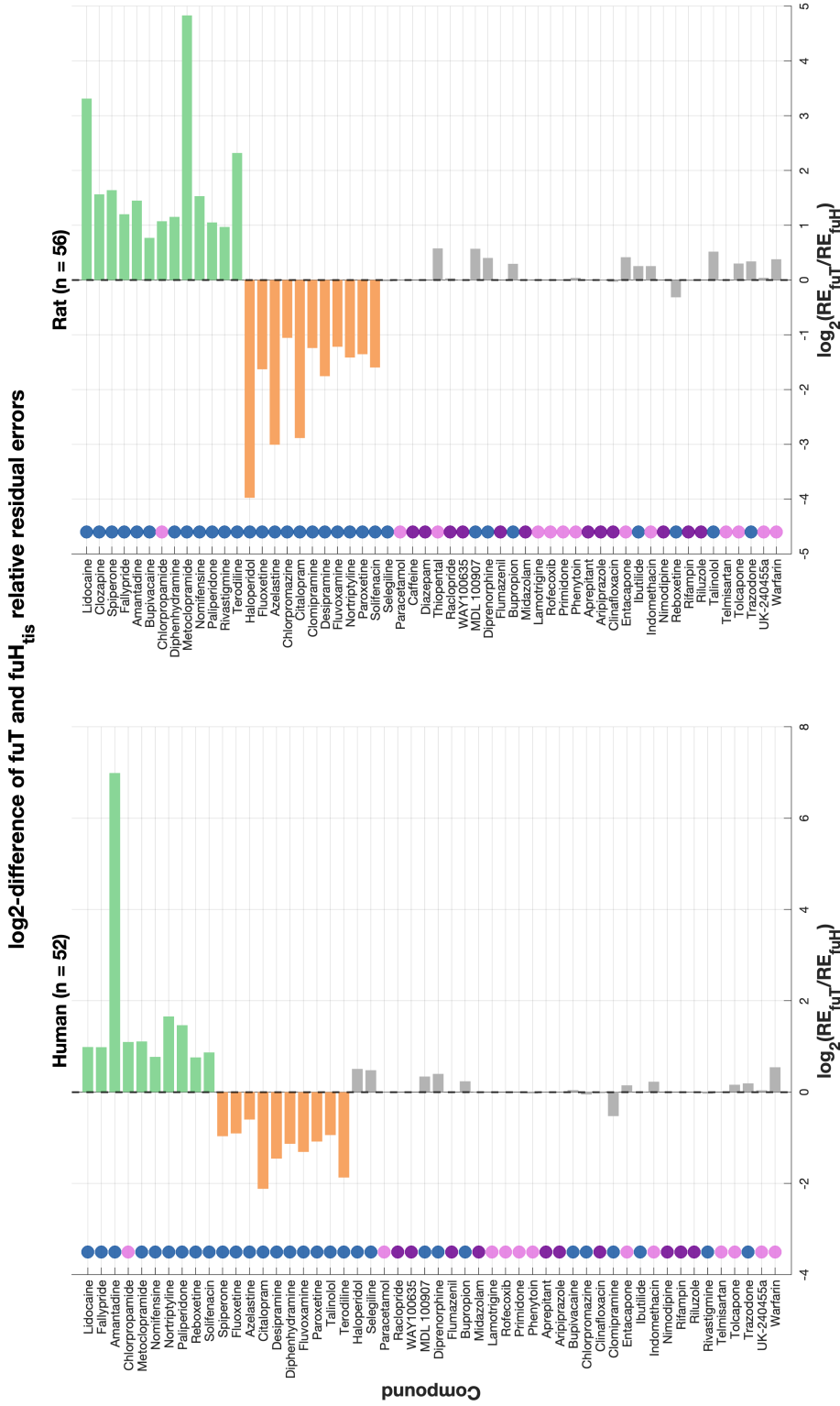


Figure 4.4: Log₂-difference in relative errors between fuH_{tis} and fuT predictions vs. experimental fuH_{exp(w)} in rats (n = 56) and humans (n = 52). Experimental values are listed in Table 4.10; physiological parameters in Table 4.1. Brain lipid composition is based on Xu et al. (2020) for rats and Poulin et al. (2002) for humans, using acidic phospholipid fractions from Xu et al. (2020). Relative error (RE) is defined as the absolute difference between predicted and observed values, normalized by the observed value. A threshold $\varepsilon = 0.5850$ (50% change) is used to classify prediction differences: green bars indicate improved fuH_{tis} prediction ($\log_2(\text{RE}_{\text{fuT}}/\text{RE}_{\text{fuH}_{\text{tis}}}) > \varepsilon$), orange bars indicate worse predictions ($< -\varepsilon$), and grey bars represent no significant change (absolute $\log_2$ -difference $< \varepsilon$). Each compound is accompanied by a point color-coded by drug type (Rodgers & Rowland classification): blue for compounds with at least one cationic pKa ≥ 7 (moderate-to-strong bases, type I zwitterions, and ordinary ampholytes), purple for compounds without such cationic pKa (acids, weak bases, type II zwitterions, and ampholytes), and pink for neutral compounds.

Sensitivity analysis of acidic phospholipid fractional volume To systematically test this hypothesis, we performed a sensitivity analysis across a biologically plausible range of acidic phospholipid fractional volumes with respect to the total phospholipid fractional volume. The remaining physiological parameters followed the default values in Table 4.1, with lipid content falling in the low range—from Rodgers & Rowland (Rodgers et al., 2005; Rodgers et al., 2006) for rats and Poulin et al. (2002) for humans. In rats, once the acidic phospholipid fractional volume exceeded about 4%—corresponding to the estimate from Xu et al. (2020)—fuH_{tis} outperformed fuT, reaching up to ~45% accuracy within 2-fold deviation, ~70% within 3-fold, and over 75% within 4-fold. While Figure 4.5 shows results for the R&R brain lipid composition, this trend held across all three rat brain lipid profiles (Rodgers et al., 2005; Poulin et al., 2002; Xu et al., 2020), although absolute performance varied. For humans, a similar transition point near 4% of total phospholipid fractional volume was observed, with fuH_{tis} outperforming fuT across all fold-deviation metrics.

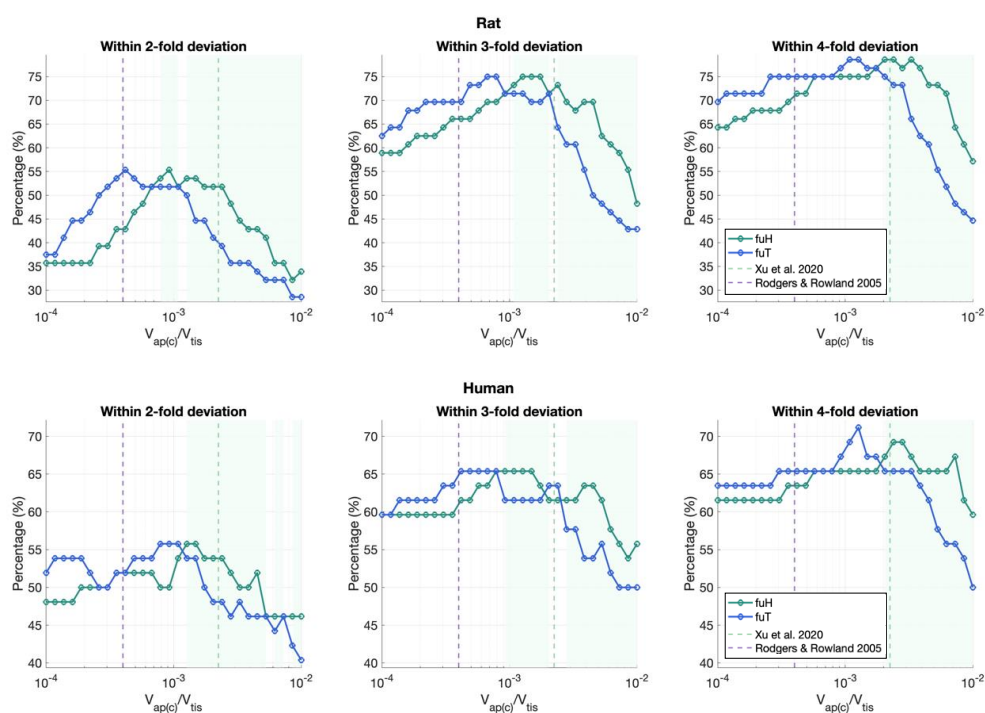


Figure 4.5: Effect of acidic phospholipid fractional volume on human and rat fuH_{tis} and fuT prediction accuracy, evaluated across different fold deviation thresholds from experimental data (Table 4.10). Vertical lines indicate the acidic-phospholipid fractions from Rodgers et al. (2005) and Xu et al. (2020). Default physiological parameters are from Rodgers et al. (2005) and Rodgers et al. (2006) for rats and Poulin et al. (2002) for humans (see Table 4.1). Accuracy for fuH_{tis} and fuT is shown in teal and blue, respectively; green shading indicates regions where fuH_{tis} outperforms fuT. The x-axis is in log scale.

Overall, these patterns suggest that the modest performance of fuH_{tis} may reflect assumptions about tissue composition—particularly uncertainty in acidic phospholipid fractional volume—rather than a limitation of the mechanistic

model. At lower acidic phospholipid volumes, fuT may coincidentally align better with homogenate data, despite assuming intact membranes and pH compartmentalization. In contrast, the homogenate model may underestimate binding due to the low availability of acidic phospholipid sites. Conversely, fuH_{tis} —designed to represent homogenate tissue conditions—becomes more predictive when acidic phospholipid content is sufficient to support electrostatic binding of basic compounds. Indeed, even after membrane disruption, acidic phospholipids remain present and available for binding in homogenate preparations. For neutral and acidic compounds, differences between methods were less pronounced, as ionization states are less influenced by phospholipid interactions under homogenization conditions. At the high end of the tested range, the drop in accuracy for both methods may reflect an overestimation of lipid binding, but fuH_{tis} still tracks better with experimental data. Overall, our results support the view that fuH_{tis} is, in principle, a mechanistically appropriate model for homogenate-based assays—provided that physiological inputs, especially acidic phospholipid content—are well characterized.

4.4 DISCUSSION

A priori predictions of brain fraction unbound are typically evaluated against homogenate data, despite the experimental bias introduced by tissue homogenization. To address this limitation, we propose a new homogenate-specific metric for *a priori* prediction of the fraction unbound in brain homogenate tissue, denoted as fuH_{tis} , and derived within the R&R framework. Our goal is to enhance the interpretation of homogenate assay measurements and, ultimately, to increase confidence in *a priori* predictions of the fraction unbound in intact brain tissue. A related but distinct approach was proposed by [Fridén et al. \(2011\)](#), who used measured values in brain homogenates as input to a mechanistic model incorporating pH partitioning to estimate the fraction unbound in intact brain tissue, validated against brain slice data. While both [Fridén et al. \(2011\)](#) and our work ultimately aim to predict fuT *in vivo*, our strategy builds trust in the mechanistic model from the bottom up: we first want to demonstrate that drug partitioning in homogenate tissue can be accurately predicted from the sum of its constituent parts—provided experimental bias is properly accounted for. Once this level of mechanistic accuracy is established for homogenates, the same principles can be extended more confidently to intact tissues.

By exploring the baseline differences with the mechanistic equations underlying the fraction unbound in intact brain tissue, our analysis confirmed that fuT and fuH_{tis} are inherently distinct. While the new metric fuH_{tis} is more appropriate for comparison with experimental homogenate data, interestingly, it can be derived from fuT by assuming a uniform pH across compartments ($pH_{iw} = pH_{cw} = pH_{hw}$) and complete membrane disruption ($K_{cw:iw} = 1$).

These transformations reflect more than methodological adjustments: they embody structural changes resulting from homogenization, which irreversibly alter drug binding and distribution by disrupting membrane integrity and physiological pH compartmentalization. Adjusting dilution factor and buffer pH cannot reconcile these differences. Importantly, ionization state played a critical role. The effect of homogenization on fraction unbound predictions varies with compound ionization class. The impact is most significant for moderate-to-strong bases and compounds with at least one cationic $\text{pK}_a \geq 7$, due to their strong affinity for acidic phospholipids in cellular membranes and their sensitivity to pH compartmentalization. In contrast, acids, neutrals, and weak bases are less affected, with observed differences mainly arising from small shifts in neutral and cationic fractions.

By design, fuH_{tis} reflects the unified pH and loss of compartmentalization in homogenate assays, while fuT incorporates physiological pH compartmentalization and cell membranes present in intact tissue. Therefore, fuH_{tis} should in principle offer a more appropriate prediction of homogenate brain tissue data. Despite this conceptual alignment, under our initial brain lipid composition assumptions, fuT predictions showed better agreement with homogenate data than fuH_{tis} in both rat and human datasets (e.g., 65.4% vs. 59.6% within a 3-fold error for humans). This unexpected result prompted further sensitivity analysis. By varying the fractional volume of acidic phospholipids ($V_{\text{ap(c)}}/V_{\text{tis}}$) across values reported in the literature, we found that fuH_{tis} matched and then exceeded fuT in predictive performance as acidic lipid content increased. Specifically, when acidic phospholipids were increased from 0.7% to 5% of total phospholipids, fuH_{tis} (human) predictions improved to 64% within 3-fold of observed values, compared to 54% for fuT . Yet, under higher acidic phospholipid conditions, we found that the predictive performance of both fuT and fuH_{tis} declined in absolute terms. This trend suggests that unexpected predictive performance may result from uncertainty in physiological parameters (especially phospholipid composition) rather than limitations in model structure. While acidic phospholipid content emerged as a key factor to account for deviations from experimental data, other sources of error—such as experimental variability or compound-specific input parameters—may also influence prediction accuracy. Nevertheless, the consistent improvement in fuH_{tis} performance under physiologically plausible lipid conditions, characterized by higher acidic phospholipid content, supports the robustness of its mechanistic foundation. Expanding this approach to incorporate more detailed lipidomic data and validate it across other tissues could enhance the applicability of mechanistic fraction unbound predictions beyond the brain, supporting broader drug development efforts.

Mechanistic limitations While the assumptions underlying fuH_{tis} and fuT are consistent with the Rodgers & Rowland mechanistic framework, they come with simplifications that may limit applicability in certain contexts, for ex-

ample the assumption of negligible intracellular protein binding and lack of subcellular compartmentalization. Our current homogenate model considers only the interstitial and cytosolic compartments. During brain homogenization, organelles such as lysosomes and mitochondria are mechanically disrupted, releasing their contents into the homogenate mixture (Abcam, 2025). Although these organelles have distinct pH values that differ from interstitial and cytosolic water, their contribution to total homogenate pH is expected to be minor, given their relatively small volume fraction and the buffering capacity of homogenization media. For this reason, intracellular compartment-specific pH effects are not explicitly included in the current framework. However, for compounds with pKa values near the homogenate pH range (7.0–7.4) (*e.g.*, weak bases and acids), even modest pH deviations—such as a 0.1–0.3 unit decrease from release of acidic lysosomal contents—could alter the fraction protonated and thereby influence tissue binding predictions.

Lysosomal sequestration is a special case of subcellular compartmentalization that may be relevant in the distribution of basic lipophilic drugs in the intact brain tissue. Even when explicitly modeled, Schmitt *et al.* (2021), however, observed only modest improvements in predicting tissue partition coefficients for such compounds. This limited impact is likely due to the relatively small lysosomal volume and to the larger contributions of lipid and acidic phospholipid binding to the overall tissue distribution—factors already captured by the underlying R&R framework. Nevertheless, future extensions of the current framework should integrate intracellular compartmentalization for predicting unbound fractions in both intact tissue (fuT) and brain homogenates (fuH_{tis}). For lysosomes specifically, fuT predictions should account for lysosomal sequestration and trapping mechanisms, whereas fuH_{tis} predictions should capture the contribution of lysosomal acidity ($pH \sim 4.5\text{--}5.0$) to the overall homogenate pH. Such refinements would provide a more comprehensive representation of pH-dependent drug partitioning in intact and homogenate brain tissues.

Another potential limitation stems from the presence of residual blood in brain homogenates, which may bias fuH_{tis} and fuT predictions for compounds with strong plasma protein binding. In such cases, overestimation can be mitigated by correcting both fuH_{tis} and fuT predictions to account for the fraction unbound in residual blood, using the fractional volume of residual blood as an additional input. Eqs. 4.25 and 4.36 and can be readily extended to incorporate these terms. However, detailed information on residual blood content is rarely reported in homogenate assays. Sensitivity analyses within realistic ranges can help assess its impact. Nevertheless, given the typically small residual blood volume (*e.g.*, $\sim 4\%$ in Brown *et al.* (1997)), improvements in prediction accuracy are likely minimal unless the compound displays exceptionally high plasma protein binding. Moreover, residual blood primarily affects compounds with low volumes of distribution (*e.g.*, acids) and has limited impact on those with high volumes (*e.g.*, bases). Since our analysis shows that homogenization

mainly affects compounds with at least one cationic $pK_a \geq 7$, such as highly basic drugs, we conclude that residual blood is unlikely to significantly alter our results. Taken together, these caveats should be considered when interpreting model predictions—particularly for compounds with known sequestration mechanisms or when applying the approach to specialized scenarios. Moreover, future work should involve testing our homogenate-specific metric using alternative tissue composition-based methods, adjusting the derivation to the specific assumptions and approximations each method makes regarding tissue constituent distribution coefficients (*e.g.*, [Schmitt \(2008\)](#), [Lukacova et al. \(2008\)](#), and [Poulin et al. \(2024\)](#)).

Takeaways The main takeaways from this work are:

- Model selection for fraction unbound evaluation should align with the experimental context. While fuT is suited for intact brain tissue or brain slice studies, fuH_{tis} is mechanistically tailored for homogenate-based assays.
- Accurate fuH_{tis} prediction relies on reliable input parameters. Beyond reproducing experimental conditions (*e.g.*, buffer pH, dilution factor), predictive accuracy is highly sensitive to physiological input—including brain lipid composition, especially acid phospholipids. This mechanistic insight strengthens the existing need for more precise and quantitative characterization of brain lipid composition.
- fuH_{tis} predictions for moderate-to-strong basic compounds are highly sensitive to acidic phospholipid variation. This pronounced sensitivity suggests that, in future studies, this metric could serve as a sentinel probe to refine model assumptions on tissue composition—specifically estimates of brain lipid composition.

Further evaluation and validation of fuH_{tis} predictions against experimental data will be a necessary step to build confidence in this homogenate-specific metric and, ultimately, improve fraction unbound estimates in intact brain tissue for CNS-active small molecules to be applied within PBPK modeling frameworks, such as the one shown in Figure 4.6.

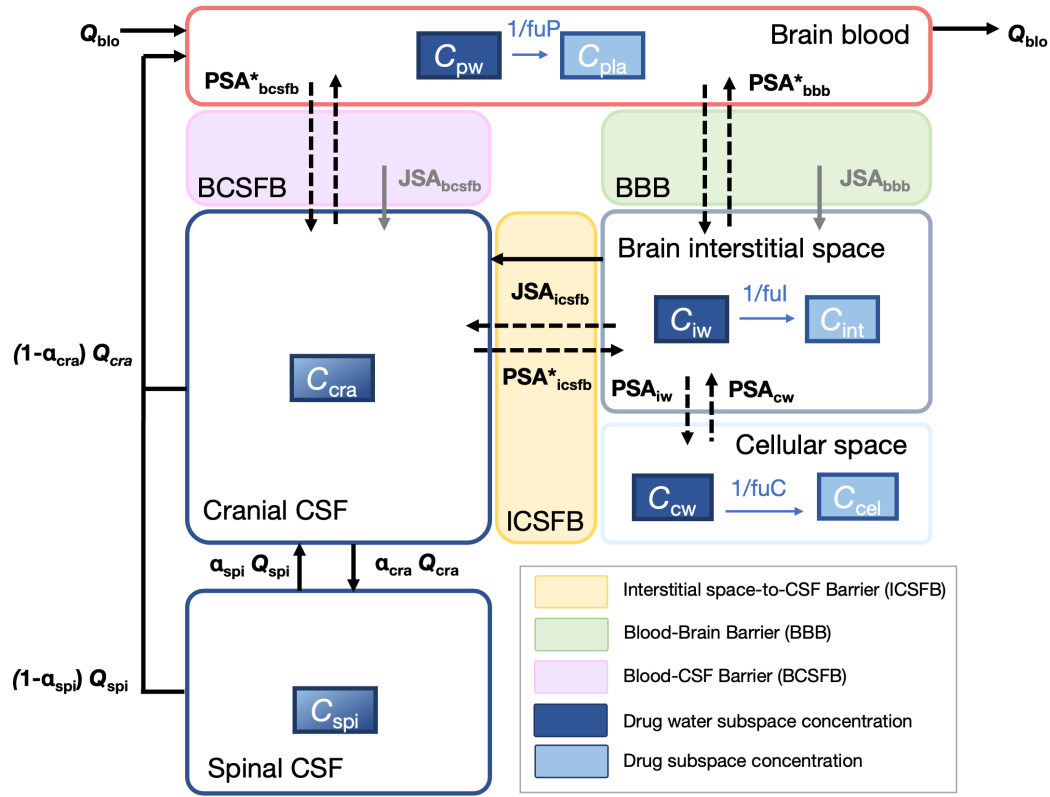


Figure 4.6: Schematic of the refined brain-CSF model derived from the 4Brain framework by [Gaohua et al. \(2016\)](#) and [Verscheijden et al. \(2019\)](#) that explicitly incorporates interstitial and cellular spaces. The model distinguishes five compartments: brain blood/microvasculature, interstitial space, cellular space, cranial CSF (ventricular and cranial subarachnoid spaces), and spinal CSF (spinal subarachnoid space). Drug distribution is governed by both convective and diffusive fluxes. Blood enters and exits the brain via the brain blood flow rate Q_{blo} , while CSF flows between cranial and spinal compartments via Q_{cra} and Q_{spi} , with respective fractions α_{cra} , $(1-\alpha_{cra})$, α_{spi} , and $(1-\alpha_{spi})$. Three key barrier-associated water fluxes are modeled: (i) JSA_{bbb} , water exchange across the blood-brain barrier (BBB); (ii) JSA_{bcsfb} , CSF production via the choroid plexus at the blood-CSF barrier (BCSFB); and (iii) JSA_{icsfb} , bulk ISF flow from the interstitial space to the cranial CSF across the interstitial-CSF barrier (ICSFB, including the ependyma and pia mater). Fluid fluxes are balanced such that water entering the interstitial space from the BBB exits into the cranial CSF via the ICSFB, and total CSF production (via BCSFB and ICSFB) equals absorption into the brain blood. Only the cranial CSF compartment exchanges solutes with the interstitial fluid; the spinal CSF exchanges only with cranial CSF. Drug permeation occurs via passive diffusion across the BBB, BCSFB, and ICSFB, with respective effective permeability-surface area products PSA^*_{bbb} , PSA^*_{bcsfb} , and PSA^*_{icsfb} . These are computed as $PSA^* = \frac{1}{2}P \cdot SA$ to account for bidirectional transport across cell layers. Passive diffusion also occurs across the brain cellular membrane (PSA_{iw} , PSA_{cw}), where only neutral species are assumed to permeate (Rodgers & Rowland assumption). Drug concentrations are tracked in water subspaces (C_{pw} , C_{iw} , C_{cw}) and in total drug subspaces (C_{pla} , C_{int} , C_{cel} , C_{cra} , C_{spi}), linked by the fractions unbound in plasma (fu_P), interstitial fluid (fu_I), cells (fu_C), and CSF (assumed to contain only unbound drug, $fu_{csf} = 1$). Active transport and brain metabolism are not included.

4.5 MATERIALS AND METHODS

R&R-BASED DERIVATION OF BRAIN FRACTIONS UNBOUND

General assumptions of tissue composition-based models We rely on general assumptions underlying mechanistic models for *a priori* prediction of brain fractions unbound, including the R&R framework. Tissue composition is assumed to be homogeneous across each compartment, with fixed fractional volumes of water, lipids, phospholipids, and proteins. The values for tissue composition are drawn from literature sources and treated as constant, without accounting for inter-individual variability or changes due to pathological conditions. In addition, each component is considered as an independent, non-saturable binding site, with no competitive nor cooperative interactions between binding partners. Partitioning is considered at steady-state conditions, such that drug concentrations in tissue compartments have reached equilibrium. Furthermore, the model assumes rapid equilibrium between ionization states, which are calculated using the Henderson–Hasselbalch equation based on the local pH of plasma, interstitial fluid, cellular environments, and homogenate tissue fluid. All binding interactions (*e.g.*, with acidic phospholipids and binding proteins) are assumed to be linear with respect to drug concentration, *i.e.*, saturation effects are not included; this assumption is generally appropriate for compounds where nonspecific binding dominates at therapeutic concentrations.

R&R assumptions and approximations In addition to general assumptions, we rely on R&R specific approximations, which we used for the standard expression of the fraction unbound in intact brain tissue and for our derivation of the fraction unbound in homogenate brain tissue. We summarize below the most relevant ones:

- Drug dissolution into water is assumed to occur in all ionization states, *i.e.*, the compound distributes into water spaces regardless of its charge. In contrast, partitioning into neutral lipid components is restricted to the neutral form of the compound.
- The interstitial fluid is primarily composed of water (> 95%) and solutes (*e.g.*, ions and proteins), *i.e.*, $isf = iw + pr(i)$. For practical PBPK modeling, the R&R methods (Rodgers et al., 2005; Rodgers et al., 2006), along with subsequent studies based on it (Lukacova et al., 2008; Schmitt, 2008; Schmitt et al., 2021), implicitly assume that the ISF is primarily aqueous, with solutes contributing minimally to its volume (Tarazi et al., 1969), and approximate the fractional volume of interstitial water relative to total interstitial fluid as $V_{iw}/V_{isf} = 1$. While explicit experimental values for this ratio are not provided in their work, the dominance of water in ISF justifies the approximation. Aligning with physiological consensus, this

assumption simplifies calculations without significantly compromising model accuracy for most tissues.

- Lysosomal trapping and intracellular pH compartmentalization are not explicitly modeled. To account for this limitation, [Schmitt et al. \(2021\)](#) provided an extension of the R&R model ([Rodgers et al., 2005](#)) for highly basic compounds by including an additional assumption, *i.e.*, the drug distributes into lysosomes in the cellular space in its neutral form. We retain the R&R assumption of absent/negligible lysosomal sequestration.
- Transport across cellular membranes is assumed to occur exclusively via passive diffusion of the neutral species, and ionized forms are not considered permeable. Indeed, the model does not account for active transport mechanisms nor tissue-specific metabolism. Drug distribution is therefore governed solely by passive processes and nonspecific binding.
- Binding interactions with interstitial proteins and cellular acidic phospholipids are modeled based on compound ionization characteristics, as detailed in Section 4.3.1.

FRACTION UNBOUND IN INTACT BRAIN TISSUE

In general, drug partitioning in the the intact tissue space is quantified by the **tissue-to-extracellular water partition coefficient** $K_{pu_{tis}}$, *i.e.*,

$$K_{pu_{tis}} := \frac{C_{tis}}{C_{ew}} \quad (4.10)$$

where the extracellular water space (ew) corresponds to the interstitial water space (iw), *i.e.*, $C_{ew} = C_{iw}$.

Derivation of the tissue-to-extracellular water partition coefficient The following mass balance equation for the interstitial and cellular tissue constituents holds:

$$\begin{aligned} A_{tis} &= A_{int} + A_{cel} = \\ &= (A_{iw} + A_{pr(i)}) + (A_{cw} + A_{pr(c)} + A_{nl(c)} + A_{np(c)} + A_{ap(c)}) (+A_{rest}) \end{aligned}$$

with the amount of drug in the intact tissue A_{tis} , in interstitial and cellular spaces A_{int} and A_{cel} , in interstitial and cellular water A_{iw} and A_{cw} , bound to binding proteins from the interstitial and cellular spaces $A_{pr(i)}$ and $A_{pr(c)}$, distributed into neutral lipids $A_{nl(c)}$, into neutral phospholipids $A_{np(c)}$, and acidic phospholipids $A_{ap(c)}$ in the cellular space. We assume that $A_{rest} = 0$, *i.e.*, the distribution in the remaining parts is negligible, including residual

vascular blood. Using $A_{\text{sub}} = V_{\text{sub}} \cdot C_{\text{sub}}$ for the individual subspace (sub) contributions (*i.e.*, iw, cw, pr, nl, ap) and dividing by V_{tis} yield

$$\begin{aligned} C_{\text{tis}} &= \frac{V_{\text{int}}}{V_{\text{tis}}} C_{\text{int}} + \frac{V_{\text{cel}}}{V_{\text{tis}}} C_{\text{cel}} = \\ &= \frac{V_{\text{iw}}}{V_{\text{tis}}} C_{\text{iw}} + \frac{V_{\text{pr(i)}}}{V_{\text{tis}}} C_{\text{pr(i)}} + \frac{V_{\text{cw}}}{V_{\text{tis}}} C_{\text{cw}} + \frac{V_{\text{pr(c)}}}{V_{\text{tis}}} C_{\text{pr(c)}} + \frac{V_{\text{nl(c)}}}{V_{\text{tis}}} C_{\text{nl(c)}} \\ &\quad + \frac{V_{\text{np(c)}}}{V_{\text{tis}}} C_{\text{np(c)}} + \frac{V_{\text{ap(c)}}}{V_{\text{tis}}} C_{\text{ap(c)}}. \end{aligned}$$

where V_{int} and V_{cel} are the volumes of the tissue-related interstitial and cellular spaces, such that $V_{\text{tis}} = V_{\text{int}} + V_{\text{cel}}$ is the tissue volume.

By dividing the equation by C_{iw} , replacing the concentration ratios $C_{\text{sub}}/C_{\text{wa}}$ with the corresponding distribution coefficients $K_{\text{sub:wa}}$, and rearranging some terms, we obtain the tissue-to-extracellular water partition coefficient as

$$\begin{aligned} K_{\text{pu}_{\text{tis}}} &:= \frac{C_{\text{tis}}}{C_{\text{iw}}} = \frac{V_{\text{int}}}{V_{\text{tis}}} \frac{C_{\text{int}}}{C_{\text{iw}}} + \frac{V_{\text{cel}}}{V_{\text{tis}}} \frac{C_{\text{cel}}}{C_{\text{iw}}} = \\ &= \frac{V_{\text{int}}}{V_{\text{tis}}} \left(\frac{V_{\text{iw}}}{V_{\text{int}}} + \frac{V_{\text{pr(i)}}}{V_{\text{int}}} K_{\text{pr(i):iw}} \right) + \frac{V_{\text{cel}}}{V_{\text{tis}}} \left(\frac{V_{\text{cw}}}{V_{\text{cel}}} + \frac{V_{\text{pr(c)}}}{V_{\text{cel}}} K_{\text{pr(c):cw}} \right. \\ &\quad \left. + \frac{V_{\text{nl(c)}}}{V_{\text{tis}}} K_{\text{nl(c):cw}} + \frac{V_{\text{np(c)}}}{V_{\text{tis}}} K_{\text{np(c):cw}} + \frac{V_{\text{ap(c)}}}{V_{\text{tis}}} K_{\text{ap(c):cw}} \right) K_{\text{cw:iw}}. \end{aligned} \quad (4.11)$$

R&R approximation of distribution coefficients Relying on the R&R methods, the distribution coefficients of the various tissue constituents are the followings.

- $K_{\text{cw:iw}}$, *i.e.*, the **cellular-to-interstitial water distribution coefficient** characterizing the exchange across the cellular membrane, is given by

$$K_{\text{cw:iw}} = \frac{\text{fn}_{\text{iw}} + \alpha(1 - \text{fn}_{\text{iw}})}{\text{fn}_{\text{cw}} + \alpha(1 - \text{fn}_{\text{cw}})} \stackrel{(\alpha=0)}{=} \frac{\text{fn}_{\text{iw}}}{\text{fn}_{\text{cw}}}. \quad (4.12)$$

Rodgers & Rowland assumed that only neutral species can cross the cellular membrane via passive diffusion, *i.e.*, $\alpha = 0$. This parameter depends exclusively on pH compartmentalization between the interstitial and cellular spaces. Other methods such as [Schmitt \(2008\)](#) assumed that neutral drug species and, to a lower extent determined by different values of $\alpha \ll 1$, also charged drug species passively diffuse across the cellular membrane.

- $K_{\text{nl(c):cw}}$, *i.e.*, the **neutral lipid-to-water distribution coefficient**, is obtained as

$$K_{\text{nl(c):cw}} = P_{\text{o:w}} \text{fn}_{\text{cw}} \quad (4.13)$$

with the underlying assumption that only the neutral species distributes in the *n*-octanol phase.

- $K_{np(c):cw}$, *i.e.*, the **neutral phospholipid-to-water distribution coefficient**, is given by a neutral logP-based parameterization

$$K_{np(c):cw} = 0.3 D_{o:w} + 0.7 fn_{cw} = (0.3 P_{o:w} + 0.7) fn_{cw}, \quad (4.14)$$

assuming that phospholipids are similar to a mixture of 30% neutral lipids and 70% water (Poulin et al., 2002), and only the neutral species distributes into the neutral phospholipids and *n*-octanol phase. The same parameterization is used to predict neutral lipid and phospholipid-to-water distribution coefficients in plasma and red blood cells, *e.g.*,

$$K_{nl(p):pw} = P_{o:w} fn_{pw} \quad \& \quad K_{np(p):pw} = (0.3 P_{o:w} + 0.7) fn_{pw} \quad (4.15)$$

- $K_{pr(c):cw}$, *i.e.*, the **cellular binding protein-to-water distribution coefficient**, is assumed to be zero:

$$K_{pr(c):cw} = 0 \quad (4.16)$$

i.e., negligible intracellular protein binding for all compounds. The same holds for the RBC-related distribution coefficient, $K_{pr(r):rw} = 0$.

- $K_{ap(c):cw}$, *i.e.*, the **cellular acidic phospholipid-to-water distribution coefficient**, is obtained by adopting a charged protein-binding parameterization:

$$K_{ap(c):cw} = \frac{V_{cel}}{V_{ap(c)}} K_{A,ap(c)^-} AP_{cel}^- fcat_{cw} = \frac{V_{tis}}{V_{ap(c)}} K_{A,ap(c)^-} AP_{tis}^- fcat_{cw}$$

with $K_{A,ap(c)^-}$ being the affinity constant associated with cellular acidic phospholipids and $AP_{cel}^- := A_{AP^-} / V_{cel}$ and $AP_{tis}^- := A_{AP^-} / V_{tis}$ being the concentrations of acidic phospholipids with reference to the brain cellular volume and tissue volume, respectively, such that $V_{tis} AP_{tis}^- = V_{cel} AP_{cel}^-$. The crucial step here consists in assuming that the affinity constant associated with acidic phospholipids is identical in RBC and cellular spaces, *i.e.*, $K_{A,ap(c)^-} = K_{A,ap(r)^-}$. This assumption is crucial given that $K_{A,ap(r)^-}$ can be inferred from *in vitro* drug-specific input parameters, *e.g.*, B:P. Relying on this assumption, we obtain

$$K_{ap(c):cw} = \frac{V_{tis}}{V_{ap(c)}} K_{A,ap(r)^-} AP_{tis}^- fcat_{cw}. \quad (4.17)$$

- $K_{pr(i):iw}$, *i.e.*, the **interstitial binding protein-to-water distribution coefficient**, is obtained by adopting the charged protein-binding parameterization previously used for acidic phospholipid binding:

$$K_{pr(i):iw} = \frac{V_{iw}}{V_{pr(i)}} \cdot K_{A,pr(i)} (1 - fcat_{iw}) PR_{isf}$$

with PR_{isf} being the total concentration of binding proteins in the interstitial fluid and $K_{A,pr(i)}$ the affinity constant associated with interstitial binding proteins. The underlying assumption is that only the non-cationic species—namely, neutral and anionic forms of the drug—are assumed to interact with binding proteins in the interstitial fluid.

By assuming $K_{A,pr(i)} = K_{A,pr(p)}$ and $PR_{isf} = \frac{V_{tis}}{V_{isf}} PR_{tis}$, the distribution coefficient can be expressed as

$$K_{pr(i):iw} = \frac{V_{tis}}{V_{pr(i)}} \frac{V_{iw}}{V_{isf}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{(1 - fcat_{iw})}{(1 - fcat_{pw})} \frac{PR_{tis}}{PR_{pla}} \quad (4.18)$$

with the ratio PR_{isf}/PR_{pla} depending on the drug type. Importantly, $K_{A,pr(p)} PR_{pla} (1 - fcat_{pw})$ can be inferred from fuP and $K_{pr(p):pw}$.

- $K_{pr(p):pw}$, *i.e.*, the **plasma binding protein-to-water distribution coefficient**, is obtained by adopting the charged protein-binding parameterization to describe the plasma protein binding within plasma water:

$$K_{pr(p):pw} = \frac{V_{pw}}{V_{pr(p)}} K_{A,pr(p)} (1 - fcat_{pw}) PR_{pla} . \quad (4.19)$$

with PR_{pla} being the total concentration of binding proteins in plasma and $K_{A,pr(p)}$ the affinity constant associated with plasma binding proteins. With some algebraic manipulations, $K_{A,pr(p)} (1 - fcat_{pw}) PR_{pla}$ can be expressed in terms of $K_{nl(p):pw}$ and $K_{np(p):pw}$ as follows:

$$\begin{aligned} K_{A,pr(p)} (1 - fcat_{pw}) PR_{pla} &= \\ &= \left(\frac{1}{fuP} - \frac{V_{nl(p)}}{V_{pla}} K_{nl(p):pw} - \frac{V_{np(p)}}{V_{pla}} K_{np(p):pw} \right) \frac{V_{pla}}{V_{pw}} - 1 . \end{aligned} \quad (4.20)$$

See Appendix B for a detailed derivation of tissue constituent distribution coefficients related to the intact brain tissue according to R&R approach.

Derivation of the fraction unbound in the intact brain tissue By definition the fraction unbound in the intact brain tissue fuT is the ratio of drug concentration in the extracellular water (here interstitial water) to drug concentration in the tissue space. Therefore, fuT is derived by

$$fuT := \frac{C_{ew}}{C_{tis}} = \frac{C_{iw}}{C_{tis}} = \frac{1}{K_{pu_{tis}}} . \quad (4.21)$$

Similarly, the fraction unbound in the interstitial space fuI is the ratio between drug concentration in the interstitial water to the drug concentration in the interstitial space, and fuC is the ratio between drug concentration in the cellular water to the drug concentration in the cellular space, *i.e.*,

$$fuI := \frac{C_{iw}}{C_{int}} \quad \& \quad fuC := \frac{C_{cw}}{C_{cel}} . \quad (4.22)$$

Therefore, by replacing the fractional concentrations in eq. 4.11 with these newly defined quantities,

$$\frac{1}{fuI} = \frac{V_{iw}}{V_{int}} + \frac{V_{pr(i)}}{V_{int}} K_{pr(i):iw} \quad (4.23)$$

$$\frac{1}{fuC} = \frac{V_{cw}}{V_{tis}} + \frac{V_{pr(c)}}{V_{tis}} K_{pr(c):cw} + \frac{V_{nl(c)}}{V_{tis}} K_{nl(c):cw} + \frac{V_{np(c)}}{V_{tis}} K_{np(c):cw} + \frac{V_{ap(c)}}{V_{tis}} K_{ap(c):cw}, \quad (4.24)$$

the fraction unbound in the intact brain tissue is obtained by the following relationship:

$$\frac{1}{fuT} = \frac{V_{int}}{V_{tis}} \frac{1}{fuI} + \frac{V_{cel}}{V_{tis}} \frac{1}{fuC} K_{cw:iw} \quad (4.25)$$

Assumption of fast exchange across the vascular endothelium

For a typical organ, the total tissue space is considered as a lumped vascular blood, interstitial and cellular space, and the exchange across the vascular endothelium is assumed to be fast compared to other PK processes. This implies that extracellular water, plasma water, and interstitial water concentrations are assumed to be identical, *i.e.*, $C_{ew} = C_{pw} = C_{iw}$. This assumption leads to

$$\frac{1}{fuT} = \left(K_{pu_{tis}} = \frac{C_{tis}}{C_{pw}} \right) = \frac{V_{int}}{V_{tis}} \frac{1}{fuI} + \frac{V_{cel}}{V_{tis}} \frac{1}{fuC} K_{pu_{cel}}. \quad (4.26)$$

with the cellular-to-plasma water distribution coefficient $K_{pu_{cel}} := C_{cw}/C_{pw} = f_{npw}/f_{ncw}$. This is the key PBPK modeling parameter predicted by Rodgers & Rowland.

The assumption of fast exchange is not valid for the brain, where the blood-brain barrier restricts the exchange between blood and brain interstitial space, *i.e.*, $C_{iw} \neq C_{pw}$. Therefore, if we consider an intact brain tissue, drug partitioning is quantified by the tissue-to-interstitial water partition coefficient—a parameter that is, in principle, distinct from tissue-to-plasma water partition coefficient. In this chapter, we focus on predicting this quantity.

FRACTION UNBOUND IN HOMOGENATE BRAIN TISSUE

As follows from Section 4.2, the drug concentration in this homogenate brain tissue C_{hom} corresponds to the volume-averaged sum of the drug concentrations in its *fictitious* interstitial and cellular spaces, expressed as:

$$C_{hom} = \frac{V_{int}}{V_{hom}} C_{int} + \frac{V_{cel}}{V_{hom}} C_{cel}, \quad (4.27)$$

where V_{int} and V_{cel} are the volumes of the homogenate tissue-related interstitial and cellular spaces, respectively, such that $V_{hom} = V_{int} + V_{cel}$ is the volume of the (undiluted) homogenate brain tissue. Due to the disruption of cell

membranes and of pH compartmentalization resulting from homogenization techniques, drug distribution in homogenate brain tissue differs from that in intact brain tissue. Therefore, a separate partition coefficient is required to describe the drug distribution in the homogenate brain tissue. To this end, we introduce the **homogenate-to-homogenate water partition coefficient** $K_{\text{hom:hw}}$ —distinct from $K_{\text{pu}_{\text{tis}}}$.

Derivation of the homogenate-to-homogenate water partition coefficient As mentioned in Section 4.2, although cell membrane disruption induced by homogenization eliminates the distinction between interstitial and cellular spaces, we can abstract and conceptually reassign tissue constituents to fictitious interstitial and cellular spaces. In other words, this abstraction enables us to express the total amount of drug in the homogenate as the sum of the drug in the homogenate-related interstitial and cellular spaces, where each space refers to the sum of the tissue constituents that originally resided in that compartment prior to homogenization. To preserve a direct link to the physiological context and facilitate comparison of our homogenate-based formulation with the standard Rodgers and Rowland equations for intact brain tissue, we retain the original notation for interstitial and cellular water (iw and cw, respectively).

In the homogenate brain tissue, we assume that the drug dissolves in homogenate water, binds to binding proteins from the interstitial and cellular spaces, and its neutral form distributes into neutral lipids and neutral and acidic phospholipids from the cellular space. Therefore, the following mass balance equation holds:

$$\begin{aligned} A_{\text{hom}} &= A_{\text{int}} + A_{\text{cel}} = \\ &= A_{\text{hw}} + A_{\text{pr(i)}} + A_{\text{pr(c)}} + A_{\text{nl(c)}} + A_{\text{np(c)}} + A_{\text{ap(c)}} (+A_{\text{rest}}) \end{aligned}$$

with the amount of drug in the homogenate A_{hom} , in the fictitious interstitial and cellular spaces A_{int} and A_{cel} , in homogenate water A_{hw} —given by the sum of interstitial and cellular amounts A_{iw} and A_{cw} , bound to binding proteins from the interstitial and cellular spaces $A_{\text{pr(i)}}$ and $A_{\text{pr(c)}}$, distributed into neutral lipids $A_{\text{nl(c)}}$, into neutral phospholipids $A_{\text{np(c)}}$, and acidic phospholipids $A_{\text{ap(c)}}$ in the cellular space. We assume that the distribution in the remaining parts, such as collagen fibers or residual blood, is negligible, *i.e.*, $A_{\text{rest}} = 0$. Using $A_{\text{sub}} = V_{\text{sub}} \cdot C_{\text{sub}}$ for the individual subspace contributions (*i.e.*, hw, pr, nl, ap), expressing C_{hw} as the volume-averaged sum of the concentrations in

the conceptual interstitial and cellular water spaces, dividing by V_{hom} , and dividing by C_{hw} yield

$$\begin{aligned} \frac{C_{\text{hom}}}{C_{\text{hw}}} &= \frac{V_{\text{int}}}{V_{\text{hom}}} \frac{C_{\text{int}}}{C_{\text{hw}}} + \frac{V_{\text{cel}}}{V_{\text{hom}}} \frac{C_{\text{cel}}}{C_{\text{hw}}} = \\ &= \frac{V_{\text{iw}}}{V_{\text{hom}}} \frac{C_{\text{iw}}}{C_{\text{hw}}} + \frac{V_{\text{pr(i)}}}{V_{\text{hom}}} \frac{C_{\text{pr(i)}}}{C_{\text{hw}}} + \frac{V_{\text{cw}}}{V_{\text{hom}}} \frac{C_{\text{cw}}}{C_{\text{hw}}} + \frac{V_{\text{pr(c)}}}{V_{\text{hom}}} \frac{C_{\text{pr(c)}}}{C_{\text{hw}}} + \frac{V_{\text{nl(c)}}}{V_{\text{hom}}} \frac{C_{\text{nl(c)}}}{C_{\text{hw}}} + \\ &+ \frac{V_{\text{np(c)}}}{V_{\text{hom}}} \frac{C_{\text{np(c)}}}{C_{\text{hw}}} + \frac{V_{\text{ap(c)}}}{V_{\text{hom}}} \frac{C_{\text{ap(c)}}}{C_{\text{hw}}}. \end{aligned}$$

Rearranging the equation above, and replacing the concentration ratios with the corresponding partition coefficients, we obtain:

$$\begin{aligned} K_{\text{hom:hw}} &= \frac{C_{\text{hom}}}{C_{\text{hw}}} = \frac{V_{\text{int}}}{V_{\text{hom}}} \frac{C_{\text{int}}}{C_{\text{hw}}} + \frac{V_{\text{cel}}}{V_{\text{hom}}} \frac{C_{\text{cel}}}{C_{\text{hw}}} = \\ &= \frac{V_{\text{int}}}{V_{\text{hom}}} \left(\frac{V_{\text{iw}}}{V_{\text{int}}} K_{\text{iw:hw}} + \frac{V_{\text{pr(i)}}}{V_{\text{int}}} K_{\text{pr(i):hw}} \right) + \frac{V_{\text{cel}}}{V_{\text{hom}}} \left(\frac{V_{\text{cw}}}{V_{\text{cel}}} K_{\text{cw:hw}} + \right. \\ &\quad \left. + \frac{V_{\text{pr(c)}}}{V_{\text{cel}}} K_{\text{pr(c):hw}} + \frac{V_{\text{nl(c)}}}{V_{\text{cel}}} K_{\text{nl(c):hw}} + \frac{V_{\text{np(c)}}}{V_{\text{cel}}} K_{\text{np(c):hw}} + \frac{V_{\text{ap(c)}}}{V_{\text{cel}}} K_{\text{ap(c):hw}} \right). \end{aligned} \quad (4.28)$$

R&R-based approximations of homogenate-related distribution coefficients Relying on the R&R methods, the distribution coefficients of the various tissue constituents related to homogenate water are listed as follows.

- The absence of the cellular water-to-interstitial water distribution coefficient reflects cell membrane and pH compartmentalization disruption due to tissue homogenization. Formally, the distribution coefficient between any water-containing subspace and the homogenate water equals 1. For example, by following R&R approximation, the cellular water-to-homogenate water distribution coefficient $K_{\text{cw:hw}}$ is given by

$$K_{\text{cw:hw}} = \frac{f_{\text{n}_{\text{hw}}}}{f_{\text{n}_{\text{cw}}}} \stackrel{(f_{\text{n}_{\text{cw}}}=f_{\text{n}_{\text{hw}}})}{=} 1$$

since all water phases share the same pH in the homogenate tissue.

- $K_{\text{nl(c):hw}}$ and $K_{\text{np(c):hw}}$, *i.e.*, the **cellular neutral (phospho-)lipid-to-homogenate water distribution coefficients**, are given by

$$K_{\text{nl(c):hw}} = P_{\text{o:w}} f_{\text{n}_{\text{hw}}} \quad \& \quad K_{\text{np(c):hw}} = (0.3 P_{\text{o:w}} + 0.7) f_{\text{n}_{\text{hw}}}. \quad (4.29)$$

- $K_{\text{pr(c):hw}}$, *i.e.*, the **cellular binding protein-to-homogenate water distribution coefficient**, is assumed to be zero based on R&R assumptions.

- $K_{ap(c):hw}$, *i.e.*, the **cellular acidic phospholipid-to-homogenate water distribution coefficient**, is obtained by adopting the charged protein-binding parameterization:

$$\begin{aligned} K_{ap(c):hw} &= \frac{V_{cel}}{V_{ap(c)}} K_{A,ap(c)^-} AP_{cel}^- fcat_{hw} = \\ &= \frac{V_{hom}}{V_{ap(c)}} K_{A,ap(c)^-} AP_{hom}^- fcat_{hw} . \end{aligned}$$

with $AP_{hom}^- := A_{AP}^- / V_{hom}$. Due to cell lysis, the acidic phospholipid concentration in the cellular space AP_{cel}^- redistributes within the homogenate brain tissue. The partition coefficient relies on the assumption that there is a complete recovery of accessible binding sites of acidic phospholipids upon homogenization and the total amount of binding sites remains the same, *i.e.*,

$$V_{hom} AP_{hom}^- = V_{cel} AP_{cel}^- = V_{cw} AP_{cw}^- .$$

Finally, by assuming $K_{A,ap(c)^-} = K_{A,ap(r)^-}$, we obtain

$$K_{ap(c):hw} = \frac{V_{hom}}{V_{ap(c)}} K_{A,ap(r)^-} AP_{hom}^- fcat_{hw} . \quad (4.30)$$

As previously mentioned for the derivation of the fraction unbound in the intact tissue, this final step is critical as $K_{A,ap(r)^-}$ can be inferred from *in vitro* drug-specific input parameters.

- $K_{pr(i):hw}$, *i.e.*, the **interstitial binding protein-to-homogenate water distribution coefficient**, is obtained by applying a charged protein-binding parameterization:

$$K_{pr(i):hw} = \frac{V_{hw}}{V_{pr(i)}} K_{A,pr(i)} (1 - fcat_{hw}) PR_{htf} \quad (4.31)$$

with htf being the homogenate tissue fluid, *i.e.*, the fluid containing tissue water and binding proteins, such that $htf = hw + pr = iw + pr(i) + cw + pr(c) = isf + cw$, with the interstitial fluid including interstitial water and binding proteins and the cellular counterpart including only cellular water (no cellular binding proteins). Moreover, $PR_{htf} = A_{PR(i)} / V_{htf}$ is the total concentration of binding proteins in the homogenate tissue fluid, and $A_{PR(i)}$ is the total amount of binding proteins (originating from the interstitial space). By assuming $K_{A,pr(i)} = K_{A,pr(p)}$ and $PR_{htf} = \frac{V_{hom}}{V_{htf}} PR_{hom}$, from eq 4.31, we obtain

$$K_{pr(i):hw} = \frac{V_{hom}}{V_{pr(i)}} \frac{V_{hw}}{V_{htf}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{(1 - fcat_{hw})}{(1 - fcat_{pw})} \frac{PR_{hom}}{PR_{pla}} \quad (4.32)$$

where

- $K_{A,pr(p)} PR_{pla} (1 - f_{cat_{pw}})$ can be inferred from fuP following eq. 4.20;
- the fractional volume of homogenate water to interstitial fluid $\frac{V_{hw}}{V_{htf}}$ is given by:

$$\frac{V_{hw}}{V_{htf}} = \frac{V_{iw} + V_{cw}}{V_{isf} + V_{cw}} = \frac{\frac{V_{iw}}{V_{isf}} + \frac{V_{cw}}{V_{iw}} \frac{V_{iw}}{V_{isf}}}{1 + \frac{V_{cw}}{V_{iw}} \frac{V_{iw}}{V_{isf}}} = \frac{V_{iw}}{V_{isf}} \frac{1 + \frac{V_{cw}}{V_{iw}}}{1 + \frac{V_{cw}}{V_{iw}} \frac{V_{iw}}{V_{isf}}};$$

If V_{iw}/V_{isf} is approximated to unity as in R&R methods, $V_{hw}/V_{htf} = 1$;

- the ratio PR_{hom}/PR_{pla} depends on the drug type, analogously to PR_{tis}/PR_{pla} .

See Appendix B for a detailed derivation of tissue constituent distribution coefficients related to the homogenate brain tissue, based on the R&R approach.

Derivation of the fraction unbound in the homogenate brain tissue We define the fraction unbound in the homogenate brain tissue fuH_{tis} , specifically referring to the homogenate water concentration, as

$$fuH_{tis} := \frac{C_{hw}}{C_{hom}} = \frac{1}{K_{hom:hw}}.$$

We also define two additional quantities:

$$fuH_{int} := \frac{C_{hw}}{C_{int}} \quad \& \quad fuH_{cel} := \frac{C_{hw}}{C_{cel}}, \quad (4.33)$$

i.e., the fractions unbound in the homogenate-related interstitial and cellular spaces, calculated as the ratios of the drug concentration in the homogenate water to the fictitious interstitial or cellular drug concentrations. Therefore, by replacing the concentration ratios in eq. 4.28 with the newly defined quantities,

$$\frac{1}{fuH_{int}} = \frac{V_{iw}}{V_{int}} K_{iw:hw} + \frac{V_{pr(i)}}{V_{int}} K_{pr(i):hw} \quad (4.34)$$

$$\begin{aligned} \frac{1}{fuH_{cel}} &= \frac{V_{cw}}{V_{cel}} K_{cw:hw} + \frac{V_{pr(c)}}{V_{cel}} K_{pr(c):hw} + \frac{V_{nl(c)}}{V_{cel}} K_{nl(c):hw} + \\ &+ \frac{V_{np(c)}}{V_{cel}} K_{np(c):hw} + \frac{V_{ap(c)}}{V_{cel}} K_{ap(c):hw}, \end{aligned} \quad (4.35)$$

the fraction unbound in the homogenate brain tissue is derived as

$$\boxed{\frac{1}{fuH_{tis}} = \frac{V_{int}}{V_{hom}} \frac{1}{fuH_{int}} + \frac{V_{cel}}{V_{hom}} \frac{1}{fuH_{cel}}} \quad (4.36)$$

EXPERIMENTAL FRACTION UNBOUND IN HOMOGENATE BRAIN TISSUE

In equilibrium dialysis of homogenate brain tissues, the compounds are usually spiked into brain homogenates, dialyzed, and diluted by a dilution factor. The resulting fraction unbound of the diluted brain homogenate $\text{fuH}_{\text{exp(d)}}$ is given by

$$\text{fuH}_{\text{exp(d)}} := \frac{C_{\text{buffer}}}{C_{\text{hom}}}, \quad (4.37)$$

where C_{buffer} is the concentration of the compound in the buffer. Fractions unbound are known to increase with dilution, therefore the fraction unbound $\text{fuH}_{\text{exp(d)}}$ requires scaling to the fraction unbound in undiluted homogenate brain tissue $\text{fuH}_{\text{exp(u)}}$. There are different methods to address this problem, *e.g.*, “equivalent dilution of plasma, linear back-extrapolation based on the extent of dilution or minimizing the overall extent of dilution” (Kalvass et al., 2002). We adopt the approach proposed by Romer et al. (1979) relying on the following nonlinear relationship:

$$\text{fuH}_{\text{exp(u)}} := \frac{C_{\text{hw}}}{C_{\text{hom}}} = \frac{1}{1 + \text{DF} \left(\frac{1}{\text{fuH}_{\text{exp(d)}}} - 1 \right)}, \quad (4.38)$$

where DF is the dilution factor. See the box below for the scaling derivation. The experimental fraction unbound in homogenate brain tissues $\text{fuH}_{\text{exp(u)}}$ corresponds to the fraction of drug that is not bound to proteins or other components in both the interstitial and intracellular spaces of the brain with pH homogenization to pH_{hw} . Thus, $\text{fuH}_{\text{exp(u)}}$ is the experimental quantity we compare to our predictions.

Derivation of undiluted fraction unbound in homogenate brain tissue To derive eq. 4.38, we follow the approach proposed by Romer et al. (1979) and explained by Kalvass et al. (2002).

In general, the relationship between binding component concentration and the fraction unbound is given by

$$\text{fu} = \frac{K_d}{K_d + B}, \quad (4.39)$$

where B is the concentration of nonspecific binding components and K_d is the dissociation constant. Going from the diluted to the undiluted experimental conditions, we obtain two different concentrations of nonspecific binding components, B_d and B_u , respectively, and the corresponding fractions unbound, $\text{fuH}_{\text{exp(d)}}$ and $\text{fuH}_{\text{exp(u)}}$. The dilution factor DF is the ratio of these two nonspecific binding component concentrations, *i.e.*,

$$\text{DF} = \frac{B_u}{B_d}. \quad (4.40)$$

Rearranging eq. 4.39 and relying on the fact that the dissociation constant does not change, we obtain:

$$1 = \frac{fuH_{\text{exp}(u)} B_u (1 - fuH_{\text{exp}(d)})}{fuH_{\text{exp}(d)} B_d (1 - fuH_{\text{exp}(u)})}. \quad (4.41)$$

By substituting DF and deriving $fuH_{\text{exp}(u)}$, we obtain eq. 4.38.

Estimating homogenate tissue pH We assume that the pH of the undiluted homogenate is the same as the pH of the diluted homogenate, pH_{hw} , calculated as the weighted average of the hydrogen ion concentrations of the interstitial, cellular, and buffer spaces as follows:

$$pH_{\text{hw}} = -\log_{10} \left(\frac{(DF - 1) 10^{-pH_{\text{buffer}}} + \frac{V_{\text{cw}}}{V_{\text{tis}}} 10^{-pH_{\text{cw}}} + \frac{V_{\text{iw}}}{V_{\text{tis}}} 10^{-pH_{\text{iw}}}}{DF - 1 + \frac{V_{\text{iw}} + V_{\text{cw}}}{V_{\text{tis}}}} \right), \quad (4.42)$$

where $DF = V_{\text{diluted hom}} / V_{\text{tis}}$. In general, the aqueous phase pH of a compartment is given by

$$pH_{\text{wa}} = -\log_{10} \left(\frac{\text{n. of moles of hydrogen ions in the water space}}{V_{\text{wa}}} \right).$$

The dilution changes the hydrogen ion concentrations by introducing more volume at a specific pH and resulting in a pH that is closer to that of the buffer. Therefore, if DF is large enough, the homogenate pH can be assumed as equal to the buffer pH.

COMPOUND SELECTION AND ASSOCIATED PHYSICOCHEMICAL PARAMETERS

The compound dataset includes 56 small molecules selected to cover a range of physicochemical values relevant to CNS drug disposition. In particular, the selection includes acidic, basic, and neutral compounds, with a distribution that reflects the higher prevalence of basic molecules observed among marketed CNS drugs (Manallack, 2007). Key physicochemical and species-specific pharmacokinetic parameters (*i.e.*, pKa, logP, B:P, fuP) were primarily sourced from GastroPlusTM (Simulations Plus, 2023) (ADMET predictor) for rats and humans, cross-checked against DrugBank and ChEMBL for consistency, and curated literature datasets (Rodgers et al., 2005; Rodgers et al., 2006; Di et al., 2011; Mamada et al., 2021; Rodgers et al., 2007). For example, B:P and fuP values were all derived from GastroPlusTM (Simulations Plus, 2023), except for nortriptyline, for which the human B:P was taken from Mamada et al. (2021), and warfarin, for which the rat B:P was obtained from Sawada et al. (1985). These exceptions were made due to inconsistencies in the experimental inputs resulting in negative association constants to proteins or acidic phospholipids,

i.e., $K_{A,ap-}$ or $K_{A,pr}$, respectively. Rodgers & Rowland (Rodgers et al., 2005; Rodgers et al., 2006) set binding constants to zero in such cases.

In the case of ampholytes, an additional parameter—the tautomeric constant K_t —was set to ∞ for zwitterionic ampholytes and to zero for ordinary ones. For zwitterions, particular care was taken in interpreting pKa values, following the clarifications provided by Zheng et al. (2024). As shown in Table 4.9 and further explained in the box below, where necessary, adjustments were made to correct common misinterpretations in the reported pKa values. That is, pKa values sourced from GastroPlusTM (Simulations Plus, 2023) were reordered to ensure $pK_{a2} > pK_{a1}$, correcting a common inversion seen in public databases such as ChEMBL and DrugBank (Zheng et al., 2024). Additionally, for ampholytes with a predominant ionization state at physiological pH, the drug ionic label—a model input to the Rodgers & Rowland tissue binding model used to indicate whether the compound is predominantly neutral, acidic, basic, or zwitterionic—was updated accordingly. Specifically, this label helps distinguish between the two drug categories: compounds with at least one cationic pKa larger or equal 7 and the others.

Misinterpretation of zwitterionic pKas.

For zwitterionic ampholytes, conventional labeling of pKa values can be misleading. As highlighted by Zheng et al. (2024), anionic (acidic) and cationic (basic) pKa values are frequently mislabeled (*i.e.*, inverted) for zwitterions, even in curated databases such as Drugbank and ChEMBL. For example, one might find an “anionic” pKa listed as smaller than a “cationic” one. These inverted values contradict fundamental electrostatic principles that state that anionic pKas should be larger than cationic ones for ampholytes. Such errors disrupt the relationship between microscopic protonation states and the macroscopic K_A parameters—the only parameters that can be experimentally measured. This misinterpretation introduces systematic biases into predictive models of ionization and partitioning, thereby highlighting the need for rigorous curation and interpretation of input pKa data.

Table 4.8: **Physicochemical and pharmacokinetic properties of selected CNS compounds** for 56 drugs, sourced from GastroPlus™ software ([Simulations Plus, 2023](#)) and the literature.

Drug	Abbr.	MW	Anionic pKa		Cationic pKa		Ionic label	logP	B:P		fuP	
			pKa _{ani1}	pKa _{ani2}	pKa _{cat1}	pKa _{cat2}			Human	Rat	Human	Rat
Lidocaine	LID	0.2343	11.978		7.871		Ordinary ampholyte	2.267	0.8420	1.1686	0.5806	0.5486
Haloperidol	HAL	0.3759			8.230		Base	3.907	0.8652	1.3444	0.1097	0.1558
Selegiline	SEL	0.1873			7.782		Base	2.598	0.9513	1.2457	0.3147	0.4581
Paracetamol	PAR	0.1512	10.060	12.17			Diprotic acid	0.449	1.0106	0.9647	0.6872	0.4431
Caffeine	CAF	0.1942			1.429		Base	1.290	1.0766	0.8013	0.8469	0.6793
Clozapine	CLO	0.3268			7.673	5.919	Diprotic base	3.637	0.8390	1.4830	0.0865	0.0887
Diazepam	DIA	0.2847			3.163		Base	2.808	0.7707	1.0527	0.0906	0.0964
Thiopental	THI	0.2423	5.266	10.10			Diprotic acid	2.735	0.7823	0.6467	0.1162	0.1930
Spiperone	SPI	0.3955			8.040	1.876	Diprotic base	2.782	0.7823	1.3700	0.1483	0.1724
Raclopride	RAC	0.3472	9.174		6.010		Zwitterion	3.218	1.0920	0.8452	0.0889	0.0973
WAY100635	WAY	0.4226			6.157	2.558	Diprotic base	3.561	0.6813	0.8996	0.0487	0.1039
MDL 100907	MDL	0.3735			8.620		Base	3.590	0.8141	1.2440	0.1283	0.2566
Diprenorphine	DIP	0.4256	10.420		9.630		Ordinary ampholyte	3.694	0.9862	1.0190	0.1685	0.3102
Fallypride	FAL	0.3645	11.450		8.609		Ordinary ampholyte	3.093	0.7586	1.0420	0.2654	0.4547
Flumazenil	FLU	0.3033			1.431		Base	1.390	0.7707	0.8538	0.2443	0.2915
Bupropion	BUP	0.2397			8.600		Base	3.191	1.0540	1.2770	0.3652	0.2545
Fluoxetine	FLX	0.3093			9.666		Base	4.395	0.8778	1.4230	0.1470	0.0878
Midazolam	MID	0.3258			5.28	1.193	Diprotic base	2.730	0.7804	0.9821	0.0640	0.0625
Lamotrigine	LAM	0.2561					neutral	1.870	1.1340	1.2290	0.1518	0.1845
Rofecoxib	ROF	0.3144					neutral	-0.290	0.8201	1.1260	0.0729	0.0738

Drug	Abbr.	MW	Anionic pKa		Cationic pKa		Ionic label	logP	B:P		fuP	
			pKa _{ani1}	pKa _{ani2}	pKa _{cat1}	pKa _{cat2}			Human	Rat	Human	Rat
Primidone	PRI	0.2183					neutral	0.512	0.8493	0.8925	0.5654	0.4210
Phenytoin	PHE	0.2523	8.146	11.64			Diprotic acid	2.157	0.7405	0.9986	0.1531	0.0922
Amantadine	AMA	0.1513			10.040		Base	2.263	1.4080	1.2000	0.7306	0.5533
Aprepitant	APR	0.5344	9.033	11.14	3.853	0.333	Ordinary ampholyte	4.041	0.7281	0.8789	0.0604	0.0722
Aripiprazole	ARI	0.4484			6.710		Base	4.9	0.7186	1.1040	0.0424	0.0580
Azelastine	AZE	0.3819			8.538	0.855	Diprotic base	3.781	0.8266	1.3260	0.1511	0.0881
Bupivacaine	BUV	0.2880			8.130		Base	3.410	0.7907	1.0620	0.1369	0.2146
Chlorpromazine	CHLZ	0.3189			8.934	1.807	Diprotic base	5.369	0.8274	1.2920	0.0618	0.1352
Chlorpropamide	CHLM	0.2767	4.944	10.79			Diprotic acid	2.287	0.6886	0.6941	0.0997	0.0577
Citalopram	CIT	0.3244			9.360		Base	4.1	0.8451	1.2450	0.2320	0.3049
Clinafloxacin	CLI	0.3658	8.894		5.840		Zwitterion	-0.245	1.3240	0.8755	0.4872	0.3485
Clomipramine	CLO	0.3149			9.200		Base	5.190	0.8650	1.2620	0.0674	0.0980
Desipramine	DES	0.2664			10.400		Base	4.9	0.9283	1.3210	0.1027	0.1357
Diphenhydramine	DIP	0.2554			8.870		Base	3.650	0.8573	1.2380	0.2826	0.2974
Entacapone	ENT	0.3053	6.090				acid	2.5	0.7097	0.7342	0.0586	0.1675
Fluvoxamine	FLU	0.3183			8.860		Diprotic base	2.8	1	1.1560	0.2708	0.2447
Ibutilide	IBU	0.3846			8.520		Base	4.720	0.9790	0.9503	0.2024	0.3466
Indomethacin	IND	0.3578	3.754				Acid	4.089	0.6755	0.6822	0.0403	0.0435
Metoclopramide	MET	0.2998	11.900		9.053		Ordinary ampholyte	2.281	0.9917	1.1460	0.4408	0.4588
Nimodipine	NIM	0.4184	11.150		-0.989		Ordinary ampholyte	3.2	0.7193	0.6444	0.0875	0.0889
Nomifensine	NOM	0.2383			8.152		Diprotic base	2.940	1.1500	1.5790	0.2593	0.3687
Nortriptyline	NOR	0.2634			9.774		Base	4.551	1.5000	1.2510	0.0460	0.1104
Paliperidone	PAL	0.4265			7.916	1.643	Diprotic base	2.254	0.8836	1.1380	0.2187	0.2026

Drug	Abbr.	MW	Anionic pKa		Cationic pKa		Ionic label	logP	B:P		fuP	
			pKa _{ani1}	pKa _{ani2}	pKa _{cat1}	pKa _{cat2}			Human	Rat	Human	Rat
Paroxetine	PAX	0.3294			9.604		Base	3.430	0.9603	1.4620	0.1470	0.1900
Reboxetine	REB	0.3134			7.679		Base	3.003	0.7931	1.1860	0.1400	0.2170
Rifampin	RIF	0.8230	7.757		6.711		Zwitterion	2.950	0.7572	1.2170	0.1885	0.1145
Riluzole	RIL	0.2342			4.574		Base	3.1	1.1830	0.8911	0.1344	0.0726
Rivastigmine	RIV	0.2503			8.812		Base	2.8	0.8518	1.2370	0.5915	0.5809
Solifenacin	SOL	0.3625			8.575		Base	4.227	0.7751	1.3300	0.1079	0.1591
Talinolol	TAL	0.3635	10.880	11.55	9.160		Ordinary ampholyte	3.3	0.7792	1.2750	0.4042	0.2037
Telmisartan	TEL	0.5146	5.860				Acid	6.660	0.6831	0.8712	0.0394	0.0306
Terodiline	TER	0.2815			10.250		Base	5.430	0.8875	1.1560	0.1887	0.0733
Tolcapone	TOL	0.2732	4.653	10.32			Diprotic acid	3.639	0.7356	0.7643	0.0394	0.0635
Trazodone	TRA	0.3719			7.115	0.971	Diprotic base	2.680	0.7946	1.1130	0.0598	0.0951
UK-240455a	UK	0.3682	8.432	10.22			Acid	0.270	0.7873	0.9182	0.5063	0.5386
Warfarin	WAR	0.3083	5.090				Acid	3.282	0.6788	0.5800	0.0534	0.0499

Table 4.9: Details of ionic labeling for the ampholytic compounds in the dataset. This table presents the ampholytic drugs in the dataset, with a focus on justifying their assigned ionic labels. (*) The predominant state column indicates the major ionization state at physiological pH (7.1–7.4). Zwitterions whose pKa window does not span this pH range were labeled as acids or bases according to their predominant state at physiological pH. Ordinary ampholytes that are predominantly neutral or weakly anionic at this pH were classified as neutral compounds; all others retained their original label as ordinary ampholytes.

Drug	Anionic pKa (pKa ₂)	Cationic pKa (pKa ₁)	Ampholyte	Predominant state*	Ionic label
Rifampin	7.757	6.711	Zwitterionic	Zwitterionic	Zwitterion
Clinafloxacin	8.894	5.838	Zwitterionic	Zwitterionic	Zwitterion
Raclopride	9.174	6.010	Zwitterionic	Zwitterionic	Zwitterion
Telmisartan	5.860	3.620	Zwitterionic	Anionic	Acid
Ibutilide	9.993	8.520	Zwitterionic	Cationic	Base
Diprenorphine	10.420	9.630	Ordinary	Cationic	Ordinary ampholyte
Lidocaine	11.978	7.871	Ordinary	Cationic	Ordinary ampholyte
Fallypride	11.450	8.609	Ordinary	Cationic	Ordinary ampholyte
Metoclopramide	11.900	9.053	Ordinary	Cationic	Ordinary ampholyte
Talinolol	[10.880, 11.550]	9.160	Ordinary	Cationic	Ordinary ampholyte
Bupivacaine	11.770	7.623	Ordinary	Cationic	Ordinary ampholyte
Aripiprazole	12.090	6.710	Ordinary	Cationic	Ordinary ampholyte
Aprepitant	[9.033, 11.140]	[0.333, 3.850]	Ordinary	Neutral to weakly anionic	Neutral
Nimodipine	11.150	-0.988	Ordinary	Neutral to weakly anionic	Neutral

EXPERIMENTAL DATASETS FROM BRAIN HOMOGENATE ASSAYS

The experimental data used for evaluating predictions of fraction unbound in intact and homogenate brain tissue, fuT and fuH_{tis} , was obtained via equilibrium dialysis in human and rat brain homogenates. These experimental values, $fuH_{exp(u)}$, are reported in Table 4.10 for our selected set of small molecules, along with their original literature sources. Figure 4.7 compares the experimental fuH values in rat and human brain homogenates for 52 compounds, showing a strong correlation between the two species, *e.g.*, $\sim 78.8\%$ within 2-fold error. This cross-species consistency supports the species-independence assumption, further corroborated by Di et al. (2011).

Although the dataset considered here includes brain homogenate data for both rat and human tissue, such human data are generally scarce in the broader literature compared to rat data. This scarcity stems from the limited availability of fresh human brain tissue and ethical constraints on its collection. Consequently, available human data typically derives from post-mortem samples, which may not fully preserve physiological conditions, potentially introducing

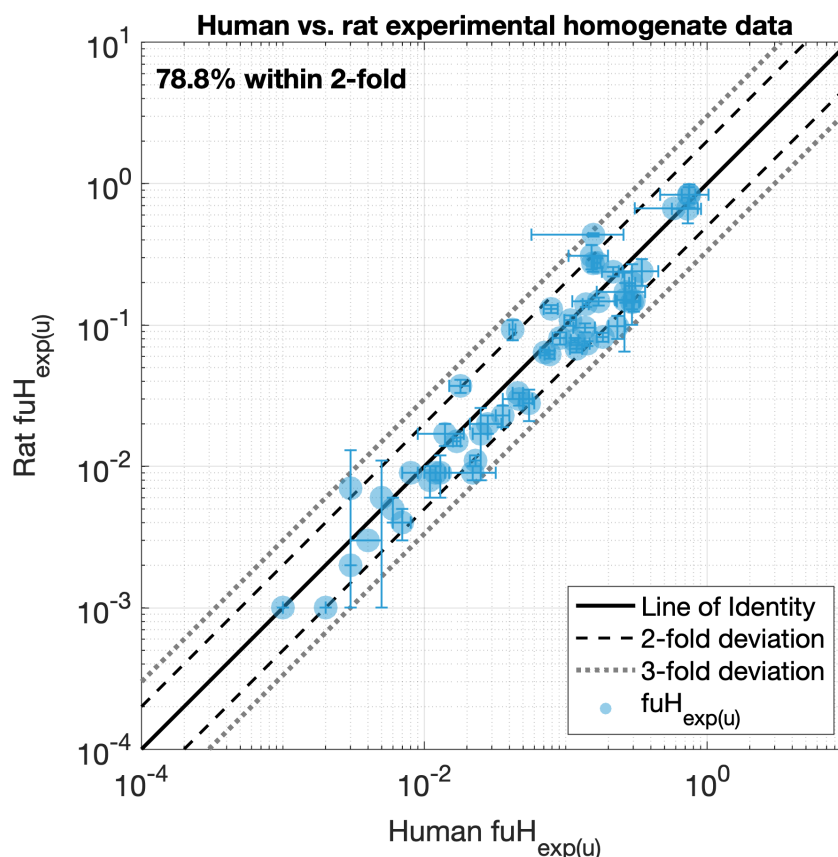


Figure 4.7: Data of human and rat brain fraction unbound for $n = 52$ compounds. Axes represent $\log_{10}$ -scale values of experimental fraction unbound in rat and human brain tissue, respectively.

artifacts that affect binding characteristics (*e.g.*, prolonged storage may exacerbate cellular lysis or protein denaturation, skewing binding measurements). Nonetheless, two key studies have provided valuable human brain homogenate data. [Summerfield et al. \(2008\)](#) used pre-frozen post-mortem human brain tissue to evaluate 18 compounds via equilibrium dialysis. Their methodology, optimized for limited sample availability, prioritizing throughput and consistency, albeit with potential limitations due to freeze-thaw effects. All human fraction unbound measurements were derived from homogenates, aligning with our focus on method-specific predictions (as confirmed in personal correspondence with Dr. Summerfield). Similarly, [Di et al. \(2011\)](#) performed broader cross-species analyses, including human homogenate samples, to assess the validity of species-independence assumptions for fuH across seven species and strains and large compound sets. In our evaluation, we compared our predicted fuH_{tis} values directly against the human data reported in these two studies ([Summerfield et al., 2008](#); [Di et al., 2011](#)). While acknowledging the inherent limitations of these datasets, this approach prioritizes direct comparison with available human data. For rat predictions, we leveraged extensive and physiologically representative datasets available. This strategy ensures that model evaluation is grounded in the most robust and context-appropriate

data available for each species. Given both the scarcity of human homogenate data and the central role of rodent-derived data in preclinical workflows, an alternative evaluation strategy for our human predictions could have involved assessing generalizability through comparison with rat data, grounded in the species-independence assumption. Given our goal of evaluating fuH predictions and not further assessing species-independence of fraction unbound, we chose to provide a conservative and direct assessment of model performance in predicting human brain binding.

Table 4.10: Experimental values of the fraction unbound in undiluted brain homogenates $fuH_{exp(u)}$ for selected CNS compounds. Data—mean values and standard deviations (SD), where available—were obtained via equilibrium dialysis in human and rat brain homogenates, as reported in the reported references. (a) [Di et al. \(2011\)](#), (b) [Summerfield et al. \(2008\)](#), (c) [Culot et al. \(2013\)](#), (d) [Longhi et al. \(2011\)](#), (e) [Fridén et al. \(2010\)](#)

Drug	Abbr.	Human			Rat		
		$fuH_{exp(u)}$	(SD)	Ref.	$fuH_{exp(u)}$	(SD)	Ref.
Lidocaine	LID	0.303	0.034	(a)	0.148	0.024	(a)
Haloperidol	HAL	0.023	0.002	(b)	0.011	0.001	(b)
Selegiline	SEL	0.142	0.006	(b)	0.074	0.003	(b)
Paracetamol	PAR	0.747	0.28	(b)	0.833	0.152	(b)
Caffeine	CAF	-	-	-	0.500	0.118	(c)
Clozapine	CLO	-	-	-	0.0094	0.0003	(c)
Diazepam	DIA	-	-	-	0.036	0.005	(d)
Thiopental	THI	-	-	-	0.160	0.0087	(e)
Spiroperone	SPI	0.018	0.003	(b)	0.037	0.004	(b)
Raclopride	RAC	0.079	0.009	(b)	0.130	0.008	(b)
WAY100635	WAY	0.139	0.007	(b)	0.141	0.007	(b)
MDL 100907	MDL	0.108	0.010	(b)	0.108	0.009	(b)
Diprenorphine	DIP	0.152	0.047	(b)	0.308	0.061	(b)
Fallypride	FAL	0.216	0.036	(b)	0.239	0.019	(b)
Flumazenil	FLU	0.744	0.080	(b)	0.835	0.106	(b)
Bupropion	BUP	0.265	0.099	(b)	0.171	0.027	(b)
Fluoxetine	FLX	0.007	0.001	(b)	0.004	0.001	(b)
Midazolam	MID	0.036	0.004	(b)	0.023	0.004	(b)
Lamotrigine	LAM	0.156	0.004	(b)	0.273	0.034	(b)
Rofecoxib	ROF	0.167	0.009	(b)	0.275	0.022	(b)
Primidone	PRI	0.733	0.171	(b)	0.662	0.137	(b)
Phenytoin	PHE	0.183	0.017	(b)	0.082	0.006	(b)
Amantadine	AMA	0.171	0.06	(a)	0.147	0.010	(a)
Aprepitant	APR	0.001	0	(a)	0.001	0	(a)
Aripiprazole	ARI	0.002	0	(a)	0.001	0	(a)
Azelastine	AZE	0.013	0.002	(a)	0.009	0.003	(a)
Bupivacaine	BUV	0.232	0.032	(a)	0.098	0.018	(a)
Chlorpromazine	CHLZ	0.003	0	(a)	0.002	0	(a)
Chlorpropamide	CHLM	0.583	0.274	(a)	0.668	0	(a)
Citalopram	CIT	0.047	0.011	(a)	0.030	0.003	(a)
Clinfloxacin	CLI	0.157	0.100	(a)	0.435	0.012	(a)
Clomipramine	CLO	0.004	0.001	(a)	0.003	0	(a)

Desipramine	DES	0.011	0	(a)	0.008	0.002	(a)
Diphenhydramine	DIP	0.042	0.002	(a)	0.093	0.015	(e)
Entacapone	ENT	0.025	0.003	(a)	0.017	0.009	(a)
Fluvoxamine	FLU	0.022	0.010	(a)	0.009	0.001	(a)
Ibutilide	IBU	0.091	0.010	(a)	0.081	0.008	(a)
Indomethacin	IND	0.055	0.005	(a)	0.028	0.007	(a)
Metoclopramide	MET	0.295	0.036	(a)	0.145	0.045	(a)
Nimodipine	NIM	0.008	0.001	(a)	0.009	0	(a)
Nomifensine	NOM	0.071	0.005	(a)	0.064	0.003	(a)
Nortriptyline	NOR	0.006	0	(a)	0.005	0.001	(a)
Paliperidone	PAL	0.116	0.007	(a)	0.078	0.003	(a)
Paroxetine	PAX	0.005	0	(a)	0.006	0.005	(a)
Reboxetine	REB	0.046	0.004	(a)	0.033	0	(a)
Rifampin	RIF	0.119	0.012	(a)	0.068	0.004	(a)
Riluzole	RIL	0.012	0.002	(a)	0.009	0.001	(a)
Rivastigmine	RIV	0.345	0.108	(a)	0.241	0.052	(a)
Solifenacin	SOL	0.017	0.001	(a)	0.015	0.001	(a)
Talinolol	TAL	0.137	0.024	(a)	0.096	0.007	(a)
Telmisartan	TEL	0.014	0.005	(a)	0.017	0.003	(a)
Terodiline	TER	0.028	0.007	(a)	0.020	0.003	(a)
Tolcapone	TOL	0.003	0	(a)	0.007	0.006	(a)
Trazodone	TRA	0.077	0.006	(a)	0.062	0.004	(a)
UK-240455a	UK	0.260	0.037	(a)	0.150	0.085	(a)
Warfarin	WAR	0.295	0.015	(a)	0.199	0.070	(a)

CONCLUSIONS

This thesis addressed two open questions in the development of CNS-targeted therapies: (i) how to mechanistically characterize aSyn aggregation under physiologically relevant *in vitro* conditions, and (ii) how to improve the comparison between mechanistic predictions of unbound drug fraction and experimental measurements in brain homogenate. Although the two projects operate at different biological and methodological levels, both aim to enhance the reliability and interpretability of models used in QSP and PBPK frameworks for neurodegenerative diseases. The following sections discuss the contributions of each project individually, highlighting their results and limitations and elaborating on potential directions for future work.

MODELING ASYN AGGREGATION

Following a comprehensive review of the literature, we developed a mechanistic model of aSyn aggregation that integrates both protein-only and lipid-mediated pathways. Rooted in chemical kinetics and calibrated against diverse *in vitro* experimental data, the model captures essential features of aggregation dynamics, including oligomer formation, fibril growth, and lipid interactions, under near-physiological conditions, and provides mechanistic insights into the dynamics of short- and long-lived oligomers. It can also be used as a virtual lab to simulate the effects of PD risk factors or candidate drugs, enabling mechanistic exploration of pharmacological perturbations. In this context, it can serve as a modular component in QSP/PBPK platforms for studying disease progression and therapeutic intervention in synucleinopathies, particularly Parkinson's disease.

Although our model provides a detailed mechanistic description of aSyn aggregation under controlled *in vitro* conditions, its translatability to *in vivo* systems remains a limitation. As thoroughly discussed in Chapter 3, the aggregation kinetics and the relative contribution of individual microscopic steps—such as secondary nucleation—are highly sensitive to solution conditions, including pH, lipid composition, and seeding levels, all of which differ between experimental setups and physiological environments (Buell et al., 2014; Gaspar et al., 2017; Dear et al., 2024). Nonetheless, several model components are supported by data that fall within physiologically relevant ranges (Rabe et al., 2013; Wilhelm et al., 2014; Dear et al., 2024; Xu et al., 2024; Horne et al., 2023). These observations suggest that the core mechanisms identified—oligomeric conversion, fibril elongation, and lipid-driven secondary nucleation—likely retain

their relevance beyond *in vitro* systems. A key caveat, however, is the substantial difference between the rapid aggregation observed *in vitro* (hours to days) and the decade-long progression of Parkinson's disease (Bakshi et al., 2019). This timescale discrepancy may be only apparent. As highlighted by Huang et al. (2025), neurodegenerative disorders such as PD exhibit “a characteristic long, slow initial phase followed by rapid progression” at the cellular and tissue levels. The slow phase may correspond to a gradual buildup of the diseased cellular state shaped by the stochastic nature of nucleation events—consistent with the sporadic nature of PD—and by cellular thresholds for functional impairment (e.g., disruption of the degradation machinery) (Meisl, 2024). In contrast, intercellular propagation of pathological states unfolds more rapidly, driven either by prion-like spread of pathological aSyn, or gradual disruption of cellular homeostasis, or selective vulnerability of neuronal circuits (Braak et al., 2002; Brundin et al., 2010; Poewe et al., 2017). Together, these processes result in decade-long disease onset and development. Fast protein aggregation kinetics occurring *in vivo*, limited by the slow buildup of cellular burden, could fit naturally within this framework. From a modeling perspective, frameworks based on slow-fast dynamical systems and multiscale approaches, e.g., (Tora et al., 2025), could combine fast local kinetics at the molecular level, rate-limiting cell-autonomous pathological processes, and (relatively) rapid intercellular and regional propagation. A further limitation of our current work is that it does not yet incorporate aSyn synthesis or degradation pathways, limiting its applicability to the whole-cell level. Moreover, although lipid-mediated aggregation is included, the model does not account for additional biological processes such as oxidative stress and neuroinflammation. These limitations constrain its immediate translatability to *in vivo* systems but also help define clear priorities for future development.

Future directions The model is flexible enough to accommodate additional biological layers, including intracellular and multicellular processes. Given the central role of impaired degradation mechanisms in Parkinson's disease (as discussed in Chapter 2), a logical next step is to incorporate synthesis and clearance pathways. This could begin with size-dependent first-order degradation reactions (Thompson et al., 2021; Pigolotti et al., 2013) and gradually evolve into more detailed representations of proteasomal and lysosomal pathways. Eventually, more detailed mechanistic models—drawing on existing work by Raichur et al. (2006), Sneppen et al. (2009), and Ouzounoglou et al. (2014)—could support simulation of feedback loops between aggregation and proteostasis collapse, aligning the model more closely with disease-relevant scenarios. A crucial challenge will be determining the appropriate level of complexity. Modeling choices should be informed by data availability, prioritizing variables with observable readouts. Astrocyte-mediated degradation of aSyn, for instance, represents a promising hypothesis (Yang et al., 2022; Tsunemi et al., 2020) that could be explored as more data—suitable for modeling—

emerge. Once degradation mechanisms are incorporated, the model would be well-positioned for sensitivity analysis not only across aggregation pathways but also within degradation modules. This analysis would pinpoint vulnerable reactions in proteasomal and lysosomal pathways and identify mechanisms as potential pharmacological targets, thus supporting both mechanistic understanding and target prioritization. Future work could also incorporate the mechanisms of action of specific therapeutic candidates, assessing their potential to modulate toxic species formation.

A natural extension is the development of cell-level models. Our central working assumption—supported by recent studies (Meisl et al., 2022; Sinnige, 2022)—is that the molecular mechanisms underlying *in vitro* aSyn aggregation assays remain relevant in a cell-level context and *in vivo*. Accordingly, when adapting the model to simulate aSyn turnover in cellular systems, the core mechanistic structure can be retained, while kinetic rates are adjusted based on data from cell-based experiments, ideally using human iPSC-derived neurons. Such models would enable more physiologically relevant simulations of intracellular aSyn turnover and allow direct testing of *in vitro*–*in vivo* translatability hypothesis. This would, in turn, establish a productive feedback loop between *in silico* modeling and experimental validation. Building on a cellular framework, the model could be adapted to simulate aSyn dynamics across different cell types, such as neurons and astrocytes (Sorrentino et al., 2019), using a shared mechanistic backbone. Exploring how aggregation and degradation kinetics vary between these cell types could yield insights into the selective vulnerability observed in PD. To further extend the spatial dimension, the intracellular aggregation module could be coupled with a simplified representation of extracellular propagation, such as the infective model of prion-like spreading proposed by Zhdanov (2023). This would allow simulation of pathogenic intercellular transmission between cells and, possibly, across brain regions.

To better reflect the multifactorial nature of neurodegeneration, future efforts should also focus on integrating aSyn aggregation with other relevant biological processes, such as oxidative stress, neuroinflammation, and broader metabolic dysfunctions (Bloomingdale et al., 2022b). As discussed in Chapter 2 and in Bakshi et al. (2019), multiple mathematical models exist that address these interactions individually and others that integrate multiple submodules related to PD relevant pathways and share aSyn as a critical component (Raichur et al., 2006; Menon et al., 2024). In our literature review, we pointed out a specific modeling opportunity: relatively few models focusing on multiple biological processes incorporate a detailed representation of aSyn aggregation kinetics. In this direction, further extensions could link our aSyn aggregation model to oxidative stress, *e.g.*, based on prior work by Cloutier et al. (2012b) and Cloutier et al. (2012a). Incorporating reactive oxygen species would allow simulation of the feedback loop between redox imbalance and aSyn misfolding. Even a minimal ROS module could help generate testable

hypotheses about oxidative stress as a driver or consequence of aggregation. In parallel, neuroinflammation could be included via a mechanistic representation of microglial activation and cytokine signaling. This would reflect the increasingly recognized role of neuroinflammation in PD pathophysiology (Ransohoff, 2016; Bloomingdale et al., 2022b). Finally, although current understanding of the physiological functions of aSyn remain limited as well as related data, modeling them alongside aSyn pathological behavior could help clarify the transition from controlled turnover to de-regulated toxic accumulation.

A key long-term goal is to integrate our model of intracellular aSyn aggregation into broader QSP-PBPK platforms for neurodegenerative disease, incorporating multicompartmental brain structures and clinically accessible readouts, such as plasma and CSF aSyn concentrations. While some recent efforts have modeled specific biological processes related to protein aggregates into minimal PBPK or QSP models (Geerts et al., 2023b; Geerts et al., 2023a; Ivanova et al., 2024), they often rely on coarse-grained aggregation modules or on kinetic parameters derived from heterogeneous and sometimes non-physiological scenarios. In contrast, our model offers a mechanistically detailed, unified, and modular representation of aSyn aggregation, calibrated against near-physiological *in vitro* data. By preserving the underlying kinetics and enabling controlled extensions (*e.g.*, the inclusion of synthesis and degradation mechanisms), it provides a consistent and adaptable foundation for integration into QSP-PBPK platforms targeting synucleinopathies. These potential developments are not intended to increase complexity arbitrarily, but rather to progressively align the model structure with biologically relevant processes. Each layer of complexity should be supported by appropriate experimental data and guided by well-defined modeling goals.

MECHANISTIC PREDICTIONS OF UNBOUND DRUG FRACTION IN BRAIN HOMOGENATES

This thesis addressed a methodological gap in PBPK modeling associated with brain homogenate assays, which are widely used in early-stage CNS drug development—namely, the mismatch between mechanistic predictions of the unbound drug fraction in brain tissue (f_uT) and experimental measurements from homogenates ($f_{uH_{exp(u)}}$). To bridge this gap, we proposed a new homogenate-specific metric, $f_{uH_{tis}}$, which adapts the Rodgers & Rowland framework to reflect the experimental conditions of equilibrium dialysis in brain homogenate assays, *i.e.*, disruption of cell membranes and of pH compartmentalization. This approach preserves the tissue constituents and binding interactions of intact brain but evaluates them within a unified pH environment. Such a mechanistic prediction of the unbound drug fraction in brain homogenates offers potentially improved alignment with experimental data and may also serve as a diagnostic tool for assessing the reliability of

physiological input parameters—particularly assumptions about brain lipid composition. In doing so, it supports both homogenate specific predictions (fuH_{tis}) and intact tissue-level estimates (fuT). Indeed, insights gained from improving fuH_{tis} can be leveraged to inform prediction of fractions unbound in interstitial and cellular compartments of the intact brain. While the proposed metric shows promise, several limitations should be acknowledged. First, fuH_{tis} was evaluated using a relatively limited dataset, which may not fully capture the diversity of compound classes. Second, key physiological parameters—particularly those related to brain lipid composition—are based on literature-derived assumptions that may not generalize across contexts or species. Finally, the current implementation relies on specific assumptions from the Rodgers & Rowland method, which is well-established but not the only available framework.

Future directions This preliminary analysis lays the foundation for multiple follow-up studies and future directions. A first step will be to improve fuH_{tis} evaluation by using a broader range of experimental brain homogenate data. This would not only improve the reliability of the metric but also test its generalizability across compound classes and species. Moreover, beyond the Rodgers & Rowland framework, future work should assess the performance of fuH_{tis} within other tissue composition-based models, each of which relies on different assumptions and approximations. Alternative models account for lysosomal trapping (Schmitt et al., 2021), intracellular protein binding (Schmitt, 2008), or different parameterizations of tissue constituent distribution coefficients. A recent R&R-based extension by Poulin et al. (2024), for instance, accentuates the influence of blood-to-plasma ratio, especially for neutral compounds. These models could be systematically tested using the same evaluation dataset employed in this thesis to determine which model structures provide the best agreement under homogenate-specific conditions. Additionally, as discussed in Chapter 4, residual blood content—a factor often overlooked—could be incorporated into future refinements of both fuH_{tis} and fuT predictions.

A particularly informative direction for future validation would involve integrating additional experimental endpoints, such as brain slice or *in vivo* microdialysis data, to test the alignment between fuT , fuH_{tis} , and their corresponding experimental counterparts. These comparisons would help confirm that fuH_{tis} performs better against homogenate data, while fuT aligns more closely with data from intact systems. If such alignment is not observed, it may indicate that central physiological assumptions—particularly those regarding lipid composition—require revision. In fact, one of the more ambitious yet testable hypotheses raised by our preliminary analysis is that fuH_{tis} could serve as a sentinel probe for brain lipid composition. Our results suggest that acidic phospholipid content has a strong impact on predictions, especially for highly basic compounds. If fuH_{tis} fails to outperform fuT in predicting homogenate data across a larger dataset, this could highlight a mismatch

between assumed and actual brain lipid profiles. In that case, fuH_{tis} could be used diagnostically to guide better characterization of brain lipidic fractional volumes—physiological input parameters in rodent and human PBPK brain models. This would foster a more iterative interaction between *in silico* modeling and experimental measurement, improving physiological foundation across preclinical and clinical species.

Despite lacking mechanistic justification, experimental measurements of $\text{fuH}_{\text{exp(u)}}$ are commonly used to inform PBPK frameworks—such as the four-compartment brain–CSF model introduced by [Gaohua et al. \(2016\)](#) and later refined by [Verscheijden et al. \(2019\)](#) (see Figure 4.6)—often serving as surrogates for fuT . As discussed in Chapter 4, this practice may introduce bias, particularly when modeling new or poorly characterized compounds. In this context, improving our model performance in predicting homogenate binding also enhances the reliability of predictions for unbound concentrations in intact brain tissue. These predictions can then be integrated into PBPK models to support simulations of brain exposure by providing estimates of unbound drug concentration in interstitial and cellular spaces. Robust *a priori* predictions of fraction unbound in brain tissues thus become essential, offering a mechanistic bridge to estimate drug exposure at the site of action in the brain.

AI-INTEGRATED MECHANISTIC MODELING: OUTLOOK FOR THIS WORK

Artificial intelligence (AI) is increasingly expected to play a key role within quantitative systems pharmacology (QSP) frameworks. For example, it can complement QSP by addressing methodological bottlenecks such as parameter estimation, model structure discovery, and literature mining ([Ribba, 2023](#); [Androulakis et al., 2025](#)). While systematic integration of these approaches into modeling workflows remains limited, they offer concrete potential to support and extend the mechanistic models developed and analyzed in this thesis. In modeling aSyn aggregation, where defining key mechanistic steps (*e.g.*, nucleation, elongation, lipid-driven processes) required extensive manual curation, generative AI approaches—including large language models—could help automate model construction by suggesting candidate reactions or alternative kinetic schemes extracted directly from literature or experimental datasets. These suggestions could then be prioritized for testing. As a complementary approach, neural symbolic regression ([Biggio et al., 2021](#)) can infer interpretable mechanistic equations from experimental input–output pairs, even under partial observability, making it well suited to propose or refine ODE formulations for aSyn aggregation and related biological processes (*e.g.*, ROS production, inflammation, and dysfunctional degradation). Surrogate models could further complement this workflow by accelerating large-scale simulations, particularly for sensitivity analyses or virtual population generation. In predicting unbound drug fractions, where uncertainties in brain lipid compo-

sition strongly influenced outcomes, hybrid mechanistic–AI approaches could help define plausible parameter ranges by learning from existing experimental data. These AI-generated ranges could then guide simulations of multiple scenarios across compounds and species, reducing the burden of exhaustive manual parameter exploration and enabling faster, more systematic testing of model predictions. Overall, integrating AI with mechanistic modeling can streamline model construction, parameterization, and exploration, ultimately supporting the deployment of these models within PBPK-QSP platforms for neurodegeneration.

BRIDGING MECHANISTIC MODELING OF INTRACELLULAR PROCESSES AND UNBOUND DRUG EXPOSURE IN THE BRAIN

A promising long-term goal is to connect the two modeling projects presented in this thesis within a single PBPK/QSP framework for neurodegeneration, with a focus on Parkinson’s disease and other synucleinopathies. On one hand, refined predictions of unbound drug fractions in brain interstitial and cellular spaces would support more reliable simulations of intracellular exposure of a pharmacologically active drug. On the other hand, this information could feed directly into a mechanistic module of aSyn aggregation kinetics, enabling simulation of intracellular target engagement and pharmacodynamic responses for candidate therapies that target reactions of aSyn aggregation cascade.

Together, these efforts underscore the importance of aligning mechanistic models with the biology they aim to represent—not only in terms of molecular processes but also in how predictions are evaluated against available data. Ultimately, modeling frameworks that are both biologically grounded and experimentally testable will be essential for supporting CNS-focused translational pharmacology.

APPENDIX

APPENDIX: A MECHANISTIC MODEL OF PURE AND LIPIDIC α -SYNUCLEIN AGGREGATION

A.1 AVERAGE FIBRIL LENGTH OF MODEL CALIBRATION SCENARIOS

The average fibril length formed *in vitro* varies significantly depending on experimental conditions, including protein concentration, solution composition (*e.g.*, pH, ionic strength), and external factors. Our model reproduces these trends across the three *in vitro* aggregation scenarios used for calibration in Figure 2 (main text), resulting in average fibril lengths consistent with experimental data (see Figure A.1, bottom rows).

- In the lipid vesicle scenario (Galvagnion et al., 2015; Dear et al., 2024), our model predicts an average fibril length up to 300 – 400 monomers per fibril under intermediate lipid-to-aSyn ratios, aligning with experimental observations by Galvagnion et al. (2015). In particular, their study reported fibrils with an average length of ~ 375 nm, corresponding to ~ 400 monomers per fibril, for an initial lipid-to-aSyn ratio of 3, given by 600 μ M DMPS lipids to 200 μ M monomers; under the same conditions, our model predicts 413 monomers per fibril.

Note that the conversion from fibril length to monomer count relies on a volume-based method used in prior literature (Galvagnion et al., 2015; Buell et al., 2014):

$$\text{monomer count per fibril} = \pi \left(\frac{\text{fibril thickness}}{2} \right)^2 \cdot \rho \cdot \frac{N_A}{M_{\text{aSyn}}} \cdot \text{fibril length}$$

Assuming a cylindrical geometry, this approach considers a fibril thickness of 5 nm (though alternative thickness values are used in other studies), monomer density of $\rho = 1.35$ g/cm³, aSyn molecular mass ($M_{\text{aSyn}} = 14.5$ kDa), and Avogadro's number N_A .

- Under constant agitation at neutral pH (Iljina et al., 2016), our model predicts that fibril length increases significantly with increasing initial monomer concentrations, ranging from hundreds to thousands of monomers per fibril. This is consistent with prior literature where, under similar conditions, long fibrils were observed varying substantially depending on the initial monomer concentration (Van Raaij et al., 2008). For example, Mao et al. (2022) observed fibrils ~ 1000 nm long, corresponding to 2000 - 4000 monomers per fibril, depending on the assumed fibril thickness (5 – 10 nm), formed under agitation at 280 μ M initial monomer concentration.

- In the case of mildly acidic pH and quiescent conditions with seeding, [Gaspar et al. \(2017\)](#) did not directly quantify fibril length in terms of monomer count, but they reported that secondary nucleation dominates at mildly acidic pH, leading to increased fibril number concentration and, thus, potentially shorter fibrils than those formed under neutral conditions. Consistent with this, our model predicts that average fibril lengths peak early during aggregation and then decrease due to secondary nucleation, particularly at higher monomer concentrations. The predicted average fibril length ranges from 30 to 100 monomers per fibril (equivalent to 20 – 80 nm, assuming a fibril thickness of ~ 5 nm). These short fibrils have a length similar to the typical length of preformed fibril seeds (PFFs) obtained after sonication and measured by atomic force microscopy (AFM), which are reported between 50 – 200 nm ([Buell et al., 2014](#); [Mahul-Mellier et al., 2015](#); [Van Raaij et al., 2008](#)), corresponding to 100 – 400 monomers per fibril assuming a thickness of 7 nm.

In this acidic pH and seeding scenario, we initialized our simulations with seed fibrils of ~ 180 monomers per fibril, consistent with [Buell et al. \(2014\)](#), who measured fibrils of 83 ± 54 nm in length with an observed mean thickness of 7 ± 2 nm.

Unlike controlled *in vitro* aSyn aggregation assays, where fibril formation can be monitored, determining the average length of aSyn fibrils *in vivo* (e.g., in post-mortem brain tissues) is far more challenging due to variability in fibril morphology and aggregation stages; for example, factors such as brain region, disease state, and individual differences influence fibril characteristics. Furthermore, fibrils *in vivo* are often embedded within cellular structures, making direct length measurements difficult with conventional imaging techniques. While several studies have provided insights into the structure and organization of these fibrils in brain tissues ([Guerrero-Ferreira et al., 2019](#); [Lewis et al., 2024](#); [Shahmoradian et al., 2019](#)), they generally focus on fibril morphology, density, and distribution rather than providing specific fibril length measurements, which remain limited in human brain tissue.

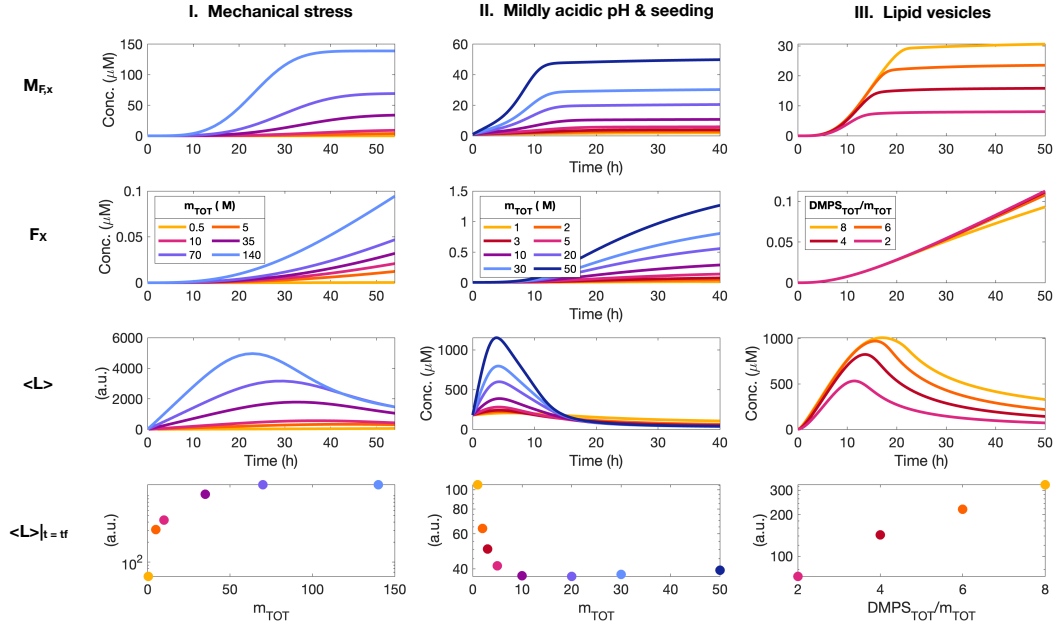


Figure A.1: Average fibril length of model calibration scenarios. The three calibration scenarios are: **I.** Mechanical stress via constant agitation (200 rpm), neutral pH, $T = 37^\circ$, smFRET (Iljina et al., 2016); **II.** Mildly acidic pH ($= 5.5$) and preformed fibril seeds ($M_{F,p} = 1 \mu\text{M}$), quiescent solution, $T = 37^\circ$, ThT dye fluorescence (Gaspar et al., 2017); **III.** DMPS SUVs, quiescent solution, neutral pH, $T = 30^\circ$, ThT dye fluorescence (Galvagnion et al., 2015). Simulations are color-coded for different initial monomer levels (scenarios I & II), and multiple lipid-to-aSyn ratios ($DMPS_{TOT}/m_{TOT}$, scenario III), with initial monomer concentration at $50 \mu\text{M}$. The first three rows show the time evolution of fibril mass and number concentrations ($M_{F,x}$ and F_x , respectively) for on pure (p) or lipidic (l) fibrils, along with average fibril length $\langle L \rangle|_t = M_{F,x}/F_x$, i.e., the mean length of all detected fibrils at a given time t . The fourth row displays the final average fibril lengths (log scale) in response to perturbation of initial conditions, either monomer or vesicle levels.

Table A.2: Parameters of chemical kinetics models associated with datasets used for calibration. (†) The authors performed the global data analysis and fitted the chemical kinetics model via the AmyloFit platform (Meisl et al., 2016); model structure and parameter estimates not available (indicated by a question mark); (*) heterogeneous primary nucleation rate proportional to bound aSyn monomer levels; (**) Michaelis-Menten kinetics for fibril elongation; (***) $k_{I1} = k_{I2} = k_I$, i.e., assuming that rate constants of fibril elongation in pure and lipidic aggregation do not differ; (§) oligomer dissociation catalyzed by fibril surfaces; note that $k_{\max,II}^* = k_{\max,II}^P$ for pure aggregation; $k_{o,II}$ and n_{o2} are the rate constant of secondary nucleation of off-pathway short-lived oligomers and the corresponding number of monomers involved.

Parameter	Our model	Iljina et al. (2016)	Dear et al. (2020b)	Gaspar et al. (2017)†	Galvagnion et al. (2015)	Dear et al. (2024)	Xu et al. (2024)
n	2	(0.9 ± 0.1)	0.9	?	/	/	2
k_n	1.21e-06 $\mu M^{-1} h^{-1}$	(4.0 ± 2.0)e-04 $\mu M^{1-n} h^{-1}$	(4.0 ± 2.0)e-04 $\mu M^{1-n} h^{-1}$	?	/	/	1.3e-10 $\mu M^{-1} h^{-1}$
n_1	2	/	/	/	0.2	0.6	/
$k_{\max,I}$	~[3, 7]e-03 $\mu M h^{-1}$	/	/	/	3.10e-06 $\mu M^{-n_1} h^{-1*}$	5.6e-04/ $k_I \mu M^{-(n_1+1)} h^{-1}$ = 7.07e-05 $\mu M^{-n_1} h^{-1}$	/
K_P	5.25	/	/	/	/	/	/
n_2	2	/	/	?	/	0	1e-03
$k_{\max,II}^P$	7.89e-03 h^{-1}	/	/	?	/	/	7.3e-05 $\mu M^{-n_2} h^{-1}$
$k_{\max,II}^I$	7.89e-04 h^{-1} (assumed)	/	/	/	/	1.5e-03/ $k_I \mu M^{-1} h^{-2}$ = 1.89e-04 h^{-1}	/
K_S	2.13	/	/	/	/	/	/
n_{o2}	/	/	/	/	/	/	1
$k_{o,II}$	/	/	/	/	/	/	1.5e-02 $\mu M^{-n_{o2}} h^{-1}$
k_d	2.4e-03 h^{-1}	/	2.4e-03 h^{-1}	/	/	/	4.4 $\mu M^{-1} h^{-1} \ddagger$
k_{c1}	[4e-02, 6.3e-01] h^{-1}	(9.5 ± 5)e-02 h^{-1}	(7.0 ± 5.0)e-04 h^{-1}	/	/	/	/
k_{c2}	[6e-03, 9.5e-02] h^{-1} (~1.5e-01 k_{c1})	(9.5 ± 5)e-02 h^{-1}	(7.0 ± 5.0)e-04 h^{-1}	/	6.84e-02 h^{-1}	/	/
k_1^{***}	7.92 $\mu M^{-1} h^{-1}$	(9.0 ± 7.0)e-02 $\mu M^{-1} h^{-1}$	7.92 $\mu M^{-1} h^{-1}$	?	7.92 $\mu M^{-1} h^{-1}$ (with $K_E = 125 \mu M^{**}$)	not specified 7.92 $\mu M^{-1} h^{-1}$ assumed	1.2e01 $\mu M^{-1} h^{-1}$
K_D	3.8e-01 μM	/	/	/	(3.8 ± 1.3)e-01 μM	3.8e-01 μM	/
γ	30	/	/	/	28.2 ± 0.8	28	/
M_s	6e03	/	/	/	6e03	6e03	/
y	457	/	/	/	/	360	/
k_{on}	9.5 $k_1 = 75.24 \mu M^{-1} h^{-1}$	/	/	/	/	≥ 7.5 $k_1 = 59.40 \mu M^{-1} h^{-1}$ & ≤ 16 $k_1 = 126.72 \mu M^{-1} h^{-1}$	/

A.3 SHORT- AND LONG-LIVED OLIGOMERS: METHOD-DEPENDENT DETECTION AND IMPLICATIONS FOR MODEL CALIBRATION

Table A.3: **Comparison of experimental studies and methodologies highlights technique-dependent detection of aSyn oligomeric species.** Systematic analysis of five studies shows that the relative mass of observed oligomer populations (1.5% to ~ 100% at peak) depends primarily on the detection approach employed, particularly whether sample modifications are required, rather than solution conditions. Yellow-highlighted studies provided experimental data for our model calibration; (*) first quantitative FRET-based study; (**) physiologically relevant conditions; FRET: Förster Resonance Energy Transfer; SEC: size exclusion chromatography; μ FFE: microchip free-flow electrophoresis; EPR: electron paramagnetic resonance.

Publications		Cremades et al. (2012)	Iljina et al. (2016)*	Arter et al. (2020)	Zurlo et al. (2021)	Xu et al. (2024)
Techniques		single molecule FRET/quantitative SEC	single molecule FRET	multispectral μ FFE	continuous wave EPR	single molecule microfluidics
Sample modifications		✓	✓	✓	None	None
Solution conditions		agitation	agitation	agitation	agitation	cytosolic pH and ionic strength**
Temporal resolution		seconds-minutes	seconds- minutes	seconds	milliseconds	milliseconds
Relative mass	oligomer	~ 1.5%	1-2%	1.5 - 2%	~ 40%	~ 90%-100%

VALIDATION ON FIBRIL MASS DATA AND DISCREPANCIES IN OLIGOMER MASS MEASUREMENTS FROM XU ET AL. (2024)

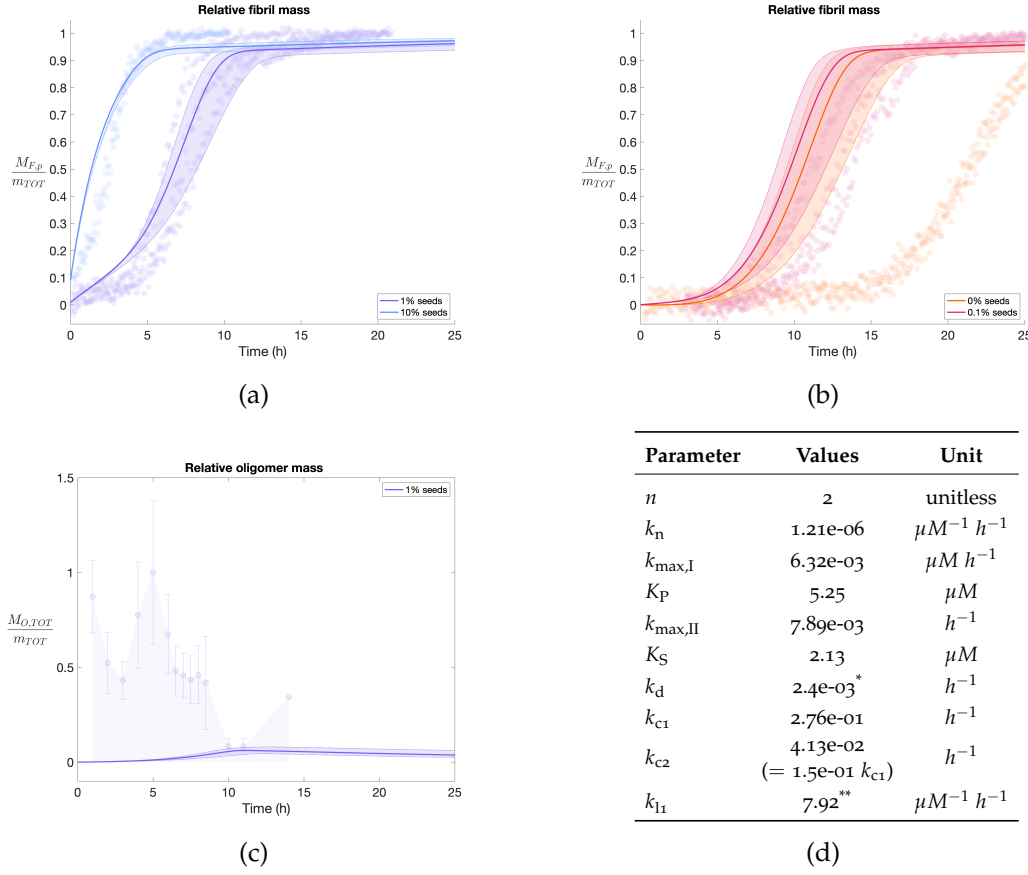


Figure A.2: **Model validation on aSyn fibril kinetics under physiological solution conditions with cytosolic ionic strength and neutral pH (Xu et al., 2024).** Data points correspond to ThT-derived experimental measurements, *i.e.*, fibril mass ($M_{F,P}$) normalized to total protein content $m_{TOT} = 100 \mu\text{M}$. Model predictions (solid lines) obtained by using parameter estimates from prior calibration: (a) model simulations of fibril mass formation for high seeding conditions (1% and 10% seeds); (b) model misfits for 0% and 0.1% seeded aggregation; (c) simulated long-lived oligomer concentrations against available data points of short-lived oligomers for 0% and 1% seeded aggregation, with orange and purple shading highlighting the discrepancy between transient and stable population levels. Lower and upper bands constraining the shaded areas in bottom panels represent the 5th and 95th percentiles of the simulations for the fitted variables from virtual populations of parameter estimates. (d) Parameter estimates derived from calibration on aSyn aggregation under mildly acidic pH & preformed fibril seeds. (*) Cremades et al. (2012) and Dear et al. (2020b); (**) Buell et al. (2014).

ADDITIONAL CALIBRATION ON SHORT-LIVED OLIGOMER AND FIBRIL
MASS DATA FROM XU ET AL. (2024)

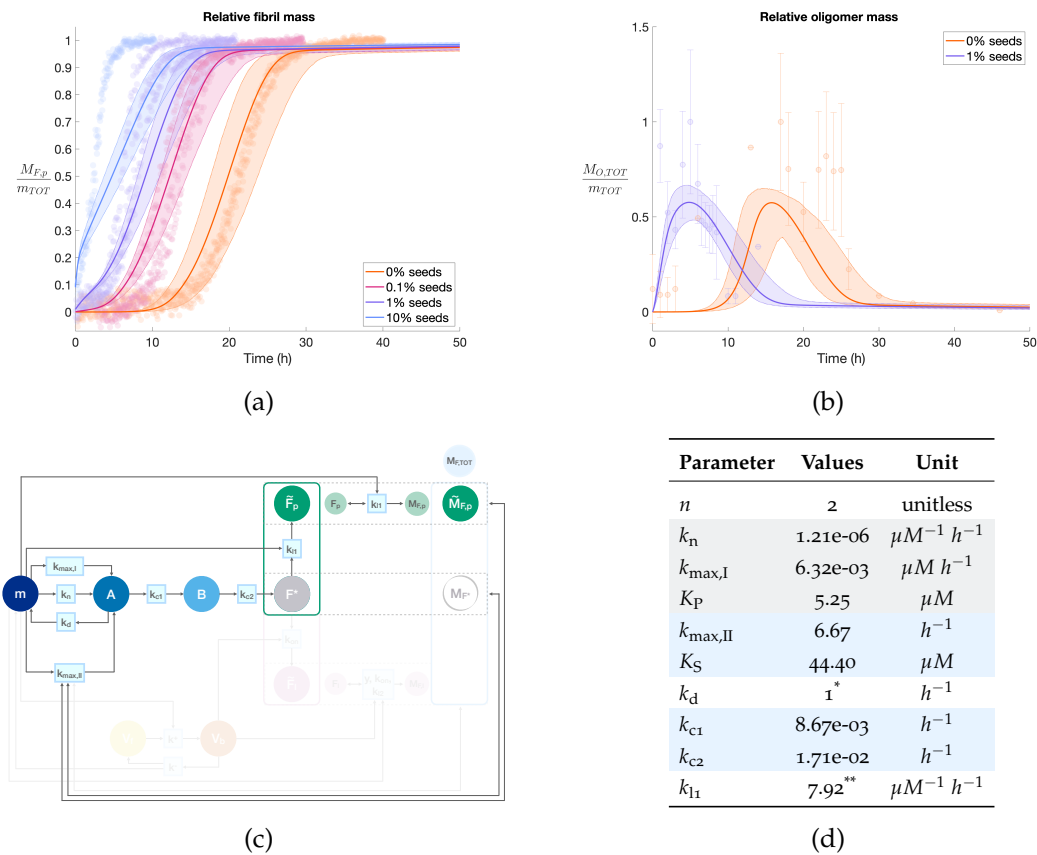


Figure A.3: Quantitative analysis of aSyn aggregation kinetics under physiological solution conditions with cytosolic ionic strength and neutral pH (Xu et al., 2024). Model simulations (solid lines) compared with experimental measurements (dots), *i.e.*, total oligomer mass ($M_{O,TOT}$) and fibril mass ($M_{F,P}$) measured by single molecule microfluidics and ThT dye fluorescence, respectively, and normalized to total protein content $m_{TOT} = 100 \mu M$; low experimental accuracy of oligomer measurements indicated by the error bars (standard deviation) results from a yet-to-optimize protocol and reflects current detection limitations. Model calibration using combined fibril and oligomer measurements from 0% (orange) and 1% (purple) seeding conditions: (a) model fits against fibril mass measurements for 0% - 1% seeding conditions - misfits for the 10% scenario; (b) oligomer dynamics with time-shifted peaks. Lower and upper bands constraining the shaded areas in bottom panels represent the 5th and 95th percentiles of the simulations for the fitted variables from virtual populations of parameter estimates. (c) Model diagram representing the specific system associated with pure protein aggregation used for calibration. (d) Tables of parameter estimates. Estimated parameters are highlighted in light blue. Parameters highlighted in light grey are carried over from prior calibrations. Remaining parameters are fixed to literature values. (*) Xu et al. (2024); (**) Buell et al. (2014).

A.4 EXPLORING THE INTERPLAY OF PURE AND LIPIDIC ASYN AGGREGATION AND THE IMPACT OF LIPID-TO-ASYN RATIOS

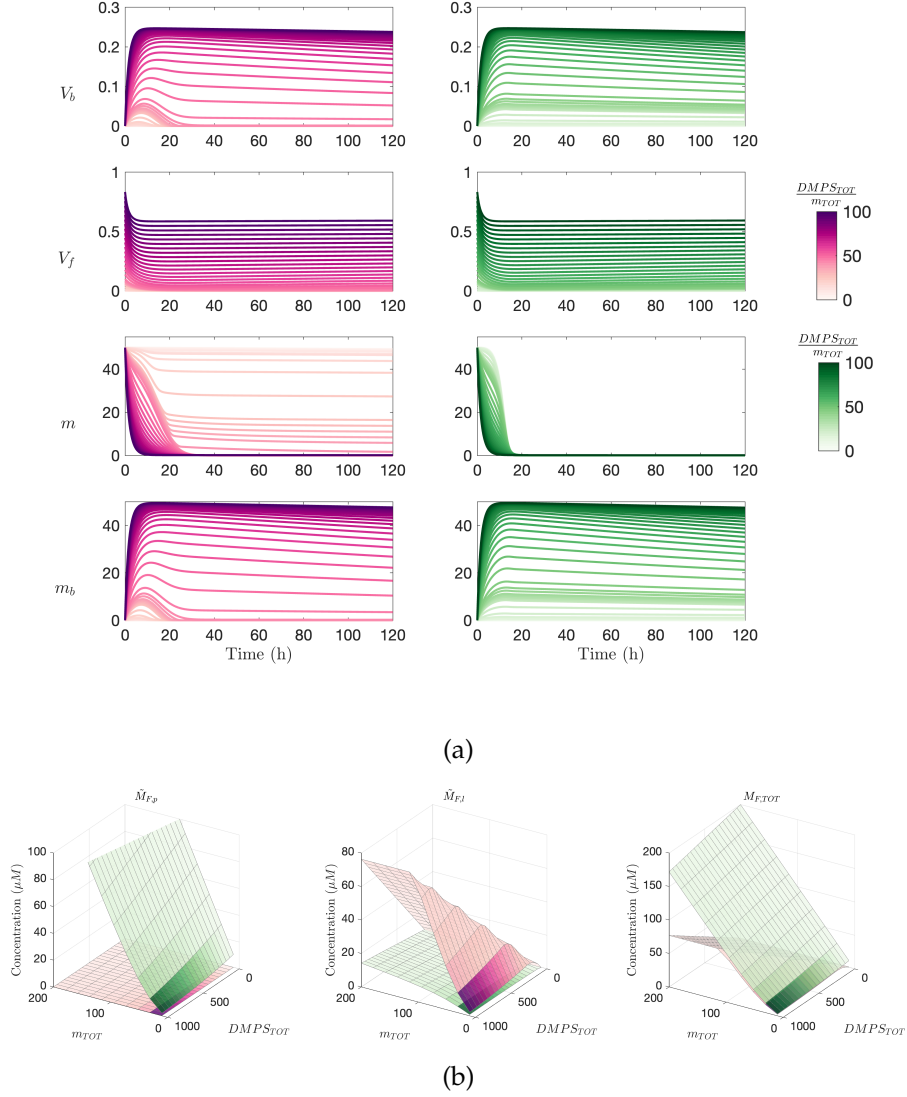


Figure A.4: **The impact of lipid-to-aSyn ratio on pure and lipidic aggregation (pure secondary nucleation considered).** Pink and green shades denote lipid-mediated aggregation in isolation and in combination with pure protein aggregation, respectively. Concentrations (μM), time (hours). (a) Deterministic simulations of aSyn oligomer and fibril formation (number and mass concentrations) under varying lipid concentrations ($DMPS_{TOT}$) with a fixed initial monomer concentration $m_{TOT} = 50 \mu M$. Column one represents simulations with pure aSyn aggregation turned off, while column two depicts simulations with both pathways active, assuming equal rate constants for pure protein and lipidic fibril formation ($k_{I1} = k_{I2}$) and secondary nucleation only on pure fibrils (*i.e.*, $k_{max,II}^p \neq 0$) - not on lipidic fibril and newly generate fibril surfaces (*i.e.*, $k_{max,II}^l = k_{max,II}^* = 0$); (b) variations in the plateau level of pure protein, lipidic, and total fibril mass concentrations in response to different combinations of free monomers and lipidic vesicles in solution over a prolonged period ($t_f = 120$ hours).

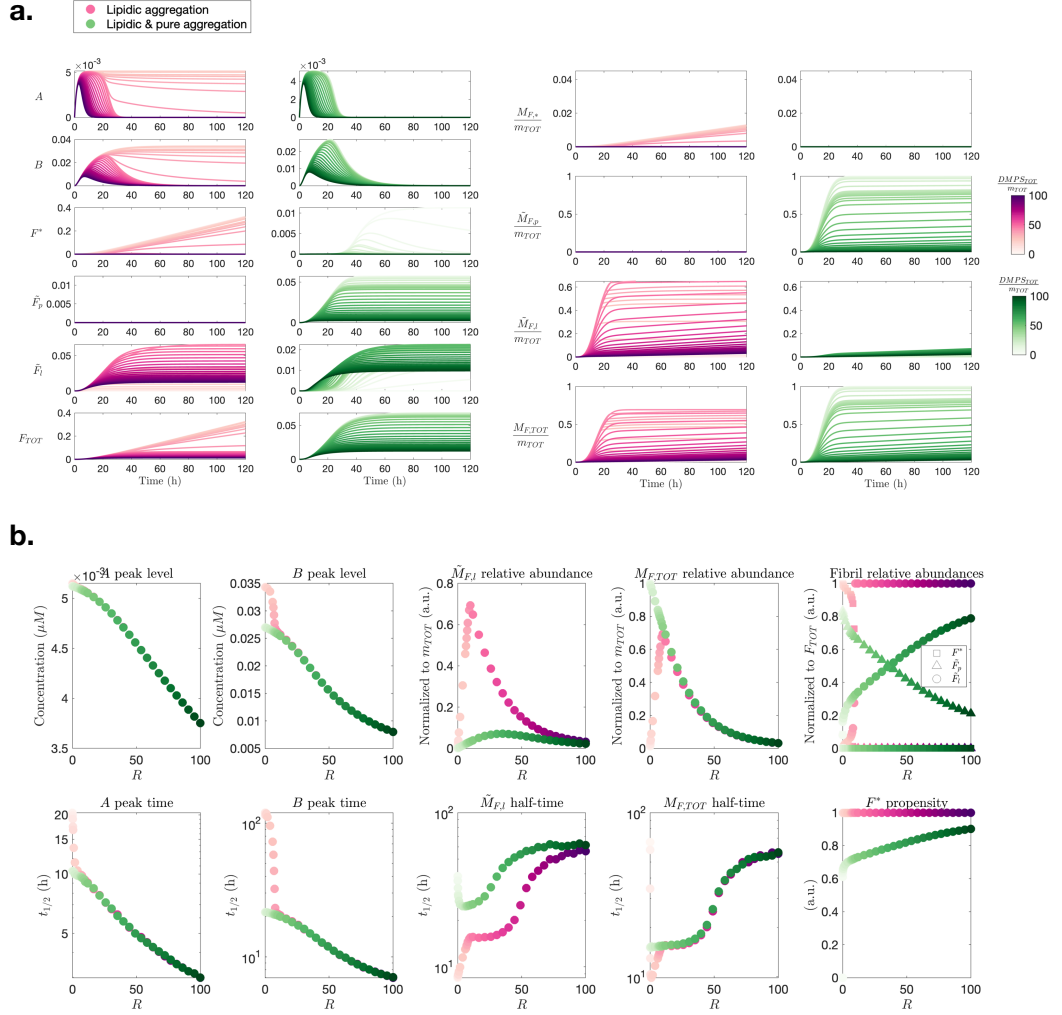


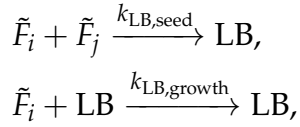
Figure A.5: The impact of lipid-to-aSyn ratio on pure and lipidic aggregation (no secondary nucleation). Pink and green shades denote lipid-mediated aggregation in isolation and in combination with pure protein aggregation, respectively. Concentrations (μM), time (hours). **(a)** Deterministic simulations of aSyn oligomer and fibril formation (number and mass concentrations) under varying lipid concentrations ($DMPSTOT$) with a fixed initial monomer concentration $m_{TOT} = 50 \mu M$. Column one represents simulations with pure aSyn aggregation turned off, while column two depicts simulations with both pathways active, assuming equal rate constants for pure protein and lipidic fibril formation ($k_{11} = k_{12}$) and no secondary nucleation mechanism, in particular $k_{max,II}^P = 0$ as well as $k_{max,II}^L = k_{max,II}^P = 0$; **(b)** metrics including oligomeric peak time and level, total fibril abundance and half-times, Lewy body levels, and propensity for lipidic aggregation over pure protein aggregation; peak times and half-times are in log scale. Each point corresponds to a deterministic simulation colored according to the subplot above, with simulations ending at $t_f = 120$ hours.

A.5 DERIVATION OF LEWY BODY COMPOSITION INDEXES

The Lewy body composition indexes defined in the main text - I^p , I^l , and I^* (which sum to I^{self}), along with I^{cross} - are derived from the following reactions describing the formation and growth of *in vitro* Lewy body-like inclusions. In this model, the inclusions are represented by their number concentration (LB) and mass concentration (M_{LB}), such that:

- Inclusion seeds form at a rate $k_{\text{LB,seed}}$ via interactions between two fibrils of any type: pure ($\tilde{F}_p$), lipidic ($\tilde{F}_l$), or newly generated (F^*).
- Once formed, inclusions grow at a rate $k_{\text{LB,growth}}$ by incorporating additional fibrils of any type, one at a time.

That is,



where $i, j \in \{p, l, *\}$; $\tilde{F}_* = F^*$ in our notation.

The corresponding ODEs for inclusion formation and growth are:

$$\begin{aligned}\frac{d\text{LB}}{dt} &= k_{\text{LB,seed}} \left(\sum_{i,j \in \{p,l,*\}} \tilde{F}_i^2 + 2 \sum_{i,j \in \{p,l,*\}, i \neq j} \text{AUC}(\tilde{F}_i \tilde{F}_j) \right) = k_{\text{LB,seed}} F_{\text{TOT}}^2, \\ \frac{dM_{\text{LB}}}{dt} &= k_{\text{LB,seed}} M_{F,\text{TOT}}^2 + k_{\text{LB,growth}} F_{\text{TOT}} M_{\text{LB}}.\end{aligned}$$

Here, the expression simplifies to F_{TOT} when accounting for the order of interactions between different fibril types.

Due to the lack of sufficient data to constrain the rate constants $k_{\text{LB,seed}}$ and $k_{\text{LB,growth}}$, we cannot fully characterize the time evolution of LB and M_{LB} as we do for aSyn species in the main text. However, we can approximate the inclusion number concentration as follows:

$$\begin{aligned}\frac{d\text{LB}(t)}{dt} &= k_{\text{LB,seed}} F_{\text{TOT}}(t)^2 \\ \Leftrightarrow \text{LB}(t) &= \int_0^t k_{\text{LB,seed}} F_{\text{TOT}}(\tau)^2 d\tau \propto \int_0^t F_{\text{TOT}}(\tau)^2 d\tau = \text{AUC}(F_{\text{TOT}}^2).\end{aligned}$$

Thus, LB is proportional to the area under the curve (AUC) of F_{TOT}^2 , where $F_{\text{TOT}} = \tilde{F}_p + \tilde{F}_l + F^*$.

Based on this, we define the self- and cross-contribution indexes in terms of AUCs of individual fibril contributions and their interactions. These indexes are normalized by $\text{AUC}(F_{\text{TOT}}^2)$:

$$I^p := \frac{\text{AUC}(\tilde{F}_p^2)}{\text{AUC}(F_{\text{TOT}}^2)}, \quad I^l := \frac{\text{AUC}(\tilde{F}_l^2)}{\text{AUC}(F_{\text{TOT}}^2)},$$

$$I^* := \frac{\text{AUC}(F_*^2)}{\text{AUC}(F_{\text{TOT}}^2)}, \quad I^{\text{cross}} := \frac{2 \sum_{i,j \in \{p,l,*\}} \text{AUC}(\tilde{F}_i \tilde{F}_j)}{\text{AUC}(F_{\text{TOT}}^2)}.$$

providing a way to quantify fibril-fibril interactions contributing to Lewy-body-like inclusion formation.

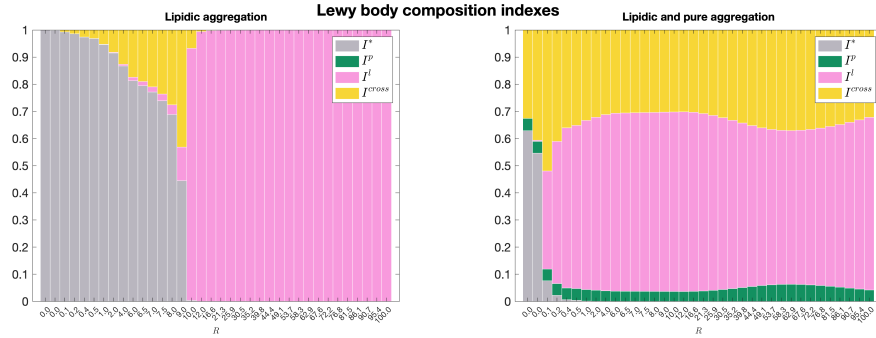


Figure A.6: **The impact of lipid-to-aSyn ratio (R) on Lewy body composition indexes.** We examine two scenarios: lipid-mediated aggregation alone and in combination with pure protein aggregation, under varying lipid concentrations (DMP_{TOT}) while keeping the initial monomer concentration fixed at $m_{\text{TOT}} = 50 \mu\text{M}$. When both pathways are active, we assume equal rate constants for pure protein and lipidic fibril formation ($k_{11} = k_{12}$) and restrict secondary nucleation to pure fibrils ($k_{\text{max,II}}^p \neq 0$, $k_{\text{max,II}}^l = k_{\text{max,II}}^* = 0$). The color-coded Lewy body composition indexes (I^p , I^l , I^* , and I^{cross}) correspond to the scenarios shown in Figure 4 (main text).

A.6 LIPID-TO-ASYN RATIO: A PROXY FOR SNCA MUTATIONS AND AGING

We simulated a scenario mimicking a damaged dopaminergic neuron flooded with aSyn aggregates, introducing increasing concentrations of either free monomers or lipidic vesicles to vary the lipid-to-aSyn ratio. The initial state involved preformed pure fibril seeds and relatively high initial oligomer concentrations (*i.e.*, $m = 35 \mu\text{M}$, $M_{F,p} = 1 \mu\text{M}$, and $M_A + M_B = 4\% m_{\text{TOT}}$ (Lorenzen et al., 2014; Ouzounoglou et al., 2014)). Here, both pure and lipidic pathways were activated. We relied on the second parameter combination for pure protein aggregation and on the third set of parameter estimates for lipidic aggregation (see Table 1, main text); for secondary nucleation, we assumed $k_{\text{max,II}}^* = 0$ and $k_{\text{max,II}}^l$ to be one order of magnitude slower than $k_{\text{max,II}}^p$.

Figure A.7 (column 1) shows aSyn aggregation dynamics after sufficient time for free monomer depletion and aggregate accumulation. Increasing the concentration of free monomers available for vesicle binding and fibril elongation

reduces the lipid-to-protein ratio at $t = t^*$, *i.e.*, $R|_{t=t^*} = \text{DMPS}_{t^*} / m_{t^*}$. At lower concentrations of added free monomers (dark green curves), aggregation proceeds without hindrance. However, above a certain threshold ($\sim 10 \mu\text{M}$), additional free monomers leads to new oligomeric peaks and higher fibril concentration, in particular for pure fibrils. Therefore, when the lipid-to-protein ratio is sufficiently low, even a small perturbation of aSyn monomer level can significantly enhance aggregation. Lipidic fibril concentration decreases for high free monomer levels, and while lipidic fibril mass stabilizes at a high plateau, pure protein fibril concentration scales linearly with monomer concentration.

Perturbing aggregation after a prolonged period presents challenges due to the need for sufficient free monomers to drive the process. If lipid-to-protein ratio changes depend solely on free lipid vesicle concentration, protein aggregation remains unaffected regardless of added vesicles concentration. The window for effective perturbation arises only when changes in DMPS concentration occur early in the process, *e.g.*, around $t = 3$ hours. Thus, the critical factor for effectively perturbing aggregation lies in the timing of intervention. Yet, as shown in Figure A.7 (second column), early vesicle addition determines only a slight decrease in the number concentration of aggregates, with trends in mass concentrations consistent with prior observations related to varying lipid levels.

Comparing the two scenarios revealed distinct aggregation kinetic signatures. In Figure A.7, we varied the free vesicle amount to align with the range of lipid-to-protein ratio explored in the first case, with lighter curves representing lower ratios. Reducing free vesicle concentration, rather than increasing free monomer level, generally slows oligomer formation but results in higher oligomeric peaks and lower total fibril mass concentrations (with relatively low half-times). Additionally, regardless of added vesicle concentrations, the model suggests that lipidic fibrils contribute to nearly 70% of Lewy body-like inclusion formation. Conversely, adding high amounts of free monomers can result in a more heterogeneous composition, balancing self- and cross-contributions, though with a predominant lipidic contribution at high monomer concentrations. These results reflect the tendency of newly formed fibrils to undergo lipidic over pure aggregation within the observed time frame. The model predicts a high F^* propensity across the explored range of lipid-to-protein ratios, except in scenarios involving high concentrations of additional free monomers.

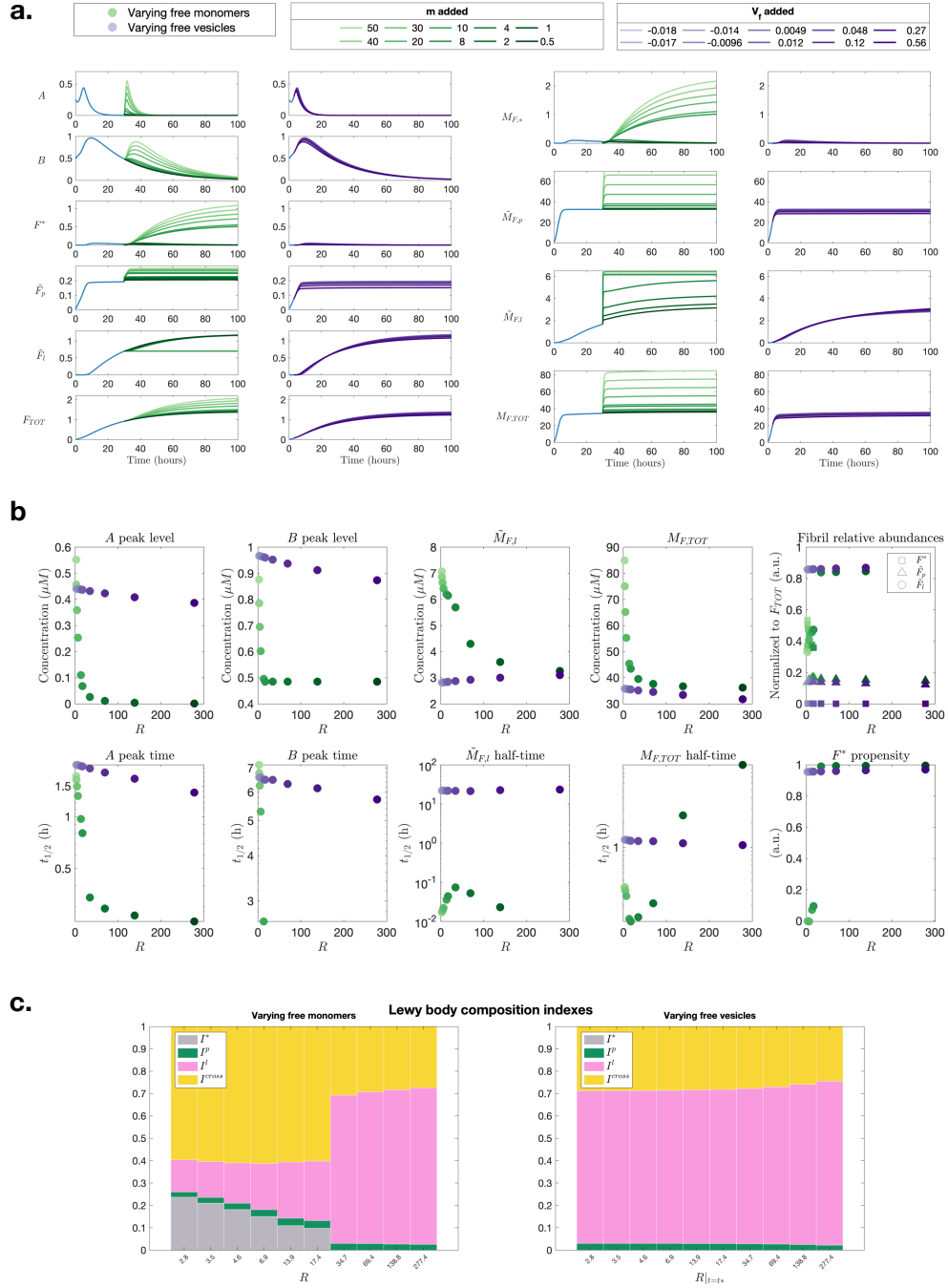


Figure A.7: Pure protein and lipid-mediated α Syn aggregation triggered by preformed fibril seeds and high oligomeric levels and modulated by varying lipid-to-protein ratios. Concentrations (μM), time (hours). **(a)** Time evolution of type-A and type-B oligomers, newly generated fibrils, and pure protein and lipidic fibrils (both number and mass concentrations) modulated by increasing concentrations of free monomers at $t = 30$ hours (column one) and varying amounts of free vesicles at $t = 3$ hours (column two). The blue curve represents α Syn aggregate dynamics when triggered by high initial concentrations of oligomers and pure protein fibrils, *i.e.*, $m = 35 \mu\text{M}$, $M_{F,P} = 1 \mu\text{M}$, and $M_A + M_B = 4\% m_{\text{TOT}}$, with $M_A = 0.5M_B$, and an initial lipid concentration of $200 \mu\text{M}$. Deterministic simulations are color-coded based on lipid-to-monomer ratios - green and purple curves for the first and second columns, respectively; **(b)** metrics summarizing the system behavior, *i.e.*, oligomeric peak time and level, total fibril abundance and half-times, relative abundance of fibril number concentrations, and propensity for lipidic aggregation over pure protein aggregation. Each point is associated with the deterministic simulation of the corresponding color in the subplot above. **(c)** Color-coded Lewy body composition indexes (I^P , I^I , I^* , and I^{cross}) corresponding to the scenarios of varying free monomer (first column) and free lipid vesicles (second column).

APPENDIX: MECHANISTIC PREDICTION OF UNBOUND DRUG FRACTION IN BRAIN HOMOGENATES

The following explanation of drug-specific input parameters and how distribution coefficients for tissue constituents and tissue subspaces in intact and homogenate brain tissues are derived is based entirely on the Rodgers & Rowland framework and relevant literature. With appropriate adaptations, it follows the notation and conceptual setting introduced in a draft chapter of an upcoming book on PBPK modeling, as referenced in the Introduction.

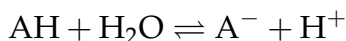
B.1 DRUG-SPECIFIC INPUT PARAMETERS

Ionization: The acidic dissociation constant and ionization fractions The ionization state of a drug critically influences its pharmacokinetic and pharmacodynamic properties. Over 95% of drugs contain ionizable functional groups, predominantly basic, with a smaller proportion being acidic and only a minority lacking ionizable moieties entirely (Di et al., 2015). Ionization directly affects properties such as lipophilicity, solubility, membrane permeability, and tissue distribution (Manallack, 2007). The acid dissociation constant, K_A , is, therefore, a key parameter in ADME prediction, as it quantifies the tendency of a compound to donate a proton, and thus governs its ionization state in different physiological environments. As previously mentioned, this parameter is used as an input to *a priori* prediction methods for tissue partitioning.

Acid-base fundamentals and pH. According to the Brønsted-Lowry theory, acids are proton donors and bases are proton acceptors. Ampholytes inherently have the capacity to act as both acid and base, though the degree to which they donate or accept protons depends quantitatively on environmental factors such as pH. In aqueous systems, the acidity of a solution is quantified by pH, defined as $\text{pH} = -\log_{10}[\text{H}^+]$, where $[\text{H}^+]$ approximates the activity of hydronium ions ($[\text{H}_3\text{O}^+]$). Under physiological conditions, ionization equilibria are pH-dependent: at low pH, bases tend to protonate, while acids remain largely uncharged; at high pH, acids deprotonate to form anions. For amphoteric species, this behavior becomes more complex, especially for zwitterionic compounds exhibiting dual protonation states.

The acid dissociation constant K_A . Consider a drug dissolved in a physiological fluid (interstitial, intracellular, or plasma water) or a buffered aqueous solution

at a constant pH. The dissociation of a monoprotic acid (AH) into its conjugate base (A^-) and a proton is represented as:



with the equilibrium constant K_A defined as: $K_A := [A^-][H^+]/[AH]$. This constant quantifies the amount of drug ionization in this water/buffer with a given pH.

The parameter pKa is the negative base-10 logarithm of K_A :

$$pKa = -\log_{10} K_A . \quad (B.1)$$

Therefore, the lower the pKa, the stronger the acid and the weaker the conjugate base. For bases, pKa values typically refer to their conjugate acids. It is essential to distinguish between anionic (acidic) and cationic (basic) pKa values, specifying whether a pKa pertains to an acid or its conjugate base (Zheng et al., 2024). Thus, an ionizable drug is characterized by one or more pKa values and its ionization type (acid, base, zwitterion). Monoprotic acids or bases have a single pKa value. This parameter can be determined *in vitro* or *in silico*. While *in silico* methods reduce inter-laboratory variability, they introduce variability across prediction models. Nonetheless, they are widely adopted due to their convenience and scalability.

Neutral and ionized fractions f_n , f_{cat} , f_{ani} . In physiological fluids or buffered solutions, the free form of a drug can exist as neutral (n), anionic (ani), cationic (cat), or zwitterionic (zwi) species. The total free drug concentration C_{wa} is:

$$C_{wa} = C_{wa,n} + C_{wa,ani} + C_{wa,cat} + C_{wa,zwi} , \quad (B.2)$$

where the net neutral concentration $C_{wa,nn}$ is the sum of the neutral and zwitterionic concentrations. The respective fractions are defined as:

$$f_n := \frac{C_{wa,n}}{C_{wa}} , \quad f_{zwi} := \frac{C_{wa,zwi}}{C_{wa}} , \quad f_{cat} := \frac{C_{wa,cat}}{C_{wa}} , \quad f_{ani} := \frac{C_{wa,ani}}{C_{wa}} , \quad (B.3)$$

such that the fraction net neutral is $f_{nn} = f_n + f_{zwi}$, and the sum of all fractions satisfies the relationship $f_{nn} + f_{cat} + f_{ani} = 1$.

Henderson-Hasselbalch (or buffer) equation. The pH of the solution and the drug pKa value determine the speciation into neutral, anionic and cationic drug species via the Henderson-Hasselbalch equation (Henderson, 1908; Hasselbalch, 1916). For a monoprotic acid (AH) with anionic pKa, it holds

$$pH = \log_{10} \left(\frac{[A^-]}{[AH]} \right) + pKa \quad i.e. \quad \frac{[A^-]}{[AH]} = 10^{pH-pKa} . \quad (B.4)$$

This equation enables to determine the corresponding neutral and ionized fractions for various drug types. For instance, for a monoprotic acid:

$$f_n = \frac{C_{wa,n}}{C_{wa}} = \frac{C_{wa,n}}{C_{wa,n} + C_{wa,ani}} = \frac{1}{1 + \frac{C_{wa,ani}}{C_{wa,n}}} = \frac{1}{1 + \frac{[A]}{[D]}} = \frac{1}{1 + 10^{pH-pK_a}} .$$

Generalizing for compounds with multiple pKas, we obtain:

- for acids with anionic **pKa** = (pKa₁, pKa₂, ..., pKa_n) in increasing order:

$$\begin{aligned} f_n &= \frac{1}{1 + \sum_{i=1}^n 10^{\sum_{j=1}^i (pH-pK_{a_j})}} , \\ f_{cat} &= f_{zwi} = 0 , \\ f_{ani} &= 1 - f_n . \end{aligned} \quad (B.5)$$

- for bases with cationic **pKa** = (pKa₁, pKa₂, ..., pKa_n) in decreasing order:

$$\begin{aligned} f_n &= \frac{1}{1 + \sum_{i=1}^n 10^{\sum_{j=1}^i (pK_{a_j}-pH)}} , \\ f_{ani} &= f_{zwi} = 0 , \\ f_{cat} &= 1 - f_n . \end{aligned} \quad (B.6)$$

- for neutrals,

$$f_n = 1 , \quad f_{ani} = f_{cat} = f_{zwi} = 0 ; \quad (B.7)$$

- For ampholytes with anionic **pKa_{ani}** = (pKa_{ani,1}, pKa_{ani,2}, ..., pKa_{ani,n}) listed in increasing order, and cationic **pKa_{cat}** = (pKa_{cat,1}, pKa_{cat,2}, ..., pKa_{cat,m}) listed in decreasing order, where all cationic pKa values are strictly smaller than the anionic pKa values, the ionization fractions can be calculated as:

$$\begin{aligned} f_{nn} &= \frac{1}{1 + \sum_{i=1}^n 10^{\sum_{j=1}^i (pH-pK_{a_{ani,j}})} + \sum_{l=1}^m 10^{\sum_{l=1}^i (pK_{a_{cat,l}}-pH)}} , \\ f_{ani} &= \frac{\sum_{i=1}^n 10^{\sum_{j=1}^i (pH-pK_{a_{ani,j}})}}{1 + \sum_{i=1}^n 10^{\sum_{j=1}^i (pH-pK_{a_{ani,j}})} + \sum_{l=1}^m 10^{\sum_{l=1}^i (pK_{a_{cat,l}}-pH)}} , \\ f_{cat} &= \frac{\sum_{l=1}^m 10^{\sum_{l=1}^i (pK_{a_{cat,l}}-pH)}}{1 + \sum_{i=1}^n 10^{\sum_{j=1}^i (pH-pK_{a_{ani,j}})} + \sum_{l=1}^m 10^{\sum_{l=1}^i (pK_{a_{cat,l}}-pH)}} . \end{aligned} \quad (B.8)$$

These expressions describe the fractions of the net neutral (fnn), anionic (fani), and cationic (fcac) species. Only the total net neutral fraction, however, can be derived without ambiguity. In particular, we cannot distinguish between neutral species in their uncharged or zwitterionic

forms without additional information, that is, the tautomeric equilibrium constant K_τ . When K_τ is known:

$$\begin{aligned} \text{fn} &= \frac{\text{fnn}}{1 + K_\tau} , \\ \text{fzwi} &= \text{fnn} - \text{fn} . \end{aligned}$$

The tautomeric constant K_τ ranges from 0 to ∞ , with $K_\tau = 0$ representing conventional (uncharged) ampholytes and $K_\tau = \infty$ representing zwitterionic ampholytes. In the latter case, the interconversion between anionic and cationic states occurs exclusively via the zwitterionic form.

For detailed derivations of these formulas and further explanation, we refer the reader to [Henderson \(1908\)](#), [Hasselbalch \(1916\)](#), and [Zheng et al. \(2024\)](#).

Lipophilicity: the n -octanol:water partition coefficient Lipophilicity refers to the affinity of a compound for nonpolar lipid over polar aqueous phases, and it affects properties such as membrane permeability, protein binding, and tissue partitioning. Two commonly used parameters quantify lipophilicity: the n -octanol:water distribution coefficient ($D_{\text{o:w}}$) and the partition coefficient ($P_{\text{o:w}}$).

- The distribution coefficient $D_{\text{o:w}}$ accounts for the ionization state of the compound at a given pH (typically pH 7.4) ([Di et al., 2015](#)) and is defined as the ratio of the total concentration in the n -octanol phase to the total concentration in the aqueous phase with the specific pH:

$$D_{\text{o:w}} := \frac{C_{\text{octanol}}}{C_{\text{wa}}} .$$

- The partition coefficient $P_{\text{o:w}}$ is measured under conditions (pH) where the compound exists entirely in its neutral form. It represents the ratio between the total concentration in the n -octanol phase and the neutral concentration in the aqueous phase:

$$P_{\text{o:w}} := \frac{C_{\text{octanol}}}{C_{\text{wa},n}} .$$

$P_{\text{o:w}}$ is widely used as a pH-independent measure of intrinsic lipophilicity ($P_{\text{o:w}} > 1$) or hydrophilicity ($P_{\text{o:w}} < 1$).

Typically, the decadic logarithmic form of these two quantities is reported, $\log D_{\text{o:w}}$ and $\log P_{\text{o:w}}$ (or simply $\log D$ and $\log P$, or $\text{clog} D$ and $\text{clog} P$ if predicted *in silico*). The relationship between $P_{\text{o:w}}$ and $D_{\text{o:w}}$ (at a specific pH) depends on the compound ionization profile at the aqueous pH. In general, the distribution coefficient can be expressed as:

$$D_{\text{o:w}} = P_{\text{o:w}} \text{fn}_{\text{wa}} + P_{\text{o:w}}^- \text{fani}_{\text{wa}} + P_{\text{o:w}}^+ \text{fcats}_{\text{wa}} ,$$

where $P_{o:w}^-$ and $P_{o:w}^+$ are the partition coefficients of the anionic and cationic species, and fn_{wa} , $fani_{wa}$, and $fcats_{wa}$ are the fractions of neutral, anionic, and cationic species in the aqueous phase at pH_{wa} . A common assumption is that only the neutral species partitions into the *n*-octanol phase, implying $P_{o:w}^- = P_{o:w}^+ = 0$ (Rodgers et al., 2005; Rodgers et al., 2006). Under this simplification, the relationship reduces to:

$$D_{o:w} = P_{o:w} fn_{wa} . \quad (B.9)$$

Plasma partitioning Two key PK parameters characterize how drugs distribute within blood: the fraction unbound in plasma (fuP) and the blood-to-plasma concentration ratio (B:P).

Fraction unbound in plasma. The fraction unbound in plasma fuP represents the proportion of drug not bound to plasma proteins, hence available for distribution into tissues, metabolism, or pharmacological activity. As such, it is a critical PK parameter influencing all aspects of ADME, as well as pharmacodynamics (PD) behavior. By definition, $fuP := C_{pw}/C_{pla}$ is the ratio between the concentration of drug in the plasma water and the total drug concentration in plasma. This value is typically measured using *in vitro* techniques such as equilibrium dialysis or ultrafiltration, with equilibrium dialysis widely considered the gold standard (Bohnert et al., 2013). Nevertheless, for highly bound drugs (particularly when $fuP \leq 0.01$), such measurements may suffer from experimental bias and reduced precision (Rodgers et al., 2005; Smith et al., 2010). Special attention is therefore needed when incorporating low fuP values into models or extrapolations.

Blood-to-plasma ratio. Complementing fuP, the blood-to-plasma ratio, *i.e.*, $B:P := C_{blo}/C_{pla}$, quantifies the distribution of a drug between whole blood and plasma. This ratio captures how much of the drug partitions into red blood cells versus remaining in plasma, and is crucial for predicting tissue partitioning in some *a priori* methods. While B:P can also be measured experimentally, such data are less commonly available than fuP, especially in clinical studies. Therefore, large compiled datasets (Mamada et al., 2021) and *in silico* predictions of tissue partition coefficient based on physicochemical properties and tissue composition have become useful alternatives. Specifically, in some *a priori* prediction methods such as the R&R approach (Rodgers et al., 2005; Rodgers et al., 2006), the blood-to-plasma ratio can be reformulated based on the RBC-to-plasma water distribution coefficient, $Kpu_{rbc} := C_{rbc}/C_{pw}$, such that:

$$B:P = (1 - hct) + hct \cdot fuP \cdot Kpu_{rbc} , \quad (B.10)$$

where hct is the hematocrit, *i.e.*, the fractional volume of red blood cells.

In practice, for example in the Rodgers & Rowland approach, when B:P is experimentally available, Kpu_{rbc} is back-calculated from eq. B.10 and then used

as an input to predict tissue partitioning. Where B:P data are inconsistent, $K_{pu_{rbc}}$ is predicted and B:P is re-obtained using this equation.

B.2 PARTITIONING INTO TISSUE CONSTITUENTS

CELLULAR WATER

Passive diffusion across the cellular membrane For small molecules, passive diffusion across the cellular membrane is generally the main route of cellular distribution. Drug transport by passive diffusion across the cellular membrane from the extracellular to the cellular space and vice versa can be considered a three-step process, where the compound first partitions into the membrane, diffuses across it, and is released on the other side of the membrane. This process depends on multiple factors, such as drug permeability, cellular membrane surface, drug ionization, and membrane composition. We consider a passive diffusion model relying on the following assumptions:

- the cellular space is separated by a cellular membrane with total surface area SA from the extracellular space, creating a pH compartmentalization with pH_{ew} and pH_{cw} ;
- the drug is dissolved in the water subspace of the extracellular and cellular space with concentrations C_{ew} and C_{cw} ; by definition, these are free (or unbound) concentrations;
- for ionizable drugs, the permeability P_{wa} of the free drug in the water subspace is parameterized by

$$P_{wa} := P_n \cdot (fn_{wa} + \alpha(1 - fn_{wa})) \quad (B.11)$$

with the permeability of the neutral species P_n and the ratio of ionized-to-neutral passive permeability α . Usually, the following three assumptions on α are alternatively used:

- $\alpha = 0$, if only neutral species permeate the membrane;
- $\alpha \ll 1$, if the neutral species permeates substantially faster than the ionized one(s), e.g., $\alpha = 0.001$;
- $\alpha = 1$, if both neutral and ionized species permeate equally well.

Given the complexity of certain aspects of passive diffusion, such as drug-membrane interactions, we adopt a highly simplified representation of these processes, following R&R approach. Under the above assumptions, the net transport by passive diffusion between the cellular and extracellular (*i.e.*, interstitial) spaces is given by

$$r_{diff} = -PSA_{iw} C_{iw} + PSA_{cw} C_{cw},$$

where the permeability-surface area product $PSA_{wa} = P_{wa} \cdot SA$ is determined by the surface area of the membrane SA and the permeability of the free

(neutral as well as ionized) drug in the water subspace P_{wa} . At steady state, the transport flow of passive diffusion across the cellular membrane satisfies $r_{diff} = 0$, *i.e.*,

$$r_{diff} = -PSA_{iw} C_{iw} + PSA_{cw} C_{cw} = 0. \quad (B.12)$$

Cellular water-to-interstitial water distribution coefficient By relying on eq. B.12 on the steady-state net transport flow, we obtain

$$\frac{C_{cw}}{C_{iw}} = \frac{PSA_{iw}}{PSA_{cw}}.$$

We define the cellular water-to-interstitial water distribution coefficient as $K_{cw:iw} := \frac{C_{cw}}{C_{iw}}$. Therefore, it can be expressed as

$$K_{cw:iw} = \frac{PSA_{iw}}{PSA_{cw}} = \frac{P_n \cdot (fn_{iw} + \alpha(1 - fn_{iw})) \cdot SA}{P_n \cdot (fn_{cw} + \alpha(1 - fn_{cw})) \cdot SA} = \frac{fn_{iw} + \alpha(1 - fn_{iw})}{fn_{cw} + \alpha(1 - fn_{cw})}. \quad (B.13)$$

This parameter depends exclusively on pH compartmentalization between the interstitial and cellular spaces as a function of the fractions neutral of the drug in the two spaces. Moreover, depending on the value associated with the factor α , the distribution coefficient is given by

$$K_{cw:iw} = \begin{cases} \frac{fn_{iw}}{fn_{cw}} & \text{passive diffusion of neutral species only, } \alpha = 0 \\ \frac{fn_{iw} + \alpha(1 - fn_{iw})}{fn_{cw} + \alpha(1 - fn_{cw})} & \text{passive diffusion of predominantly the neutral, } \alpha \ll 1 \\ 1 & \text{passive diffusion of neutral and charged species, } \alpha = 1. \end{cases} \quad (B.14)$$

Cellular water-to-plasma water distribution coefficient If the assumption of fast/instantaneous exchange across the vascular endothelium (*i.e.*, between the plasma and interstitial space) compared to other PK processes holds, extracellular water, plasma water, and interstitial water concentrations are assumed to be identical, *i.e.*, $C_{ew} = C_{pw} = C_{iw}$. Therefore, the parameter defined as cellular water-to-plasma water distribution coefficient

$$K_{puu_{cel}} := \frac{C_{cw}}{C_{pw}}$$

can be used to characterize the exchange across both the cellular membrane and the vascular endothelium. Here, the steady-state net transport by passive diffusion from the interstitial space to the cellular space across the cellular membrane is given by

$$r_{diff} = -PSA_{pw} C_{pw} + PSA_{cw} C_{cw} = 0,$$

Therefore, $K_{puu_{cel}}$ can be expressed as

$$K_{puu_{cel}} = \frac{PSA_{pw}}{PSA_{cw}} = \frac{P_n \cdot (fn_{pw} + \alpha(1 - fn_{pw})) \cdot SA}{P_n \cdot (fn_{cw} + \alpha(1 - fn_{cw})) \cdot SA} = \frac{fn_{pw} + \alpha(1 - fn_{pw})}{fn_{cw} + \alpha(1 - fn_{cw})}. \quad (B.15)$$

RBC water-to-plasma water distribution coefficient Considering the red blood cells, the RBC water-to-plasma water distribution coefficient defined as

$$K_{puu_{rbc}} = K_{rw:pw} := \frac{C_{rw}}{C_{pw}}, \quad (B.16)$$

characterizes the exchange across the RBC membrane between plasma and RBC water. Following the previous idea, given the steady-state net transport by passive diffusion between plasma to RBC space satisfies

$$r_{diff} = -PSA_{pw} C_{pw} + PSA_{rw} C_{rw} = 0,$$

$K_{puu_{rbc}}$ can be expressed as

$$K_{puu_{rbc}} = \frac{PSA_{pw}}{PSA_{rw}} = \frac{P_n \cdot (fn_{pw} + \alpha(1 - fn_{pw})) \cdot SA}{P_n \cdot (fn_{rw} + \alpha(1 - fn_{rw})) \cdot SA} = \frac{fn_{pw} + \alpha(1 - fn_{pw})}{fn_{rw} + \alpha(1 - fn_{rw})}. \quad (B.17)$$

Homogenate subspace water-to-homogenate water distribution coefficient Due to cell membrane and pH compartmentalization disruption during homogenization, any homogenate subspace water-to-homogenate water distribution coefficient satisfies the following relationship:

$$K_{wa:hw} = 1. \quad (B.18)$$

with $wa \in \{cw, iw\}$.

NEUTRAL LIPIDS AND NEUTRAL PHOSPHOLIPIDS

To derive the neutral lipid-to-water distribution coefficient $K_{nl:wa}$, it is assumed that the following identity holds:

$$K_{nl:wa} = D_{o:w} = P_{o:w} fn_{wa} + P_{o:w}^- fani_{wa} + P_{o:w}^+ fcat_{wa}, \quad (B.19)$$

relying on eq B.9 in Section B.1. In addition, we assume that only the neutral species distributes in the n -octanol phase, resulting in $P_{o:w}^- = P_{o:w}^+ = 0$, and ultimately

$$K_{nl:wa} = P_{o:w} fn_{wa}. \quad (B.20)$$

If we assume that phospholipids are similar to a mixture of 30% neutral lipids and 70% water (Poulin et al., 2002) and only the neutral species distributes into the neutral phospholipids and n -octanol phase, it follows that

$$K_{np:wa} = 0.3 D_{o:w} + 0.7 f_{nwa} = (0.3 P_{o:w} + 0.7) f_{nwa} . \quad (B.21)$$

We adopt this neutral logP-based parametrization to predict neutral lipid and phospholipid-to-water distribution coefficients in plasma and cellular space for both intact and homogenate tissues. By following this approach, we obtain:

$$K_{nl(p):pw} = P_{o:w} f_{npw} \quad \& \quad K_{np(p):pw} = (0.3 P_{o:w} + 0.7) f_{npw} \quad (B.22)$$

$$K_{nl(c):cw} = P_{o:w} f_{ncw} \quad \& \quad K_{np(c):cw} = (0.3 P_{o:w} + 0.7) f_{ncw} \quad (B.23)$$

$$K_{nl(c):hw} = P_{o:w} f_{nhw} \quad \& \quad K_{np(c):hw} = (0.3 P_{o:w} + 0.7) f_{nhw} \quad (B.24)$$

ACIDIC PHOSPHOLIPIDS

To describe drug binding to acidic phospholipids in either cellular, RBC, or homogenate spaces, we adopt a non-saturable protein binding model in which

- acidic phospholipids exist at a constant total concentration AP^- with one accessible binding site, either dissolved in the fluid compartment (volume V), or positioned such that its binding site remains accessible from the compartment;
- no competing binders—no proteins or other binding components interact with the drug;
- reversible binding following a first-order kinetics with affinity constant K_{A,ap^-} .
- only the cationic drug species can interact with acidic phospholipids, with $f_{cat_{wa}}$ denoting the fraction cationic in the aqueous space.

Therefore, at binding equilibrium, the following relationship holds:

$$C_{ap} = K_{A,ap^-} \cdot AP^- \cdot f_{cat_{wa}} \cdot C_u \quad (B.25)$$

where $C_{ap} := A_{ap}/V$ and $C_u := A_u/V$ represent the unbound (or free) and AP^- -bound drug concentrations, with A_u and A_{ap} being the corresponding drug amounts. This charged protein-binding parametrization enables prediction of distribution coefficients for acidic phospholipids in RBCs, cellular space, or homogenate tissue ($K_{ap(r):rw}$, $K_{ap(c):cw}$, and $K_{ap(c):hw}$).

Red blood cell acidic phospholipid-to-water distribution coefficient Upon the charged protein-binding parametrization described above, the partition coefficient $K_{ap(r):rw}$ can be expressed as

$$K_{ap(r):rw} = \frac{V_{rbc}}{V_{ap(r)}} K_{A,ap(r)^-} AP_{rbc}^- f_{cat_{rw}}$$

with $AP_{\text{rbc}}^- := \frac{A_{\text{AP}}^-}{V_{\text{rbc}}}$ as the concentration of acidic phospholipids with reference to RBC volume. If we define $AP_{\text{tis}}^- := \frac{A_{\text{AP}}^-}{V_{\text{tis}}}$ and assume $V_{\text{tis}} AP_{\text{tis}}^- = V_{\text{rbc}} AP_{\text{rbc}}^-$, the previous equation becomes

$$K_{\text{ap(r):rw}} = \frac{V_{\text{tis}}}{V_{\text{ap(r)}}} K_{A,\text{ap(r)}} AP_{\text{tis}}^- \text{fcat}_{\text{rw}}. \quad (\text{B.26})$$

Cellular acidic phospholipid-to-water distribution coefficient To derive practical expressions for cellular acidic phospholipids distribution coefficients, we follow the same steps as for the RBC and, additionally, assume that the affinity constant associated with acidic phospholipids is the same in RBC and cellular spaces, *i.e.*, $K_{A,\text{ap(c)}}^- = K_{A,\text{ap(r)}}^-$, as proposed by Rodgers & Rowland. This assumption is crucial given that $K_{A,\text{ap(r)}}^-$ can be inferred from *in vitro* drug-specific input parameters, *e.g.*, B:P, as detailed below in Section B.4.

We define $AP_{\text{cel}}^- := A_{\text{AP}}^- / V_{\text{cel}}$ and $AP_{\text{tis}}^- := A_{\text{AP}}^- / V_{\text{tis}}$ as the concentrations of acidic phospholipids with reference to the brain cellular volume and tissue volume, respectively, such that $V_{\text{tis}} AP_{\text{tis}}^- = V_{\text{cel}} AP_{\text{cel}}^-$. By adopting the charged protein-binding parametrization described above, the partition coefficient $K_{\text{ap(c):cw}}$ can be expressed as

$$\begin{aligned} K_{\text{ap(c):cw}} &= \frac{V_{\text{cel}}}{V_{\text{ap(c)}}} K_{A,\text{ap(c)}} AP_{\text{cel}}^- \text{fcat}_{\text{cw}} \\ &= \frac{V_{\text{tis}}}{V_{\text{ap(c)}}} K_{A,\text{ap(c)}} AP_{\text{tis}}^- \text{fcat}_{\text{cw}}. \end{aligned}$$

Here, by assuming $K_{A,\text{ap(c)}}^- = K_{A,\text{ap(r)}}^-$, we obtain the cellular acidic phospholipid-to-water distribution coefficient as

$$K_{\text{ap(c):cw}} = \frac{V_{\text{tis}}}{V_{\text{ap(c)}}} K_{A,\text{ap(r)}} AP_{\text{tis}}^- \text{fcat}_{\text{cw}}. \quad (\text{B.27})$$

Cellular acidic phospholipid-to-homogenate water distribution coefficient Due to cell lysis, the acidic phospholipid concentration in the cellular space AP_{cel}^- redistributes within the homogenate brain tissue. Given $AP_{\text{hom}}^- := A_{\text{AP}}^- / V_{\text{hom}}$, we assume a complete recovery of accessible binding sites of acidic phospholipids upon homogenization and

$$V_{\text{hom}} AP_{\text{hom}}^- = V_{\text{cel}} AP_{\text{cel}}^- = V_{\text{cw}} AP_{\text{cw}}^-, \quad (\text{B.28})$$

i.e., the total amount of binding sites remains the same. Based on the charged protein-binding parametrization, the partition coefficient $K_{\text{ap(c):hw}}$ is given by

$$\begin{aligned} K_{\text{ap(c):hw}} &= \frac{V_{\text{cel}}}{V_{\text{ap(c)}}} K_{A,\text{ap(c)}} AP_{\text{cel}}^- \text{fcat}_{\text{hw}} \\ &= \frac{V_{\text{hom}}}{V_{\text{ap(c)}}} K_{A,\text{ap(c)}} AP_{\text{hom}}^- \text{fcat}_{\text{hw}}. \end{aligned}$$

Finally, by assuming $K_{A,ap(c)^-} = K_{A,ap(r)^-}$, we obtain

$$K_{ap(c):hw} = \frac{V_{hom}}{V_{ap(c)}} K_{A,ap(r)^-} AP_{hom}^- fcat_{hw}. \quad (B.29)$$

INTERSTITIAL BINDING PROTEINS

The modeling approach to derive the distribution coefficients for interstitial binding proteins relies on the charged protein-binding kinetics framework previously introduced for acidic phospholipid binding. In this context, only the non-cationic species—namely, neutral and anionic forms of the drug—are assumed to interact with binding proteins in the interstitial fluid.

Interstitial binding protein-to-water distribution coefficient Relying on the charged protein-binding parametrization, the steady-state interstitial protein binding within the interstitial fluid is given by

$$C_{pr(i),isf} = K_{A,pr(i)} (1 - fcat_{iw}) PR_{isf} \cdot C_{u,isf} \quad (B.30)$$

with

$$C_{pr(i),isf} := \frac{A_{pr(i)}}{V_{isf}}, \quad C_{u,isf} := \frac{A_{iw}}{V_{isf}} \quad (B.31)$$

and PR_{isf} being the total concentration of binding proteins in the interstitial fluid and $A_{pr(i)}$ being the total amount of interstitial binding proteins. Using $V_{isf} \cdot C_{pr(i),isf} = V_{pr(i)} \cdot C_{pr(i)}$ and $V_{isf} \cdot C_{u,isf} = V_{iw} \cdot C_{iw}$, we obtain

$$\frac{V_{pr(i)}}{V_{isf}} \cdot C_{pr(i)} = K_{A,pr(i)} (1 - fcat_{iw}) PR_{isf} \cdot \frac{V_{iw}}{V_{isf}} \cdot C_{iw} \quad (B.32)$$

or equivalently

$$K_{pr(i):iw} = \frac{C_{pr(i)}}{C_{iw}} = \frac{V_{iw}}{V_{pr(i)}} \cdot K_{A,pr(i)} (1 - fcat_{iw}) PR_{isf}. \quad (B.33)$$

Therefore, by assuming $K_{A,pr(i)} = K_{A,pr(p)}$ and $PR_{isf} = \frac{V_{tis}}{V_{isf}} PR_{tis}$, the distribution coefficient can be expressed as

$$\begin{aligned} K_{pr(i):iw} &= \frac{V_{iw}}{V_{pr(i)}} K_{A,pr(i)} (1 - fcat_{iw}) PR_{isf} \\ &= \frac{V_{iw}}{V_{pr(i)}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{(1 - fcat_{iw})}{(1 - fcat_{pw})} \frac{PR_{isf}}{PR_{pla}} \\ &= \frac{V_{tis}}{V_{pr(i)}} \frac{V_{iw}}{V_{isf}} K_{A,pr(p)} PR_{pla} (1 - fcat_{pw}) \frac{(1 - fcat_{iw})}{(1 - fcat_{pw})} \frac{PR_{tis}}{PR_{pla}}. \end{aligned} \quad (B.34)$$

with $K_{A,pr(p)} PR_{pla} (1 - fcat_{pw})$ inferred from fuP and $K_{pr(p):pw}$, based on eq. 4.20.

Interstitial binding protein-to-homogenate water distribution coefficient The homogenate tissue fluid htf is defined as the fluid containing tissue water and binding proteins, $htf = hw + pr = iw + pr(i) + cw + pr(c) = isf + cw$, with the interstitial fluid including interstitial water and binding protein and the cellular counterpart including only cellular water (no cellular binding proteins). Protein binding in homogenate tissue fluid can be assumed in steady state. We adopt the charged protein-binding parametrization to describe the interstitial protein binding within the homogenate tissue fluid:

$$C_{pr(i),htf} = K_{A,pr(i)} (1 - fcat_{hw}) PR_{htf} \cdot C_{u,htf} \quad (B.35)$$

with

$$C_{pr(i),htf} := \frac{A_{pr(i)}}{V_{htf}}, \quad C_{u,htf} := \frac{A_{hw}}{V_{htf}} = \frac{A_{iw}}{V_{htf}} + \frac{A_{cw}}{V_{htf}}, \quad (B.36)$$

$PR_{htf} = A_{PR}/V_{htf}$ being the total concentration of binding proteins in the homogenate tissue fluid, and $A_{PR(i)}$ being the total amount of binding proteins (originating from the interstitial space). Using $V_{htf} \cdot C_{pr(i),htf} = V_{pr(i)} \cdot C_{pr(i)}$ and $V_{htf} \cdot C_{u,htf} = V_{hw} \cdot C_{hw}$, we obtain

$$\frac{V_{pr(i)}}{V_{htf}} \cdot C_{pr(i)} = K_{A,pr(i)} (1 - fcat_{hw}) PR_{htf} \cdot \frac{V_{hw}}{V_{htf}} \cdot C_{hw}. \quad (B.37)$$

or equivalently

$$K_{pr(i):hw} = \frac{C_{pr(i)}}{C_{hw}} = \frac{V_{hw}}{V_{pr(i)}} \cdot K_{A,pr(i)} (1 - fcat_{hw}) PR_{htf}. \quad (B.38)$$

B.3 FRACTION UNBOUND IN TISSUE SUBSPACES

PLASMA

In plasma partitioning, it is assumed that the drug is dissolved in plasma water, binds to plasma proteins—the specific binding proteins depends on the drug type (acid, neutral, base, etc)—and distributes into plasma lipid components (e.g., neutral lipids and phospholipids) in its neutral form. Therefore, the following mass balance equation holds:

$$A_{pla} = A_{pw} + A_{pr(p)} + A_{nl(p)} + A_{np(p)} \quad (B.39)$$

with the amount of drug in plasma A_{pla} , in interstitial water A_{pw} , bound to plasma binding proteins $A_{pr(p)}$, and distributed into neutral lipids $A_{nl(p)}$ and $A_{np(p)}$. Using $A_{sub} = V_{sub} \cdot C_{sub}$ for the individual subspace contributions and dividing by V_{pla} and C_{pw} result in

$$\frac{C_{pla}}{C_{pw}} = \frac{V_{pw}}{V_{pla}} + \frac{V_{pr(p)}}{V_{pla}} \frac{C_{pr(p)}}{C_{pw}} + \frac{V_{nl(p)}}{V_{pla}} \frac{C_{nl(p)}}{C_{pw}} + \frac{V_{np(p)}}{V_{pla}} \frac{C_{np(p)}}{C_{pw}}$$

Given the fraction unbound in the plasma space $\text{fuP} := C_{\text{pw}}/C_{\text{pla}}$, we obtain the following relationship:

$$\frac{1}{\text{fuP}} = \frac{V_{\text{pw}}}{V_{\text{pla}}} + \frac{V_{\text{pr(p)}}}{V_{\text{pla}}} K_{\text{pr(p):pw}} + \frac{V_{\text{nl(p)}}}{V_{\text{pla}}} K_{\text{nl(p):pw}} + \frac{V_{\text{np(p)}}}{V_{\text{pla}}} K_{\text{np(p):pw}} \quad (\text{B.40})$$

In the absence of binding or distribution into lipids, the unbound fraction in plasma equals the ratio of plasma volume to plasma water volume ($\text{fuP} = V_{\text{pla}}/V_{\text{pw}} > 1$). For humans, $V_{\text{pw}}/V_{\text{pla}} = 0.945$ (Poulin et al., 2002) yields $\text{fuP} \approx 1.06$. Neglecting this correction can lead to inconsistencies in parameter estimation, such as obtaining negative values for the partition coefficient between plasma proteins and plasma water $K_{\text{pr(p):pw}}$.

RED BLOOD CELLS

In red blood cells (or erythrocytes) partitioning, it is assumed that the drug is dissolved in RBC water, binds to RBC proteins and distributes into RBC lipid components (*i.e.*, neutral lipids, and neutral and acidic phospholipids) in its neutral form. Therefore, the following mass balance equation holds:

$$A_{\text{rbc}} = A_{\text{rw}} + A_{\text{pr(r)}} + A_{\text{nl(r)}} + A_{\text{np(r)}} + A_{\text{ap(r)}} \quad (\text{B.41})$$

with the amount of drug in the red blood cells A_{rbc} , in RBC water A_{rw} , bound to RBC proteins $A_{\text{pr(r)}}$, and distributed into neutral lipids $A_{\text{nl(r)}}$, into neutral phospholipids $A_{\text{np(r)}}$, and acidic phospholipids $A_{\text{ap(r)}}$. By using $A_{\text{sub}} = V_{\text{sub}} \cdot C_{\text{sub}}$ for the individual subspace contributions and dividing eq. B.41 by V_{rbc} and C_{rw} , we obtain the equivalent relationship between the drug concentrations:

$$\frac{C_{\text{rbc}}}{C_{\text{rw}}} = \frac{V_{\text{rw}}}{V_{\text{rbc}}} + \frac{V_{\text{pr(r)}}}{V_{\text{rbc}}} \frac{C_{\text{pr(r)}}}{C_{\text{rw}}} + \frac{V_{\text{nl(r)}}}{V_{\text{rbc}}} \frac{C_{\text{nl(r)}}}{C_{\text{rw}}} + \frac{V_{\text{np(r)}}}{V_{\text{rbc}}} \frac{C_{\text{np(r)}}}{C_{\text{rw}}} + \frac{V_{\text{ap(r)}}}{V_{\text{rbc}}} \frac{C_{\text{ap(r)}}}{C_{\text{rw}}}$$

Under the above assumptions, the ratio of total RBC-to-RBC water concentration $\text{fuR} := C_{\text{rw}}/C_{\text{rbc}}$ can be derived as

$$\frac{1}{\text{fuR}} = \frac{V_{\text{rw}}}{V_{\text{rbc}}} + \frac{V_{\text{pr(r)}}}{V_{\text{rbc}}} K_{\text{pr(r):rw}} + \frac{V_{\text{nl(r)}}}{V_{\text{rbc}}} K_{\text{nl(r):rw}} + \frac{V_{\text{np(r)}}}{V_{\text{rbc}}} K_{\text{np(r):rw}} + \frac{V_{\text{ap(r)}}}{V_{\text{rbc}}} K_{\text{ap(r):rw}} \quad (\text{B.42})$$

INTERSTITIAL SPACE

In interstitial space partitioning, it is assumed that the drug distributes into interstitial water and binds interstitial binding proteins. Therefore, the following mass balance equation holds:

$$A_{\text{int}} = A_{\text{iw}} + A_{\text{pr(i)}} + A_{\text{rest}} \quad (\text{B.43})$$

with the amount of drug in the interstitial space A_{int} , in interstitial water A_{iw} , and bound to binding proteins from the interstitial space $A_{\text{pr(i)}}$. We assume that $A_{\text{rest}} = 0$, *i.e.*, the distribution in the remaining parts is negligible (*e.g.*, additional interstitial subspaces other than ISF, such as collagen fibers of the extracellular matrix). By using $A_{\text{sub}} = V_{\text{sub}} \cdot C_{\text{sub}}$ for the individual subspace contributions (*i.e.*, iw and pr(i)), we obtain the equivalent relationship between the drug concentrations:

$$V_{\text{int}} C_{\text{int}} = V_{\text{iw}} C_{\text{iw}} + V_{\text{pr(i)}} C_{\text{pr(i)}} \quad (\text{B.44})$$

Fraction unbound in the interstitial space of intact tissue Dividing eq. B.44 by V_{int} and C_{iw} results in

$$\frac{C_{\text{int}}}{C_{\text{iw}}} = \frac{V_{\text{iw}}}{V_{\text{int}}} + \frac{V_{\text{pr(i)}}}{V_{\text{int}}} K_{\text{pr(i):iw}}$$

By defining the fraction unbound in the interstitial space of the intact tissue of volume V_{tis} as $\text{fuI} := C_{\text{iw}}/C_{\text{int}}$, we obtain the following relationship:

$$\boxed{\frac{1}{\text{fuI}} = \frac{V_{\text{tis}}}{V_{\text{int}}} \left(\frac{V_{\text{iw}}}{V_{\text{tis}}} + \frac{V_{\text{pr(i)}}}{V_{\text{tis}}} K_{\text{pr(i):iw}} \right)} \quad (\text{B.45})$$

with the interstitial binding protein-to-water partition coefficient $K_{\text{pr(i):iw}}$ defined in eq. B.34 (or eq. 4.18).

Fraction unbound in the interstitial space of homogenate tissue Dividing eq. B.44 by V_{int} and C_{hw} results in

$$\begin{aligned} \frac{C_{\text{int}}}{C_{\text{hw}}} &= \frac{V_{\text{iw}}}{V_{\text{int}}} \frac{C_{\text{iw}}}{C_{\text{hw}}} + \frac{V_{\text{pr(i)}}}{V_{\text{int}}} K_{\text{pr(i):hw}} \\ \frac{C_{\text{int}}}{C_{\text{hw}}} &= \frac{V_{\text{iw}}}{V_{\text{int}}} K_{\text{iw:hw}} + \frac{V_{\text{pr(i)}}}{V_{\text{int}}} K_{\text{pr(i):hw}} \end{aligned}$$

By defining the fraction unbound in the fictitious interstitial space of this homogenate tissue of volume V_{hom} as

$$\text{fuH}_{\text{int}} := C_{\text{hw}}/C_{\text{int}}, \quad (\text{B.46})$$

we obtain the following relationship:

$$\boxed{\frac{1}{\text{fuH}_{\text{int}}} = \frac{V_{\text{hom}}}{V_{\text{int}}} \left(\frac{V_{\text{iw}}}{V_{\text{hom}}} K_{\text{iw:hw}} + \frac{V_{\text{pr(i)}}}{V_{\text{hom}}} K_{\text{pr(i):hw}} \right)} \quad (\text{B.47})$$

with the interstitial binding protein-to-homogenate water partition coefficient $K_{\text{pr(i):hw}}$ defined in eq. B.38 (or eq. 4.32) and the interstitial water-to-homogenate water partition coefficient $K_{\text{iw:hw}} = 1$ (see eq. B.18).

CELLULAR SPACE

In cellular partitioning, it is assumed that the drug is dissolved in cellular water, binds to cellular binding proteins and distributes into cellular lipid components (*i.e.*, neutral lipids, and neutral and acidic phospholipids) in its neutral form. Therefore, the following mass balance equation holds:

$$A_{\text{cel}} = A_{\text{cw}} + A_{\text{nl(c)}} + A_{\text{np(c)}} + A_{\text{ap(c)}} + A_{\text{pr(c)}} + A_{\text{rest}} \quad (\text{B.48})$$

with the amount of drug in the cellular space A_{cel} , in cellular water A_{cw} , bound to binding proteins from the cellular space $A_{\text{pr(c)}}$, and distributed into neutral lipids $A_{\text{nl(c)}}$, into neutral phospholipids $A_{\text{np(c)}}$, and acidic phospholipids $A_{\text{ap(c)}}$. We assume that $A_{\text{rest}} = 0$, *i.e.*, the distribution in the remaining cellular parts is negligible. By using $A_{\text{sub}} = V_{\text{sub}} \cdot C_{\text{sub}}$ for the individual subspace contributions (*i.e.*, cw, pr(c), nl(c), ap(c)) and dividing by V_{cel} and C_{cw} , we obtain the equivalent relationship between the drug concentrations:

$$V_{\text{cel}} C_{\text{cel}} = V_{\text{cw}} C_{\text{cw}} + V_{\text{pr(c)}} C_{\text{pr(c)}} + V_{\text{nl(c)}} C_{\text{nl(c)}} + V_{\text{np(c)}} C_{\text{np(c)}} + V_{\text{ap(c)}} C_{\text{ap(c)}} + V_{\text{pr(c)}} C_{\text{pr(c)}} + V_{\text{rest}} C_{\text{rest}}. \quad (\text{B.49})$$

Fraction unbound in the cellular space of intact tissue Dividing eq. B.49 by V_{cel} and C_{cw} results in

$$\frac{C_{\text{cel}}}{C_{\text{cw}}} = \frac{V_{\text{cw}}}{V_{\text{cel}}} + \frac{V_{\text{nl(c)}}}{V_{\text{cel}}} K_{\text{nl(c):cw}} + \frac{V_{\text{np(c)}}}{V_{\text{cel}}} K_{\text{np(c):cw}} + \frac{V_{\text{ap(c)}}}{V_{\text{cel}}} K_{\text{ap(c):cw}} + \frac{V_{\text{pr(c)}}}{V_{\text{cel}}} K_{\text{pr(c):cw}}.$$

By defining the fraction unbound in the interstitial space of the intact tissue of volume V_{tis} as $\text{fuC} := C_{\text{cw}}/C_{\text{cel}}$, we obtain the following relationship:

$$\boxed{\frac{1}{\text{fuC}} = \frac{V_{\text{tis}}}{V_{\text{cel}}} \left(\frac{V_{\text{cw}}}{V_{\text{tis}}} + \frac{V_{\text{nl(c)}}}{V_{\text{tis}}} K_{\text{nl(c):cw}} + \frac{V_{\text{np(c)}}}{V_{\text{tis}}} K_{\text{np(c):cw}} + \frac{V_{\text{ap(c)}}}{V_{\text{tis}}} K_{\text{ap(c):cw}} + \frac{V_{\text{pr(c)}}}{V_{\text{tis}}} K_{\text{pr(c):cw}} \right)} \quad (\text{B.50})$$

with the cellular distribution coefficients $K_{\text{nl(c):cw}}$, $K_{\text{np(c):cw}}$, $K_{\text{ap(c):cw}}$, and $K_{\text{pr(c):cw}} = 0$ in eqs. B.23, B.27 (or eqs. 4.13, 4.14, 4.17, 4.16 in the main text).

Fraction unbound in the cellular space of homogenate tissue Dividing eq. B.49 by V_{cel} and C_{hw} results in

$$\frac{C_{\text{cel}}}{C_{\text{hw}}} = \frac{V_{\text{cw}}}{V_{\text{cel}}} K_{\text{cw:hw}} + \frac{V_{\text{nl(c)}}}{V_{\text{cel}}} K_{\text{nl(c):hw}} + \frac{V_{\text{np(c)}}}{V_{\text{cel}}} K_{\text{np(c):hw}} + \frac{V_{\text{ap(c)}}}{V_{\text{cel}}} K_{\text{ap(c):hw}} + \frac{V_{\text{pr(c)}}}{V_{\text{cel}}} K_{\text{pr(c):hw}}.$$

By defining the fraction unbound in the fictitious cellular space of this homogenate tissue of volume V_{hom} as $\text{fuH}_{\text{cel}} := C_{\text{hw}}/C_{\text{cel}}$, we obtain the following relationship:

$$\frac{1}{\text{fuH}_{\text{cel}}} = \frac{V_{\text{hom}}}{V_{\text{cel}}} \left(\frac{V_{\text{cw}}}{V_{\text{hom}}} K_{\text{cw:hw}} + \frac{V_{\text{nl(c)}}}{V_{\text{hom}}} K_{\text{nl(c):hw}} + \frac{V_{\text{np(c)}}}{V_{\text{hom}}} K_{\text{np(c):hw}} + \frac{V_{\text{ap(c)}}}{V_{\text{hom}}} K_{\text{ap(c):hw}} + \frac{V_{\text{pr(c)}}}{V_{\text{hom}}} K_{\text{pr(c):hw}} \right) \quad (\text{B.51})$$

with the distribution coefficients $K_{\text{nl(c):hw}}$, $K_{\text{np(c):hw}}$, $K_{\text{ap(c):hw}}$, $K_{\text{pr(c):hw}}$ defined in Section B.2, and the cellular water-to-homogenate water partition coefficient $K_{\text{cw:hw}} = 1$ (see eq. B.18).

B.4 FRACTION UNBOUND IN BLOOD

Peripheral blood is typically considered as a lumped compartment including peripheral plasma and red blood cells with the following assumptions:

- distribution into other cellular components of the blood (white blood cells, platelets) is negligible;
- the exchange across the cellular membrane of red blood cells is not rate-limiting compared to other PK processes. It is characterised by the RBC water-to-plasma water distribution coefficient $K_{\text{puu}_{\text{rbc}}}$ (defined by eq. B.17). This assumption is typically made for small molecules due to their fast distribution in red blood cells.

In blood partitioning, the following mass balance equation holds:

$$A_{\text{blo}} = A_{\text{pla}} + A_{\text{rbc}} \quad (\text{B.52})$$

with the amount of drug in blood A_{blo} , in plasma A_{pla} , and in RBC A_{rbc} . Using $A_{\text{sub}} = V_{\text{sub}} \cdot C_{\text{sub}}$ for the individual subspace contributions and dividing by V_{blo} and C_{pw} result in

$$\frac{C_{\text{blo}}}{C_{\text{pw}}} = \frac{V_{\text{pla}}}{V_{\text{blo}}} \frac{C_{\text{pla}}}{C_{\text{pw}}} + \frac{V_{\text{rbc}}}{V_{\text{blo}}} \frac{C_{\text{rbc}}}{C_{\text{rw}}} \frac{C_{\text{rw}}}{C_{\text{pw}}}.$$

Under these assumptions, the ratio of total blood-to-plasma water concentration $\text{fuB} := C_{\text{pw}}/C_{\text{blo}}$ can be derived as

$$\frac{1}{\text{fuB}} = \frac{V_{\text{pla}}}{V_{\text{blo}}} \frac{1}{\text{fuP}} + \frac{V_{\text{rbc}}}{V_{\text{blo}}} \frac{1}{\text{fuR}} K_{\text{puu}_{\text{rbc}}}.$$

Defining the hematocrit hct as the volume fraction of erythrocytes in blood $V_{\text{rbc}}/V_{\text{blo}}$ results in

$$\frac{1}{\text{fuB}} = (1 - \text{hct}) \frac{1}{\text{fuP}} + \text{hct} \frac{1}{\text{fuR}} K_{\text{puu}_{\text{rbc}}} \quad (\text{B.53})$$

Alternatively, fuB can be derived as the ratio of two *in vitro* experimental assay readouts, fuP and B:P, as

$$\text{fuB} := \frac{C_{\text{pw}}}{C_{\text{blo}}} = \frac{\text{fuP}}{\text{B:P}}. \quad (\text{B.54})$$

Relationship between $K_{A,\text{ap}(r)^-}$ and B:P, fuP, and hct Given B:P, fuP, and hct as input parameters, we can derive the partition coefficient $K_{A,\text{ap}(r)^-}$ as follows:

$$K_{A,\text{ap}(r)^-} = \left[\frac{\text{B:P} - (1 - \text{hct}_{\text{B:P}})}{\text{fuP} \text{hct}_{\text{B:P}}} \frac{1}{K_{\text{puu}_{\text{rbc}}}} - \frac{V_{\text{rw}}}{V_{\text{rbc}}} - \frac{V_{\text{pr}(r)}}{V_{\text{rbc}}} K_{\text{pr}(r):\text{rw}} \right. \\ \left. - \frac{V_{\text{nl}(r)}}{V_{\text{rbc}}} K_{\text{nl}(r):\text{rw}} - \frac{V_{\text{np}(r)}}{V_{\text{rbc}}} K_{\text{np}(r):\text{rw}} \right] \frac{1}{\text{AP}_{\text{rbc}}^- \text{fcat}_{\text{rw}}} \quad (\text{B.55})$$

where $\text{hct}_{\text{B:P}}$ denotes the hematocrit of the blood used in the *in vitro* assay to determine B:P. Since $K_{A,\text{ap}(r)^-}$ is non-negative, the drug-specific input parameters, such as fuP and B:P, are assumed to be not independent.

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DECLARATION

I, Elena Righetti, confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Trento, Academic year 2023 - 2024

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