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Igor Camargo Fontana

Glutamate transport dysfunction in neurological disorders: from cellular models to in vivo neuroimaging

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Igor Camargo Fontana

Glutamate transport dysfunction in neurological disorders: from cellular models to in vivo neuroimaging

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Orientador (Supervisor): Prof. Dr. Eduardo Rigon Zimmer (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil)

Co-orientador (Co-advisor): Prof. Dr. Diogo Onofre Gomes de Souza (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil)

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“La vie n'est facile pour aucun de nous. Mais quoi, il faut avoir de la persévérance, et surtout de la confiance en soi. Il faut croire que l'on est doué pour quelque chose, et que, cette chose, il faut l'atteindre coûte que coûte”.
Marie S. Curie.

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PREAMBLE

Presentation

This thesis (written in British English) is organised in three parts, in accordance with local rules of the *Graduate Program in Biological Sciences: Biochemistry* of the Universidade Federal do Rio Grande do Sul (UFRGS):

Part I: Abstract, Introduction and Objectives.

Part II: Results presented as scientific articles. Each article corresponds to one chapter.

Part III: Discussion, conclusion, and references. References correspond to citations in Parts I and III. References in Part II are within each chapter.

Scientific contributions in the form of co-authored research articles that are not part of the main body of this thesis can be found in Annexes.

The experiments described in this thesis were performed in the Department of Biochemistry – UFRGS (Porto Alegre, RS, Brazil) and in the School of Biomedical Engineering & Imaging Sciences, St. Thomas' Hospital, King's College London (London, United Kingdom).

PREÂMBULO

Apresentação

Esta tese (escrita em inglês britânico) está organizada em três partes, de acordo com as regras do Programa de Pós-graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS):

Parte I: Resumo, Introdução e Objetivos.

Parte II: Resultados que serão apresentados na forma de artigos científicos. Cada artigo científico representa um capítulo.

Parte III: Discussão, Conclusão e Referências Bibliográficas citadas na Introdução e Discussão.

Em anexos constarão artigos científicos publicados durante a tese e que não fazem parte do corpo principal da tese.

Os experimentos descritos nessa tese foram desenvolvidos no Departamento de Bioquímica – UFRGS (Porto Alegre, RS, Brasil) e na *School of Biomedical Engineering & Imaging Sciences*, St. Thomas' Hospital, King's College London (Londres, Reino Unido).

Table of Contents (Sumário)

PART I

In Part I, the abstract, the list of abbreviations, the introduction and the objectives of this PhD thesis are described.

Abstract

For decades, evaluating the neuropathology of Alzheimer's disease (AD) was only possible trough post-mortem investigation of the brain tissue. Recent advances in positron emission tomography (PET) imaging allow for assessing the living brain in a non-invasive manner. Indeed, the use of selective PET radiotracers permits the specific examination of amyloid-β (Aβ) and tau – the AD core pathology. However, there is a consensus regarding the need for developing innovative PET radiotracers to detect AD in the initial preclinical stages. With this mind, this thesis aimed at investigating early pathophysiological changes in AD with PET imaging. For doing so, we first critically revised the Aβ oligomers (AβOs) literature – the main early toxic species of Aβ. Second, we optimised and validated a method for synthetic AβOs quality control. Third, we demonstrated that synthetic AβOs cause detectable [^{18}F]FDG PET brain hypometabolism in mice, which seems related to astrocyte reactivity. Fourth, we searched the literature for potential PET radiotracer candidate molecules with affinity to astrocyte glutamate transporters. Fifth, we designed and tested the first generation of PET radiotracers targeting glutamate transporters. Finally, we used the knowledge acquired in PET imaging research in AD to propose strategies to understand COVID-19 effects in the brain. In summary, theoretical articles (reviews, letters, and others) produced in this thesis helped to guide basic and clinical research in AD and COVID-19. In addition, our experimental findings advanced our knowledge regarding AβOs as triggers of [^{18}F]FDG PET brain hypometabolism and provided the first generation of an innovative class of PET radiotracers targeting astroglial glutamate transporter.

Keywords: Alzheimer's disease, amyloid-β, astrocytes, brain, COVID-19, gut and PET.

List of abbreviations

AD	Alzheimer's Disease
Aβ	Amyloid-β
AβOs	Aβ oligomers
PET	Positron emission tomography
AT(N)	Amyloid, Tau and Neurodegeneration
NIA-AA	National Institute on Aging and Alzheimer's Association
^{11}C	Carbon-11
^{18}F	Fluorine-18
^{14}N	Nitrogen-14
^{18}O	Oxygen-18
[^{18}F]FDG	^{18}F-Fluorodeoxyglucose
MRI	Magnetic resonance imaging
APP	Amyloid precursor protein
LMW	Low-molecular weight
HMW	High-molecular weight
CNS	Central nervous system
EAAT2	Excitatory amino acid transporter 2
SN1	System N1
SA1	System A1
SE-HPLC	Size-exclusion high-performance liquid chromatography
ICV	Intracerebroventricular
COVID-19	Coronavirus disease – 19
QC	Quality control
IWG	International Working Group
ENS	Enteric nervous system

Introduction

The brain in neurological disorders

"The brain is a complex biological organ possessing immense computational capability: it constructs our sensory experience, regulates our thoughts and emotions, and controls our actions. It is responsible not only for relatively simple motor behaviours like running and eating, but also for complex acts that we consider quintessentially human, like thinking, speaking, and creating works of art.", as described by Professor Eric Kandel [1]. Somehow, the cells responsible for these astonishing capabilities may undergo a sequence of disrupted biological processes, shifting the brain into a disordered state.

Neurological disorders are characterised by the progressive decline of structure/function which affect the brain, spinal cord, and peripheral nerves. Based on incidence and prevalence, some of the most common neurological disorders are migraine, stroke, and Alzheimer's Disease (AD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) [2, 3]. In this thesis, we will mostly focus on AD, the leading neurodegenerative disease [4].

Alzheimer's Disease

AD affects around 45 million people worldwide, a number expected to triple until 2050. Thus, it is crucial finding ways to improve AD diagnosis and, consequently, developing efficient therapies to treat or halt disease progression [4]. AD is characterised by the progressive loss of memory due to a cascade of neurodegenerative events. The biomarkers that distinguish AD pathophysiology from other causes of dementia comprise the deposition of amyloid-β (Aβ) plaques, neurofibrillary tangles composed of tau protein and neurodegeneration - the AT(N) system [5, 6]. Indeed, the AT(N) system provides a framework to define AD based on fluid and positron emission tomography (PET) and magnetic resonance imaging (MRI) biomarkers [6]. Recently, the National Institute on Aging and Alzheimer's Association (NIA-AA) proposed an

expansion towards the ATX(N) system, in which X characterises innovative biomarkers that may help to evolve our knowledge on AD definition [7].

PET imaging biomarkers in AD

PET imaging, with the application of PET radiotracers[1], enables the in vivo quantification of metabolic process (e.g., oxygen and glucose), receptors, transporters, and pathological changes, in a non-invasive way [8]. The PET radiotracers targeting the central nervous system (CNS) are usually incorporated with short half-lives radionuclides such as carbon-11 (^{11}C) and fluorine-18 (^{18}F). The ^{11}C and ^{18}F are generally produced following a cyclotron-induced transmutation nuclear reaction. In the specific case of ^{11}C, a proton bombardment (beam current) is released from the cyclotron and directed to a gas target composed of nitrogen-14 (^{14}N), subsequently, forming the ^{11}C and releasing an α-particle – this nuclear reaction reads ^{14}N(p, α)^{11}C. Similarly, the ^{18}F is generally obtained following the ^{18}O(p, *n*)^{18}F, in which the ^{18}O enriched gas target is hit by the cyclotron-produced beam current, releasing a neutron to form ^{18}F. Currently, a vast library of PET radiotracers is available to evaluate changes in the brain of AD patients, including probes that detect neuroinflammation, tau protein and Aβ peptide, cholinergic transmission, among others [9]. Nevertheless, according to the NIA-AA research framework, the three PET imaging biomarkers that provide the biological construct for defining AD are: cortical Aβ plaques PET ligand binding, cortical tau PET ligand binding and ^{18}F-Fluorodeoxyglucose ([^{18}F]FDG)[2] PET hypometabolism [6, 10]. Nonetheless, it is suggested that Aβ alone might be the earliest neuropathological change detectable in vivo [11, 12].

[1]PET radiotracers: molecules labelled with a radionuclide (e.g., carbon-11 and fluorine-18) with high affinity and selectivity to a biological target that emit positrons which can be translated to images using specific software.

[2] ^{18}F[FDG]: the most used PET radiotracer worldwide – a glucose analogue radiolabelled with fluorine-18

The Aβ peptide: from monomers, through oligomers to insoluble plaques

In 1984, Glenner & Wong purified and characterised the Aβ peptide for the first time [13]. Three years later, Kang and colleagues [14] identified the origins of the Aβ peptide, an A4 polypeptide consisting of 695 residues - the so-called amyloid precursor protein (APP). Currently, it is widely accepted that the Aβ peptide is generated following the sequential cleavage of APP by β- and γ-secretase, releasing Aβ monomers, which are very prone to aggregate into soluble Aβ oligomers (AβOs), protofibrils, fibrils and insoluble Aβ plaques [15, 16]. Indeed, following the pivotal work by Hardy & Higgins in 1992 proposing the amyloid cascade hypothesis, the Aβ plaques have been spotted as the primary culprits of neurodegeneration in AD [17]. In fact, this hypothesis has been constantly challenged [18] and reviewed [19]. In this context, it seems that prior to the formation of insoluble Aβ plaques, the soluble AβOs could be the main causative of synaptic dysfunction and neuronal death in AD [20, 21].

Soluble AβOs as the main toxic entities in AD

Over the last three decades, the use of synthetic Aβ in experimental models of AD helped uncovering multiple mechanisms of toxicity played by AβOs, at the cellular and molecular level (See Chapter I: "Amyloid-β oligomers in cellular models of Alzheimer's disease") [22]. The soluble AβOs can be divided into two metastable forms: the low- and the high-molecular weight (LMW and HMW) oligomers. The LMW AβOs are typically expressed as trimers and tetramers (~ 13.5 kDa), while the HMW AβOs may range from 40 to 150 kDa [23, 24]. Interestingly, LMW and HMW AβOs differentially impact synapses and memory [25]. Indeed, AβOs interact directly or indirectly with a wide range of pre- and post-synaptic receptors and transporters, activating a cascade of downstream events which in turn culminates in neuroinflammation, oxidative stress, glutamatergic excitotoxicity, and neuronal death [26-

29]. In fact, it is suggested that AβOs might impact glutamate transport, inducing glutamatergic excitotoxicity, in the early stages of AD, prior to the formation of Aβ plaques and neurodegeneration [21, 30, 31].

Glutamate transport impairment induced by AβOs

Glutamate is the main excitatory neurotransmitter in the CNS, and it is mostly synthesised via the glutamate-glutamine cycle. Under physiological conditions, a pre-synaptic neuron releases glutamate in the synaptic cleft, which binds to a wide range of ionotropic and metabotropic glutamatergic receptors (Figure 1A) [32, 33]. The remaining glutamate in the extracellular space is then captured via the excitatory amino acid transporter 2 (EAAT2), mainly localised in the membrane of astrocytes [34]. The glutamate is recycled into glutamine in the astrocytic intracellular space and released via the system N1 (SN1) transporter. The neuronal cell via the system A1 (SA1) takes up the glutamine to continue this fine-tuned cycle [35].

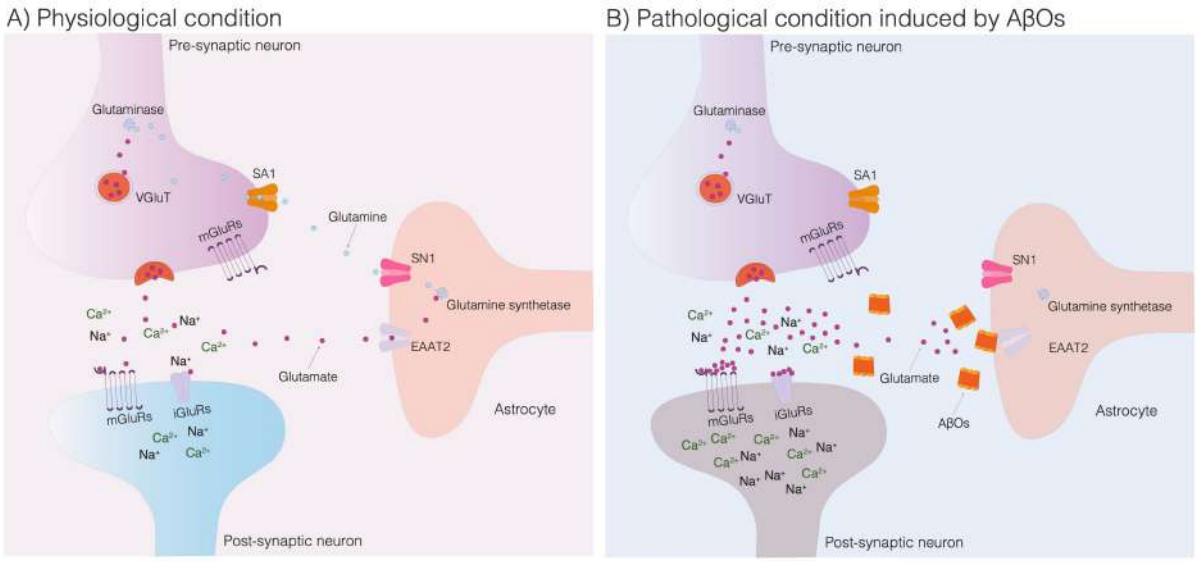

Figure 1: Illustration of glutamatergic neurotransmission in physiological and pathological conditions. A) In physiological conditions the glutamate released by the presynaptic neuron binds to postsynaptic neuronal receptors such as metabotropic and ionotropic glutamate receptors (mGluRs and iGluRs). The excessive extracellular glutamate is then recycled by the excitatory amino acid transporter 2 (EAAT2) in the membrane of astrocytes. Glutamine synthetase catalyses glutamate into glutamine which is released via the system A2 (SA2) transporter. The neuronal cells takes up the glutamine via the system A1 (SA1) transporter and the glutamine-glutamate cycle continues. B) In pathological conditions, such as exposure to soluble AβOs, the EAAT2 is internalised in the membrane of astrocytes, thus, the excessive glutamate remains in the extracellular space causing the hyperactivation of neuronal receptors leading to cell death – a phenomenon termed glutamatergic excitotoxicity.

In vitro models of AD demonstrated that AβOs internalise EAAT2 in the membrane of astrocytes, thus, leading to increased extracellular levels of glutamate in the synaptic cleft (Figure 1B) [30, 36]. In turn, glutamate hyperactivates glutamatergic receptors leading to neuronal death [29, 37]. Interestingly, data from animal models of AD corroborate with these in vitro findings, showing that memory loss and neuronal death are preceded by the AβOs-induced EAAT2 density loss in the absence of Aβ plaques [21, 30, 31, 38] (For a full review on EAAT2, see Chapter IV: "Targeting the excitatory amino acid transporter 2: a medicinal chemistry perspective").

A potential link between brain glucose uptake, EAAT2 and AβOs: what is missing?

The seminal work led by Pellerin and Magistretti [39] provided initial evidence linking glutamate uptake via the astroglial EAAT2 and glucose consumption in the brain [39]. More than two decades later, Zimmer and colleagues [40] confirmed these findings using [^{18}F]FDG PET and demonstrated that ceftriaxone, a post-translational activator of the EAAT2, modulates glucose consumption in adult rats. Thus, it is likely to suggest that EAAT2 loss could decrease brain glucose consumption [41]. Combining these findings, one could argue that if EAAT2 modulates glucose consumption and AβOs induce EAAT2 loss, AβOs might cause brain glucose hypometabolism. To challenge this hypothesis, we first optimised and validated a size-exclusion high performance liquid chromatography (SE-HPLC) method to increase experimental reproducibility in the preparation of synthetic AβOs (See Chapter II: "Rapid Size-exclusion High-Performance Liquid Chromatography Method for the Quality Control of Amyloid-β Oligomers"), then, we standardised a mouse model of the pre-amyloid phase of AD following the intracerebroventricular (icv) infusion of AβOs. In Chapter III ("Soluble amyloid-β oligomers drive brain [^{18}F]FDG PET hypometabolism in mice"), it has been demonstrated

that AβOs induce glucose hypometabolism, indexed by [^{18}F]FDG PET, in the absence of Aβ plaques.

The last piece of the puzzle, to confirm the involvement of EAAT2 in the early stages of AD, requires a refined combination of PET radiochemistry techniques to develop an EAAT2-selective PET radiotracer. The state-of-art for designing and testing a PET radiotracer targeting EAAT2 is fully described in Chapter IV: "Targeting the excitatory amino acid transporter 2: a medicinal chemistry perspective" and Chapter V: "Development of a carbon- 11 PET radiotracer targeting glutamate transporters". We believe that such PET radiotracer would serve as a proof-of-concept to confirm that alterations in the density of EAAT2 in pre-amyloid phase of AD induce [^{18}F]FDG PET hypometabolism.

PET imaging solving the mysteries of the diseased brain

The versatility of PET imaging allows for visualising pathological processes that provides us a biological signature for AD, something unimaginable for several decades [6]. In fact, the use of specific PET radiotracers to evaluate the brain functioning and metabolism has been shown extremely useful to understand the pathophysiology of many other neurological disorders [42]. Furthermore, PET imaging can be a useful tool for improving our knowledge of emerging global health problems, such as the coronavirus disease – 19 (COVID-19) pandemic (Box 1).

Box 1 | PET imaging in COVID-19

In 2020, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as the causative of COVID-19 pandemic and, until July 2021, affected more than 180 million people worldwide. Remarkably, a substantial part of COVID-19 patients reported neurological manifestation, ranging from mild to severe complications [43]. In fact, it is currently known that the SARS-CoV-2 invades the brain and infects neuronal and glial cells [44, 45]. In Chapter VI ("PET Imaging as a Tool for Assessing COVID-19 Brain Changes"), we describe a short-review article proposing PET imaging and the use of specific PET radiotracers to help understanding the pathophysiological alterations in the brain of COVID-19 patients.

This idea was well accepted by the scientific community and in Chapter VII ("About the source and consequences of 18F-FDG brain PET hypometabolism in short and long COVID-19"), we bring a Letter to the Editor, suggesting a biological interpretation for the glucose hypometabolism observed in the brain of COVID-19 patients, indexed by [^{18}F]FDG PET. In addition, due to the similarities of regional glucose hypometabolism and clinical symptoms in long-COVID-19 patients and Alzheimer's Disease, we also raise the alarm for a potential increased risk for developing neurodegenerative diseases.

Objectives

Main objective:

To investigate early pathophysiological changes in Alzheimer's disease using PET imaging biomarker perspective.

Specific objectives:

- To review the literature of soluble AβOs in in vitro models of AD to define potential AβOs targets in the early stages of AD (Chapter I).
- To optimise and validate a size-exclusion high-performance liquid chromatography (SE-HPLC) method for the quality control (QC) of synthetic AβOs to increase experimental reproducibility (Chapter II).
- To evaluate AβOs impact in the [^{18}F]FDG PET signal in mice (Chapter III).
- To review the excitatory amino acid transporter 2 as a target for the early diagnosis of AD and to define a candidate molecule for developing a PET radiotracer selective for EAAT2 (Chapter IV).
- To develop the first PET radiotracer radiolabelled with carbon-11 targeting glutamate transporters and evaluate the biodistribution in mice using microPET imaging (Chapter V).
- To propose the use of PET imaging to improve the understanding of COVID-19 neurological manifestations (Chapter VI and Chapter VII).

PART III

In this part, the discussion and final conclusions of this thesis are presented. In addition, the references used in the main body of the text are described.

Discussion

In 1898, Dr. Marie Skłodowska-Curie coined the term radioactivity to describe the rays emitted by uranium salts [51]. Dr. Curie's initial studies led to the discovery of two novel chemical elements – Polonium [52] and Radium [53]. These findings paved the way for Rutherford's work in the characterisation of the different types of radioactive decay [54]. Indeed, he demonstrated that uranium salts emitted alpha (α) radiation - doubly charged helium ions (He^{2+}) - and beta (β) radiation which, in turn, emits negative (electron) or positive (positron) particles [54]. The third type of radioactive decay, later identified by Dr. Villard, was the gamma (γ) radiation. Unlike α- and β-radiation, made of both energy and mass, γ-radiation is pure energy, so high it penetrates the skin [55].

Altogether, these historical and independent accomplishments led to the development of powerful scanners that combine positrons/electrons and γ-radiation to reproduce tomographic images of the human body [56]. Currently, this imaging procedure is known as PET. The PET imaging scanner detects γ-rays emitted in opposite directions from the positron-electron annihilation [57]. A high-resolution camera detects these γ-rays which are further reconstructed into computerised images of a specific organ or tissue [57]. Of note, the amount of energy emitted from each area can be quantified and may indicate the well-functioning of the body region imaged. Furthermore, PET distinguishes from other imaging diagnostic tools for its great specificity, sensitivity, and robustness [57, 58]. Indeed, the PET radiotracers are responsible for this exceptionality [58]. Currently, several hundreds of PET radiotracers have been developed, but still, the glucose analogue developed in the early 1970's [59] - [^{18}F]FDG – is the most widely used PET probe for diagnostic purposes [60]. In fact, [^{18}F]FDG PET added significant knowledge to the understanding of the human brain, in both health and disease [60]. More specifically, [^{18}F]FDG has provided insights on spatiotemporal metabolic changes in different neurological disorders [61]. Furthermore, current advances on PET radiochemistry

allow for the development of PET radiotracers capable of detecting disease-related processes, such as alteration in neurotransmission [62], deposition of protein aggregates [63] and cell-specific responses to pathological conditions [64]. Indeed, PET imaging has been a crucial piece of the puzzle to building up our current knowledge on AD pathophysiology [7, 65].

The 2018 NIA-AA provided the AT(N) system in which [^{18}F]FDG PET hypometabolism and cortical deposition of Aβ plaques and tau protein represent a biological construct that helps defining AD [6]. Nevertheless, the latest recommendations of the International Working Group (IWG) proposed that these biomarkers should be combine with specific AD phenotypes (e.g., amnestic syndrome of hippocampal type) for diagnosing AD. In addition, it is a consensus that the current biological model that supports the ATN system of the NIA-AA is unlikely to predict symptomatic stages and urges for the detection of biological changes, prior to the deposition of Aβ plaques, i.e., in the pre-amyloid phase [7, 66]. In this context, the astrocytes have been spotted as potential targets for biomarker development to detect early changes in AD [67, 68]. In particular, astrocytes may undergo changes in morphology, function, and metabolism, thus, assuming a reactive form – a phenomenon termed astrocyte reactivity [69]. An important work from Rodriguez-Vieitez and colleagues at Karolinska Institutet, using a multitracer PET imaging approach, demonstrated that astrocyte reactivity precedes Aβ plaques deposition in vivo, suggesting that soluble forms of Aβ (i.e., the AβOs) may dictate astrocyte function and metabolism [70]. Indeed, the review article presented in Chapter I of this thesis, proposes multiple cellular and molecular changes induced by AβOs in in vitro models of AD [22]. In fact, it seems that one of the earliest AβOs-induced changes is the mislocalisation of EAAT2 – the main glutamate transporter of the mammalian brain – in the membrane of astrocytes [38] (Figure 1).

Importantly, the EAAT2 seems to play a crucial role in maintaining proper synaptic function and brain energetic metabolism [39, 71]. In specific, it seems that glutamate uptake

via EAAT2 triggers glucose consumption in astrocytes and, consequently, stimulates aerobic glycolysis providing lactate, the main energetic substrate that astrocytes deliver to neurons (for a full review, please see Magistretti & Pellerin (1999) [72]). Corroborating with these findings, Zimmer et al. (2017) [40] demonstrated increased glucose uptake, indexed by [^{18}F]FDG PET, following EAAT2 activation [40]. Moreover, a very recent pilot clinical trial proved the potential efficacy and safety of riluzole, an FDA-approved drug positive modulator of EAAT2, in the treatment of AD [73]. The authors showed that riluzole improved [^{18}F]FDG PET signal and cognitive performance in individuals diagnosed as probable AD [73]. Thus, if glucose consumption is driven by glutamate uptake via EAAT2 and AβOs induce EAAT2 loss in the membrane of astrocytes, in this thesis, we hypothesised that in an animal model of the AD pre-amyloid phase, AβOs induce glucose hypometabolism via EAAT2 density loss.

A potential alternative to investigate the pre-amyloid stages of AD is the infusion of soluble AβOs in mice [25, 74-76]. Indeed, synthetic Aβ characterises an affordable and convenient way to obtain soluble AβOs which affect animal memory and induce multiple toxic mechanisms related to AD pathophysiology [25, 77]. Nevertheless, synthetic AβOs are very sensitive to fluctuations in physicochemical parameters such as temperature, pH, ionic strength and protein concentration [78]. In keeping with this, it is crucial employing a strong and reliable QC to characterise synthetic AβOs. In Chapter II, we described the optimisation and validation of an SEC-HPLC method that has proven to be a rapid and exceptionally reproducible technique for characterising synthetic low- and high-molecular weight AβOs [79]. Subsequently, we challenged our hypothesis that AβOs induce glucose hypometabolism in vivo, in the absence of Aβ plaques. For doing so, we applied a dual-tracer approach, using [^{18}F]FDG PET to estimate glucose metabolism and designing an innovative PET radiotracer to evaluate the density of glutamate transporters. In Chapter III, we demonstrated for the first time that AβOs cause regional [^{18}F]FDG PET hypometabolism prior to the deposition of Aβ plaques. These findings

could reconceptualise the current view of AD pathophysiological changes, in which Aβ plaques are indicated to trigger glucose hypometabolism, thus, leading to neurodegeneration [17, 80].

To demonstrate that the AβOs-induced [^{18}F]FDG PET hypometabolism could be a consequence of astroglial EAAT2 density loss, we decided to develop the first ^{11}C-radiolabelled PET radiotracer targeting glutamate transporters in vivo. Of note, a few parameters should be followed to design and test a PET radiotracer (Box 2). In this context, selecting an appropriate candidate molecule to become a PET radiotracer that has high affinity to the target is a crucial step. In Chapter IV, we reviewed the literature to reinforce that targeting EAAT2 in AD (and other neurodegenerative diseases) is a promising early biological target for diagnosis, to conduct a structure-activity relationship of the library of EAAT2-binders and, ultimately, to propose the development of a PET radiotracer targeting EAAT2 in vivo. From this work, we selected the compound termed "IF1" in Chapter V (previously synthesised by Greenfield et al. (2005) [49]) as the most appropriated molecular structure for developing a ^{11}C-radiolabelled PET radiotracer. In fact, in Chapter V we described in detail the step-by-step to obtain the PET radiotracer [^{11}C]IF1, from the radiosynthesis until the microPET biodistribution in healthy adult mice. The [^{11}C]IF1 symbolises the first generation of PET radiotracer targeting glutamate transporters in vivo. Of note, as demonstrated in our initial experimental findings in Chapter V, the [^{11}C]IF1 was unable to penetrate the BBB, thus, additional work is warranted to optimise the molecular structure and increase the ability of entering the brain to evaluate the density of EAAT2. Nonetheless, the remarkable image of the intestine obtained in the microPET imaging studies of healthy mice injected with [^{11}C]IF1 (Figure 4B in Chapter V) unravelled a new hypothesis – would glutamate transport be altered in the enteric nervous system (ENS) prior to the CNS glutamate excitotoxicity?

Box 2 | Ten rules for developing a PET radiotracer

Design:

1. **B_{max}** – concentration of the target protein in the region of interest (ROI) (nM, mol/g).
2. **Affinity** – refers to the binding of a molecule to the respective target (K_i, Kd, IC_{50}).
3. **Selectivity** – indicates how a molecule preferentially binds to one target than another similar (e.g., EAAT2 *vs* EAAT3).
4. **Easy radiosynthesis** –incorporation of a radionuclide in a molecular structure must be easy and reproducible.
5. **Accessibility** – the radiotracer must easily access its target in the ROI (e.g., if targeting a brain receptor, the radiotracer must penetrate the BBB).

Test:

1. **Signal-to-noise ratio** – comparison between a region with low concentration of the target (noise) *vs* the ROI with high concentration of the target (signal).
2. **Pharmacokinetics** – indicates whether the radiotracer binding to the target is reversible or irreversible.
3. **Pharmacology** – test the specific/non-specific binding using blocking agents.
4. **Metabolites** – following the radiotracer administration evaluate potential metabolization of the molecule collecting tissue/blood samples.
5. **Sensitivity** – how sensitive the radiotracer is to detect pathological changes compared to control.

Research from the last couple of decades indicate that neurodegenerative disease, such as PD, could have its origins in the intestine, affecting the microbiota-gut-brain axis [81-83]. In fact, in recent years a "post-amyloid era in AD" has been postulated in which peripheral alterations, commencing in the guts, are suggested to be very early biological changes in AD pathophysiology [84]. Among multiple mechanisms remain to be explored to understand how gut dysfunction may affect the CNS, it has been strongly debated that maintenance of the intestinal epithelial barrier integrity is a crucial step to avoid peripheral translocation of microbes and inflammatory cytokines to the brain [84]. Very interestingly, glutamate seems to work as the main fuel for maintaining the intestinal membranes intact [85]. In specific,

glutamate uptake via intestine glutamate transporters promotes the proliferation of intestinal epithelial cells [86]. Combining these findings, one could suggest that a specific PET radiotracer, such as [^{11}C]IF1, targeting intestine glutamate transporters could help shedding light on the involvement of glutamate uptake in the maintenance of the intestinal epithelial barrier in vivo.

Altogether, the several lines of scientific evidence discussed on the paragraphs above, indicate how powerful PET imaging can be to further our knowledge on neurological disorders that are yet to be understood. Even in situations that humankind must face extreme conditions, such as COVID-19 pandemic, PET imaging offers an unprecedent way of helping to define specific pathophysiological changes. Following the substantial part of neurological manifestations reported in COVID-19 patients, in Chapter VI, in a short-review we suggest that PET imaging and the use of specific PET radiotracers could help unrevealing the pathophysiological alterations in the brain of individuals infected with SARS-CoV-2 [87]. Indeed, [^{18}F]FDG PET enabled researchers to observed brain glucose hypometabolism following COVID-19 infection [50, 88]. A possible biological interpretation for the [^{18}F]FDG PET hypometabolism in COVID-19 was presented in Chapter VII, turning the spotlight to the astrocytes and raising the alarm for the potential risk to the development of neurodegenerative diseases.

In keeping with this, from the early discoveries in 1898 by Dr. Curie, through the development of the first PET scanners and advances in PET radiochemistry (for an example of innovative techniques for incorporation radionuclides in chemical compounds, please see Annex I), we have the chance of solving one of the major problems humankind has ever faced - dementia.

Conclusion

This thesis highlighted the potential of PET imaging in revealing detailed brain pathophysiological alterations in neurological disorders. Using a PET imaging approach, we explore promising biological targets for the early diagnosis of AD in a mouse model of the pre-amyloid phase. Following a critical review of the Aβ oligomers (AβOs) literature, we optimised and validated a method for synthetic AβOs QC. Using the synthetic AβOs we showed detectable [^{18}F]FDG PET brain hypometabolism induced by AβOs in mice, which seems related to astrocyte reactivity. Subsequently, we reviewed the literature to identify potential candidate molecules with affinity to astrocyte glutamate transporters for developing a PET radiotracer. The PET radiotracer targeting glutamate transporters was successfully designed and tested, warranting further research to optimise its use to detect early changes in AD. Lastly, the PET imaging applications in AD research helped us to suggest strategies to develop our knowledge in COVID-19 neurological manifestations. Indeed, the vast library of PET radiotracers allows for the identification of specific pathophysiological changes in neurological disorders.

Perspectives

The development of innovative biomarkers that can detect AD in the early stages, prior to the symptomatic phase is an urgent need. The constant evolution of PET radiochemistry providing novel radiosynthesis strategies allow for the design of highly selective PET radiotracers with strong sensitivity to detect pathological changes in the diseased brain. Our innovative PET radiotracer ([^{11}C]IF1) targeting glutamate transporters did not penetrate the BBB but left opened two new lines of research:

1) The fact that AβOs impact [^{18}F]FDG PET signal allows to hypothesised other PET radiotracers already available may also be affected by AβOs. With this in mind, multi-tracer strategies in the pre-amyloid phase are highly necessary.
2) The optimisation of the chemical structure of [^{11}C]IF1 (improve the BBB permeability) to investigate the potential link between AβOs, glutamate transport dysfunction and glucose hypometabolism in the pre-amyloid phase of AD.
3) The assessment of [^{11}C]IF1 to explore the potential role of intestinal glutamate uptake in maintaining the integrity of the intestine epithelial membranes, thus, providing a potential tool for studying the hypothesis of the leaky gut in AD.
4) Multi-tracer PET imaging strategies are still lacking in experimental and clinical research of COVID-19. Although the acute effects of SARS-CoV-2 in the brain seem to be transitory, several individual present long-term neurological manifestations. These individuals should be monitored with a multimodal biomarker perspective.

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