Systems pharmacology of the amyloid cascade : unfolding oligomer modulation in Alzheimer's disease

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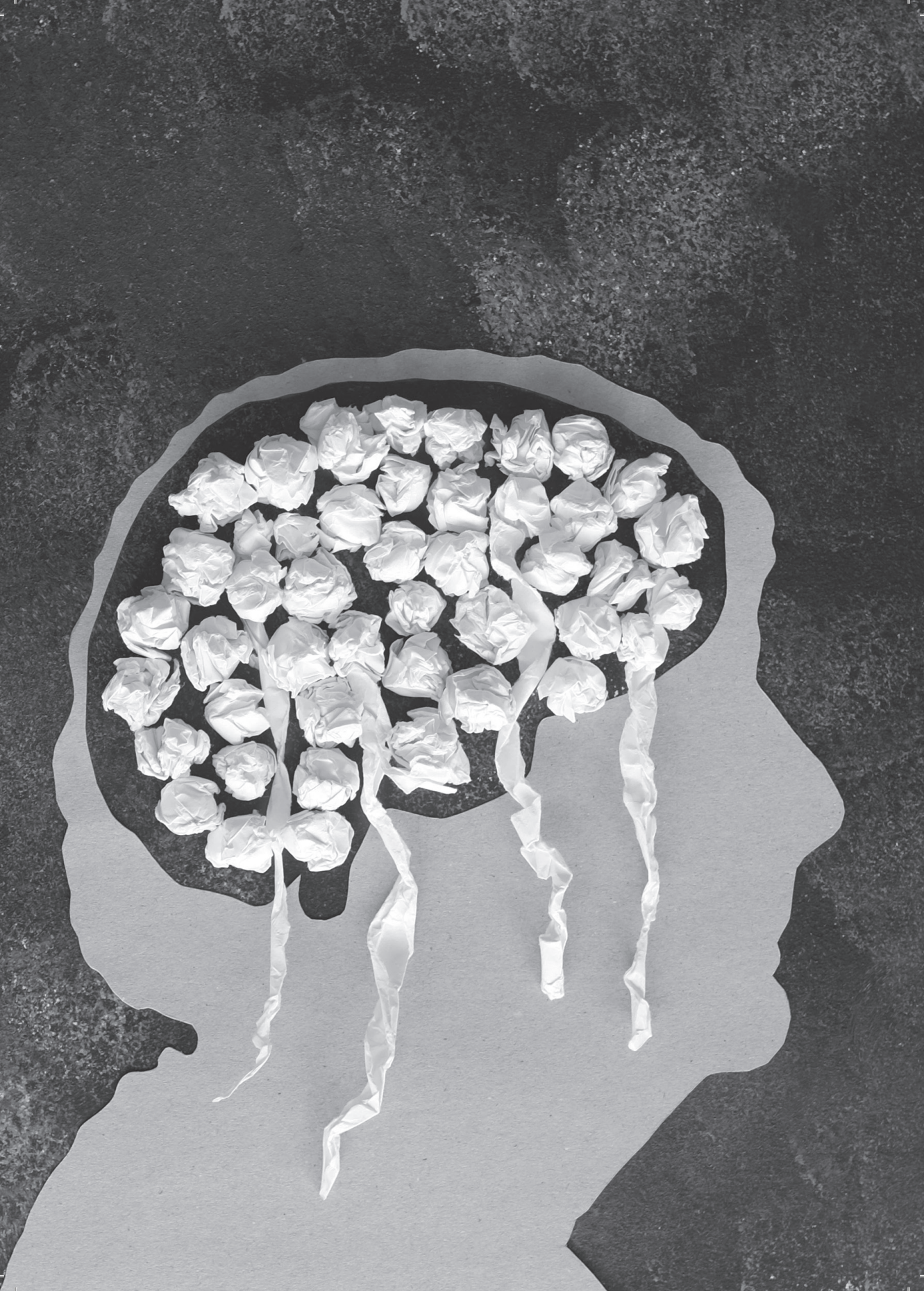

Chapter 2

Systems pharmacology approach to the modulation of oligomers in protein misfolding neurodegenerative disorders

Accumulation of protein aggregates inside or outside of neurons is the leading cause of cellular dysfunction in neurodegenerative disorders. The common cause of protein deposition and the trigger of degenerative signals in the neurons is an unusual folding of proteins, such as α-synuclein in Parkinson's disease (PD) and Huntingtin in Huntington's disease (HD) (Table 2.1). Through folding, proteins obtain a tertiary structure needed to take on their biological functions. To ensure correct folding, multiple chaperone systems are required as well as degradation pathways to destroy misfolded proteins [1]. Due to the complexity of this process, an error can disrupt protein folding causing the protein not to achieve its functional conformation, and the misfolded protein may be toxic or aggregation-prone. These early aggregates are believed to instigate toxicity in neurodegenerative disorders. The phenotypically different but biochemically similar aggregates across protein misfolding neurodegenerative diseases indicate a highly conserved molecular mechanism of pathogenesis [2]. Moreover, the same progression of neuronal death, nervous system deterioration and cognitive impairment is presented in Alzheimer's disease (AD), PD, HD, Prion disease and motor disorders, such as amyotrophic lateral sclerosis [3]. Even though major progress has been made in the unraveling of the pathogenesis of protein misfolding neurodegenerative diseases, effective treatments are still lacking.

Table 2.1: Examples of protein misfolding neurodegenerative diseases and their disease specific proteins

DISEASE	PROTEIN FEATURED	Reference
Alzheimer's disease	Amyloid-β	[4]
Parkinson's disease	α-Synuclein	[5]
Parkinson's disease dementia	α-Synuclein, Amyloid-β	[6]
Transmissible spongiform encephalopathy (Prion disease)	Scrapie prion protein (PrP^{Sc})	[7]
Huntington's disease	Huntingtin	[8]
Familial amyloid polyneuropathy	Transthyretin	[9]
Cerebral amyloid angiopathy	Amyloid-β	[10]
Amyotrophic lateral sclerosis	Superoxide dismutase 1 (SOD1)	[11]

One of the most studied protein misfolding neurodegenerative disorders is AD *(vide infra)*. Amyloid-β peptide (Aβ) is the main component of the amyloid plaques in the brain of AD patients. Soluble monomeric Aβ does not cause the reduced neuroviability; the issue begins when the Aβ peptide self-aggregates. The 'amyloid cascade hypothesis'

poses that this Aβ aggregation is the initiating mechanistic event, in which the different stages of aggregation, from soluble Aβ oligomers (Aβ_O) to insoluble fibrils in plaques, are believed to impair synaptic function and ultimately damage neurons, resulting in chronic neurodegeneration leading to cognitive impairment and finally dementia [12]. The amyloid cascade hypothesis provides a framework for other protein misfolding neurodegenerative diseases, in which pathological changes are driven by an error in protein conformation followed by abnormal assemblies.

One of the main therapeutic strategies for AD aims at Aβ reduction through either inhibition of Aβ production or enhancing of Aβ clearance. Due to the complexity of the underlying biochemical network, the effects of these interventions on the individual attributes of the APP processing pathways are difficult to predict. Furthermore, the effect on Aβ_O after inhibiting Aβ production or enhancing Aβ clearance is not fully understood. This step is essential in view of the development of disease-modifying treatments: Aβ_O concentrations in cerebrospinal fluid (CSF) may be considered as tool to monitor the effects of disease-modifying drugs.

This thesis focuses on drugs aimed at Aβ production inhibition and the potential for subsequent reduction of Aβ_O levels. In this chapter, the pathophysiology of AD is described first. Next, the amyloid cascade hypothesis and the production of Aβ through the amyloid-β precursor protein (APP) pathway are outlined. Then, the diagnosis and pharmacological treatment of AD is discussed. After that, generally used methods to detect and quantify Aβ are summarized. Finally, the use of systems pharmacology modelling to provide a quantitative understanding of the effects of drugs on the APP pathway is outlined.

Alzheimer's Disease

AD is the most prevalent form of dementia. The World Health Organization estimates that in 2015 46.8 million people worldwide were living with AD, or related dementia, and that this number will almost double every 20 years, making it the major chronic health issue of this century [13]. The prevalence of AD is rising due to the 'double ageing' process: there are relatively more and more elderly who are individually living longer.

Although AD mainly affects older people, it is not a normal part of ageing It is a chronic and progressive neurodegenerative disorder, impairing higher brain functions such as memory, thinking and personality. The neuropathology of AD involves massive neuronal cell loss and atrophy, which is especially prevalent in the cortex and hippocam-

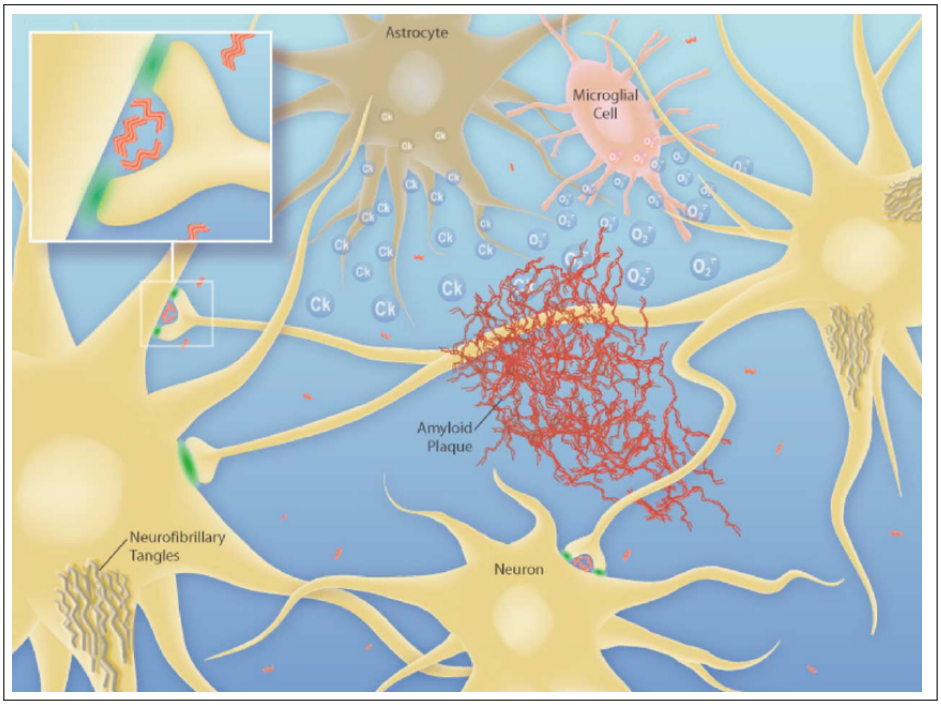

Figure 2.1: Illustration of neurofibrillary tangles within neurons and amyloid plaques [14]**.**
Green cloud: Disruption of synaptic efficacy by diffusible, low-n Aβ_O, depicted as a decrease in normal transmission at synapses; *Red*: Aβ species; *Ck*: Cytokines, released as result of activation of astrocytes; O_2: Superoxide radicals, generated by microglia.

pus, and ventricle enlargement [15,16]. Pathologically, the disease is characterized by the misfolding and abnormal assembly of two proteins, tau and a short fragment of APP, the 42-amino acid long peptide Aβ42, causing abnormal structures that cover the brains of AD patients. Hyperphosphorylated tau protein appears in neurofibrillary tangles within neurons, whereas Aβ is deposited in extracellular neuritic plaques that consist of neuron fragments surrounding a core of Aβ (Figure 2.1) [17,18,19]. The progressive accumulation of neurofibrillary tangles in neurons and amyloid fibers in neuritic plaques are two of several brain changes believed to contribute to the development of AD.

The two basic types of AD are sporadic and familial. AD generally occurs sporadic in patients over the age of 60, but there is also an early-onset phenotype afflicting patients in the 4th or 5th decade of life that develops as result of autosomal dominant inheritance [21]. Both forms of AD show similar neuropathology and altered Aβ42 kinetic rates (Figure 2.2).

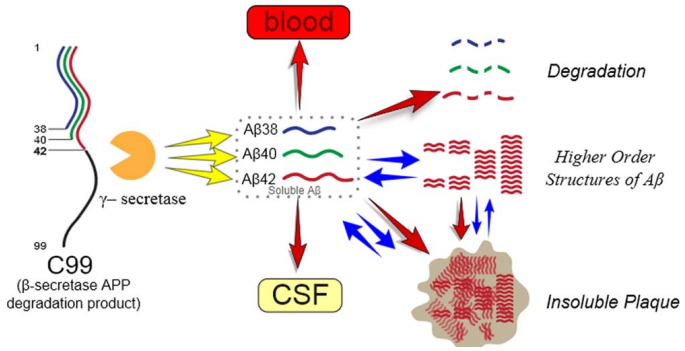

Figure 2.2: Biological model for increased Aβ42 exchange and increased irreversible loss in the presents of amyloidosis[20].
Faster irreversible loss *(red arrows)* and exchange *(blue arrows)* are present in amyloidosis.

Familial AD (FAD) mutations are found in APP as well as in the presenilin genes PS1 and PS2, genes encoding for the catalytic subunit of γ-secretase, a protease that cleaves APP and generates the Aβ peptides[22]. The FAD mutations increase the production of Aβ42, which is more neurotoxic compared to the shorter Aβ40, leading to elevated total amounts of Aβ and altering Aβ peptide ratios[23,24,22]. No mutations in the tau gene have been linked to AD[18].

In AD patients, decreased CSF Aβ42 concentrations have been consistently found. Postmortem investigations have established inverse correlations between CSF Aβ42 and neuritic plaque burden indicating that low concentrations of CSF Aβ42 are resulting from its deposition in brain parenchyma [25,26].

There are three recognized disease stages of AD: preclinical, mild cognitive impairment (MCI) and AD dementia[27] (Figure 2.3). In the preclinical stage subjects are asymptomatic and cognitively normal, but some have AD pathological changes such as Aβ accumulation and neuronal injury and dysfunction. This will eventually lead to clinical symptoms, but accumulation of Aβ begins years before the onset of clinical symptoms. The second, prodromal, stage of AD, MCI, is defined by noticeable dysfunction in memory and impairments related to cognitive function that do not meet the criteria for dementia. The patients have elevated CSF tau or signs of neuronal injury on imaging methods (positron emission tomography [PET] imaging, magnetic resonance imaging [MRI] of the brain). The final stage, dementia, is characterized by unresponsiveness, loss of mobility and control of body functions. The disease course can last 2-20 years, leading to death.

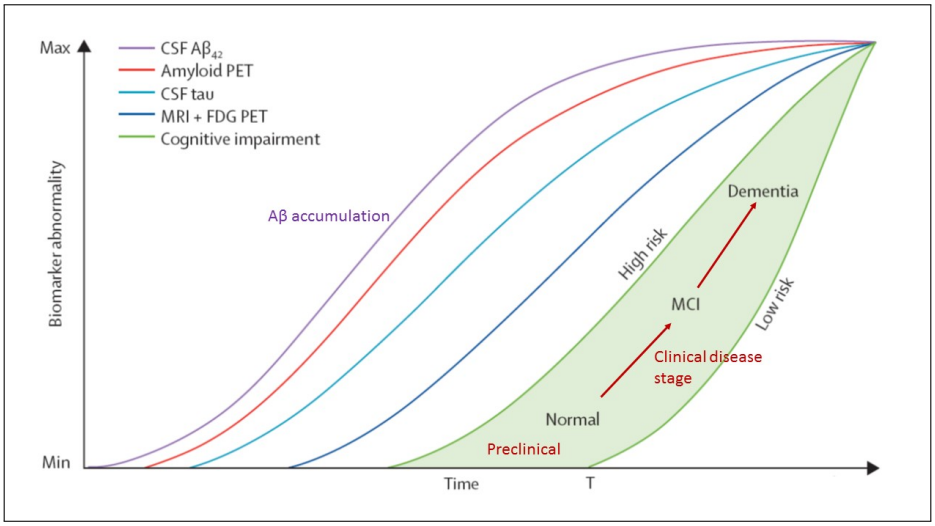

Figure 2.3: Schematic of biomarkers of the pathological cascade and clinical disease stages in AD. Adapted from Jack et al. (2013).
Cognitive impairment is illustrated as a green area with low-risk and high-risk borders.

The amyloid cascade hypothesis

The amyloid cascade hypothesis poses that Aβ levels are increased early in the disease process, forming toxic oligomers and plaques (Figure 2.4)[30,31,32,33,12,34]. These accumulate over time, interfering with the neuron-to-neuron communication at synapses and contributing to cell death, ultimately leading to cognitive and functional decline. It is generally believed that aggregated Aβ is the primary influence that is responsible for disease progression[12]. Soluble toxic Aβ oligomers have been proposed to account for the neurotoxicity of Aβ peptide[29]. Tau protein, aggregating to tangles, accumulate later than Aβ[35,36]. The AD biomarkers become abnormal sequentially, while people remain clinically asymptomatic (Figure 2.3). The amyloid cascade hypothesis is a framework for all amyloid disorders, in which protein misfolding and different stages of aggregates are the drivers of pathological changes.

APP processing pathway

Aβ exists in both soluble and fibrillar forms. Soluble Aβ is a normal metabolic product, present in cerebrospinal fluid (CSF), sera of normal individuals and patients with AD. Aβ peptide is the final product of proteolytic cleavage of the transmembrane protein APP, which is synthesized in the brain as well as in the periphery. The physiological role of

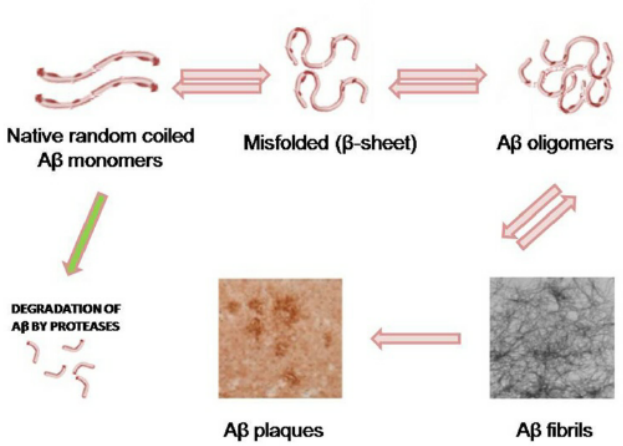

Figure 2.4: Aβ exists in various aggregation states [28].
Aβ monomers can misfold to form β-sheet structures. From the misfolded Aβ, soluble Aβ_O and insoluble amyloid fibrils are generated. These form amyloid plaques and cerebrovascular deposits in the AD brain. Aβ monomers, Aβ_O and fibrils exist in a complex equilibrium [29].

Aβ is yet to be fully elucidated. Aβ fragments have been associated with neurogenesis, anti-viral functions and pro-inflammatory response [37,38,39].

In the APP processing pathway, APP is cleaved sequentially by β-secretase (BACE1) and γ-secretase (GS) resulting in Aβ (Figure 2.5). A third secretase, α-secretase cleaves APP within the Aβ sequence generating non-amyloidogenic sAPPα and precluding Aβ generation.

In the non-amyloidogenic pathway, γ-secretase releases the so called P3 peptide. At the γ-site, APP can be cleaved at different positions, creating Aβ peptides of different amino acid forms (Aβ38-42), of which the 40-residue β peptide (Aβ40) accounts for 80-90% of the total. Aβ42 appears to be the most pathogenic, as it is more prone to aggregation and the predominant Aβ form found in amyloid plaques.

A new APP processing pathway was recently reported by Willem et al. (2015), in which sequential cleavage of APP by η-secretase and BACE1 or α-secretase leads to the formation of A$\eta - \alpha$ and A$\eta - \beta$, respectively. There may be other alternate pathways unknown at this time.

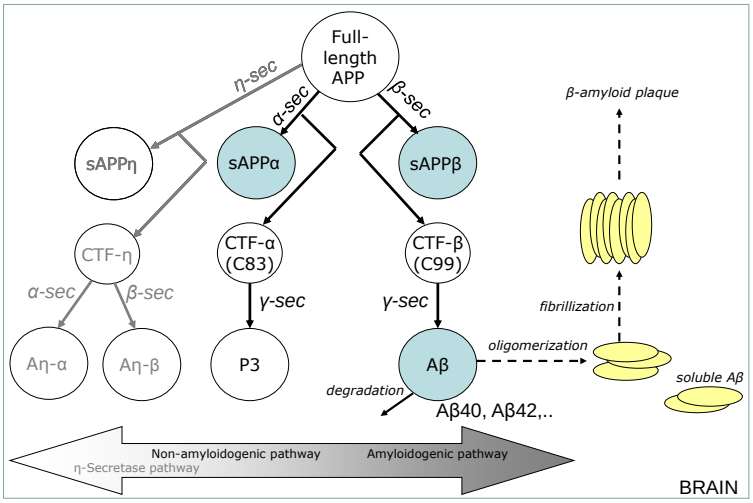

Figure 2.5: Schematic of the APP processing pathway.
In the APP processing pathway, full length APP is cleaved by BACE1 (β-sec) or α-secretase (α-sec) to form sAPPβ and C99 or sAPPα and C83. C99 is then cleaved by γ-secretase (γ-sec) to form Aβ. The amyloid hypothesis states that an imbalance in production and clearance of Aβ can result in aggregation of Aβ42 fragments into amyloid plaque. In an alternative path, APP is sequentially cleaved by η-secretase (η-sec) and BACE1 or α-secretase leading to the formation of A$\eta - \alpha$ and A$\eta - \beta$.
blue circles: APP metabolites measured in CSF.

Pharmacological treatment of AD

AD is presently incurable, as the loss of neurons is irreversible and none of the currently available treatments slow down the progression of the pathologic cascade let alone halt the disease. The FDA has only approved a few drugs to alleviate the symptoms associated with AD. The primary treatment goals of these symptomatic treatments are to enhance the quality of life and to maximize function by improving cognition, mood and behaviour. These treatments are aimed at improving processes at the end of AD's pathologic cascade. FDA approved AD medications include antidepressants, antipsychotics, cholinesterase inhibitors (e.g. Exelon (rivastigmine[41]), weak NMDA receptor antagonists (e.g. memantine), acethylcholinesterase inhibitors (e.g. Aricept (donepezil)) and other cognitive enhancers such as estrogen and vitamin E[42]. None of the treatments available slows or stops the damage to neurons that causes AD symptoms and ultimately makes the disease fatal.

Brain changes associated with AD begin before symptoms such as memory loss appear.

The dementia phase in AD may be prevented from ever developing, by treating AD early with disease modifying treatments. Disease modifying treatments are expected to be most effective during preclinical and MCI stages of AD. It is theorized that all downstream pathological processes may be prevented by lowering the levels of Aβ peptides prone to toxic aggregation, e.g. Aβ42.

Lower Aβ42 levels can be achieved by increasing Aβ42 clearance and/or decreasing Aβ42 production. The latter requires modulation of the APP processing pathway. Examples of such are immune-based therapies, designed to remove Aβ peptide from the brain[21] and inhibitors of secretases of the APP processing pathway, designed to decrease Aβ42 production[42].

To date, no disease-modifying treatment has demonstrated therapeutic benefit and to be safe[43]. Several promising BACE1 inhibitors (BACEi) have recently entered human clinical trials[44]. Given the complex pathophysiology of AD, combination disease-modifying treatment, targeting more than one pathophysiological pathway, may be necessary for effective intervention[45]. Furthermore, the appropriate target(s) may depend on the disease stage.

Diagnosing AD

Presently, AD is diagnosed after the onset of clinical manifestations. There is no single test that can verify whether a person has AD. It may be difficult to determine the exact cause of a person's dementia. Current diagnosis of AD relies on a combination of a thorough medical history, mental status checking, a physical and neurological exam, CSF biomarkers and imaging techniques such as positron emission tomography (PET) and magnetic resonance imaging (MRI)[46]. Blood tests and brain imaging are also used to eliminate other causes of dementia-like symptoms. However, the definite diagnosis of AD can only be made after the patient has died, by histological examination of brain tissue at autopsy to confirm the presence of plaques and tangles.

Three cerebrospinal fluid (CSF) biomarkers have been well established and validated: Aβ42, total Tau and phospho-Tau-181 [47]. The diagnostic validity significantly increases by the combination of these three CSF biomarkers [4]. CSF biomarkers have the potential to improve the diagnostic accuracy at the early stages of AD [48]. This is essential when treating AD early with disease-modifying treatments, to monitor the effects of drugs before clinical symptoms occur. Novel biomarkers to monitor important pathological mechanisms in AD are constantly sought. CSF Aβ_{O} has the potential to be a biomarker

of disease pathogenesis of AD, as it is related to toxicity and synaptic dysfunction.

Aβ as a biomarker

As Aβ is a central factor in AD pathogenesis, reliable detection and quantification of this peptide in biological samples is important for understanding disease progression as well as for the evaluation of therapeutic intervention targeting Aβ. Clinically and generally in *in vivo* animal work we can only measure the response in CSF. CSF is in contact with the brain and by that provides a reflection of cerebral processes. Thus, CSF Aβ serves as key biomarker for disease progression and Aβ targeted therapy.

Concentrations of Aβ peptides are typically determined using direct or sandwich enzyme linked immunosorbant assay (ELISA) systems[49]. Some of these assays are specifically constructed to measure both the first and last amino acid of the Aβ isoform of interest (e.g., Aβ1-40, Aβ1-42)[50]. There are also assays that are C-terminally end-specific but use N-terminal antibodies to capture the N-terminally truncated Aβ fragments, in addition to the full Aβ peptide[51,52].

Bateman et al. (2007) reported a method to quantify Aβ protein production and clearance rates in the CNS based on *in vivo* stable isotope labelling kinetics (SILK), immunoprecipitation of Aβ from cerebrospinal fluid, and quantitative liquid chromatography electrospray-ionization tandem mass spectrometry (LC-ESI-tandem MS). The SILK protocol has also been used to assess the effect of drugs on Aβ production. However, questions have been raised about the interpretation of the findings of the SILK protocol[54].

Modeling in Alzheimer's disease

The relationship between Aβ concentrations in CSF and the pharmacokinetics (PK) of Aβ lowering agents is complex. Preclinical selection of AD's drug candidates is based on an evaluation of the PK, pharmacodynamics (PD) and safety in *in vitro* assays and preclinical animal models. This requires a understanding of the *in vivo* pharmacology and the relevant biological system. In that respect drug development efforts for AD can benefit from modelling approaches.

PKPD modelling

PKPD modelling can be used to describe and understand the time-course of drug exposure and response after the administration of different doses or formulations of a drug

to individuals, based on the use of mathematical and statistical models. A PK model describes the relationship between the dose of a drug and the time profile of drug concentration. A PD model describes the relationship between the drug concentration and the pharmacological efficacy. An PKPD model describes the dynamics of exposure-response relationship(s) of a drug. The PD variable(s) in a PKPD model is usually a biomarker related to either efficacy or toxicity of the drug. Several studies on the PK and the PD of BACE1 and GS secretase inhibitors have been reported,[55,56,57,58,59,60,61]. Liu et al. (2013) proposed a mechanistic PKPD model of BACE1 inhibition in monkeys. They identified the β-secretase cleavage step as the rate limiting step for Aβ formation. However, their model is a simplification of the underlying system as no distinction is made between the β-secretase and γ-secretase steps and Aβ was modelled as a direct product of APP. Also, the transport of APP metabolites from brain to CSF, which may differ per species, was not taken into account. Therefore, their identified β-secretase cleavage rate reflects both transport and cleavage by sequentially β-secretase and γ-secretase. What's more, all parameters were estimated by fitting the PK and PD models to the average of the observed data at each time point, not taking into account the variability in drug concentrations and drug effects among individuals. Das et al. (2011) reported a two-compartment model describing Aβ response to GS inhibition, as observed in plasma and CSF in rhesus monkeys. Their model postulates an inhibitory mechanism of Aβ clearance by GS inhibition. However, in their model aspects of the Aβ production, transport and clearance processes were simplified. A model-based meta-analysis of published and in-house (pre-)clinical GS inhibitors data was performed by Niva et al. (2013). The production and clearance of Aβ was described with a turnover model, with a drug effect on the production rate. Tai et al.[59] also used turnover models to describe Aβ levels following GS inhibition in brain, CSF and plasma in wild type rat. They propose a quasi-static Aβ pool in the brain which does not change after short drug exposure.

It has been demonstrated that mechanism-based PKPD models have much improved properties for extrapolation and prediction[62,63]. However, the mechanistic detail of most PKPD models remains relatively limited compared to full systems biology models.

Systems biology

Systems biology is the study of biological systems, based on the understanding that these are composed of interacting parts, resulting in characteristics not found in the individual parts alone. These systems include signalling, gene regulatory, and metabolic networks[64]. An example of a signalling network is the AlzPathway, reported by Mizuno

et al. (2012). The AlzPathway is a comprehensive map of intra-, inter- and extracellular signalling pathways in AD, consisting of 1347 molecules and 1070 reactions in neuron, brain blood barrier, presynaptic, postsynaptic, astrocyte, and microglial cells and their cellular localizations (Figure 2.6). It was based on a collection of 123 review articles involving AD. The key molecules of the AlzPathway are presented in Figure 2.7, in which each reaction is decomposed into a binary relation between reactant(s) and product(s), and modifier(s) and product(s). The molecules Aβ, apolipoprotein-E, microtubule-associated protein-τ and γ-secretase were considered central in the AD-signalling network. The model was developed for both clarification of the pathogenic mechanisms of AD and identifying drug targets. In general, these type of models are used to explore and identify drug targets and potential biomarkers of disease and drug response.

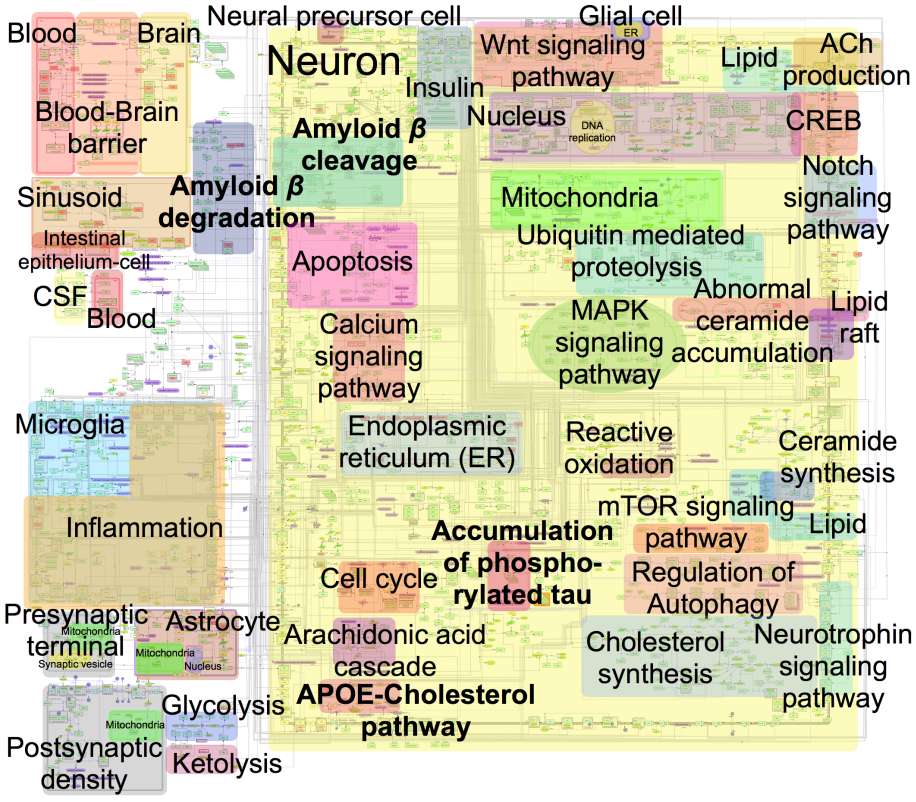

Figure 2.6: Overview of the AlzPathway map [65]

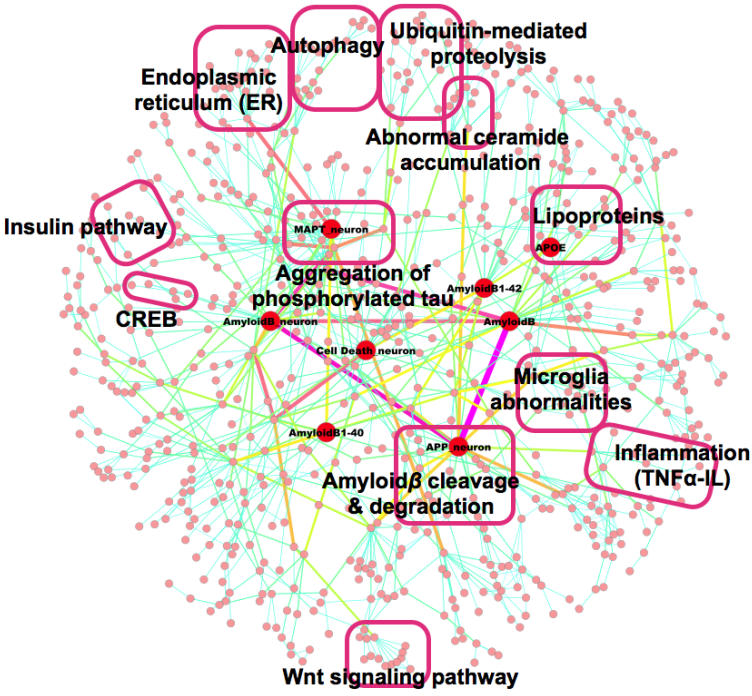

Figure 2.7: Key molecules in the AlzPathway in binary-relation notation [65].

Systems pharmacology modelling

The above mentioned reported PKPD models looked solely at the behaviour of Aβ and not at the behaviour of the APP system as a whole. The intricacy of the underlying biochemical network makes it difficult to predict the effects of drug interventions on the individual attributes of the APP processing pathway. Therefore, the understanding of the APP system is imperative to translate drug concentrations to APP pathway inhibition and to improve the prediction of drug effects on Aβ levels. Systems biology models are not concerned with pharmacology and general principles of PKPD modelling.

Systems pharmacology (SP) models integrate the best available understanding of the biology and pharmacology of the system responses. This involves computational analysis of the time course of the changes in treatment associated biomarkers on the basis of a structural mathematical model that describes the underlying biological processes, while making a strict distinction between drug-specific and systems specific parameters. In essence, SP models are mechanism-based models embedded in a systems biology

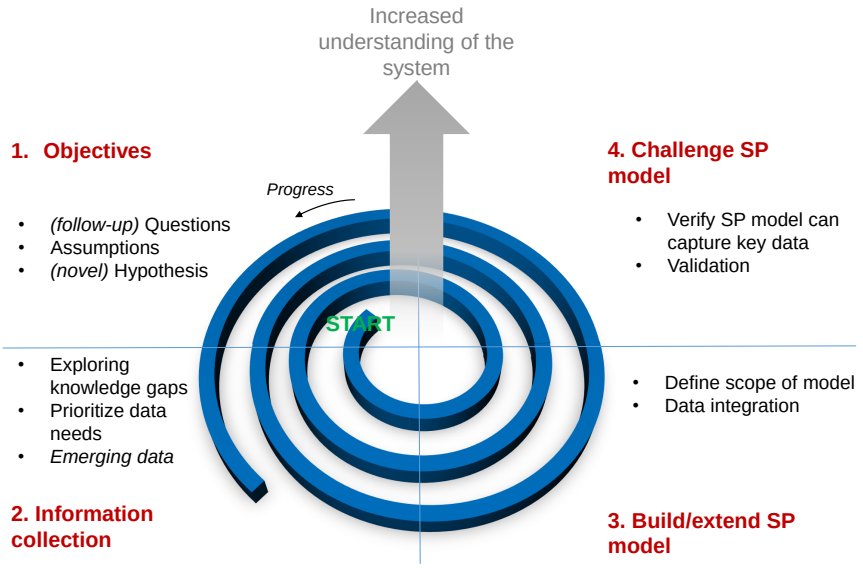

Figure 2.8: Schematic of the evolutionary process of systems pharmacology (SP) model building

framework. No SP models have been developed or applied to the pharmacological action of drugs in the APP processing pathway.

The development and implementation of a SP model is an evolutionary process, as presented in Figure 2.8. The model facilitates the integration of prior knowledge of biological systems, assumptions about the pathology and pharmacology with emerging data. In Figure 2.8, the spiral represents the iterative approach of model development. An iterative model development approach has the benefit that model updates are foreseen: before going into the first cycle of model building, it is know that another round will follow, but on a higher level, in terms of more knowledge and understanding of the system than the first time. Thus, we obtain an evolutionary improvement of the SP model and consequently of the (model based) understanding of the system.

By recognizing that building a SP model is an evolutionary process, it acknowledges the fact that at the beginning, there are knowledge gaps and that the specifications and requirements of the final SP model are not known. There could be hidden behaviours

of the systems, e.g. feedback loops, which cannot be predicted until the SP model is assembled together and parametrized. It could also be that, presuming a mechanism of action, the systems model does not fit the data. In case the model does not capture the data adequately, we can learn something. Then, the only question we need to ask is *why?* As such, a SP model is a hypothesis generating tool.

How many iteration cycles are necessary depends on how long one cycle takes, and how much time is available in order to set the SP model up to answer relevant questions that are as concise and directed as possible. A SP model is a framework for asking questions about the pharmacology of the drug in the context of the system and the disease. The scope of the model must be tailored to answering the question at hand. For some questions, it may be enough to capture the main trend of the behaviour of the system, but for other situations a more detailed model is needed. SP models can also identify the data needs and be reapplied to follow-up questions. Thus, SP models act as a central repository of (novel) hypotheses, knowledge and data.

A SP model of the APP pathway will provide a quantitative understanding of the effects of drugs on the APP processing pathway to improve the prediction and magnitude of Aβ reducing effects. Perturbing the APP system through drug interactions acting at different sequence in the APP pathway *(BACE1 and GS inhibition)* and not looking solely at the behaviour of a single biomarker, but in the context of the system, is expected to provide valuable biological insights into the APP pathway and the chances to modify AD. By using a systems model, we can learn more on the biological complexity of the APP system (e.g. resilience), and by understanding its complexity make more informed decisions concerning pharmacological intervention and support challenges in drug development.

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