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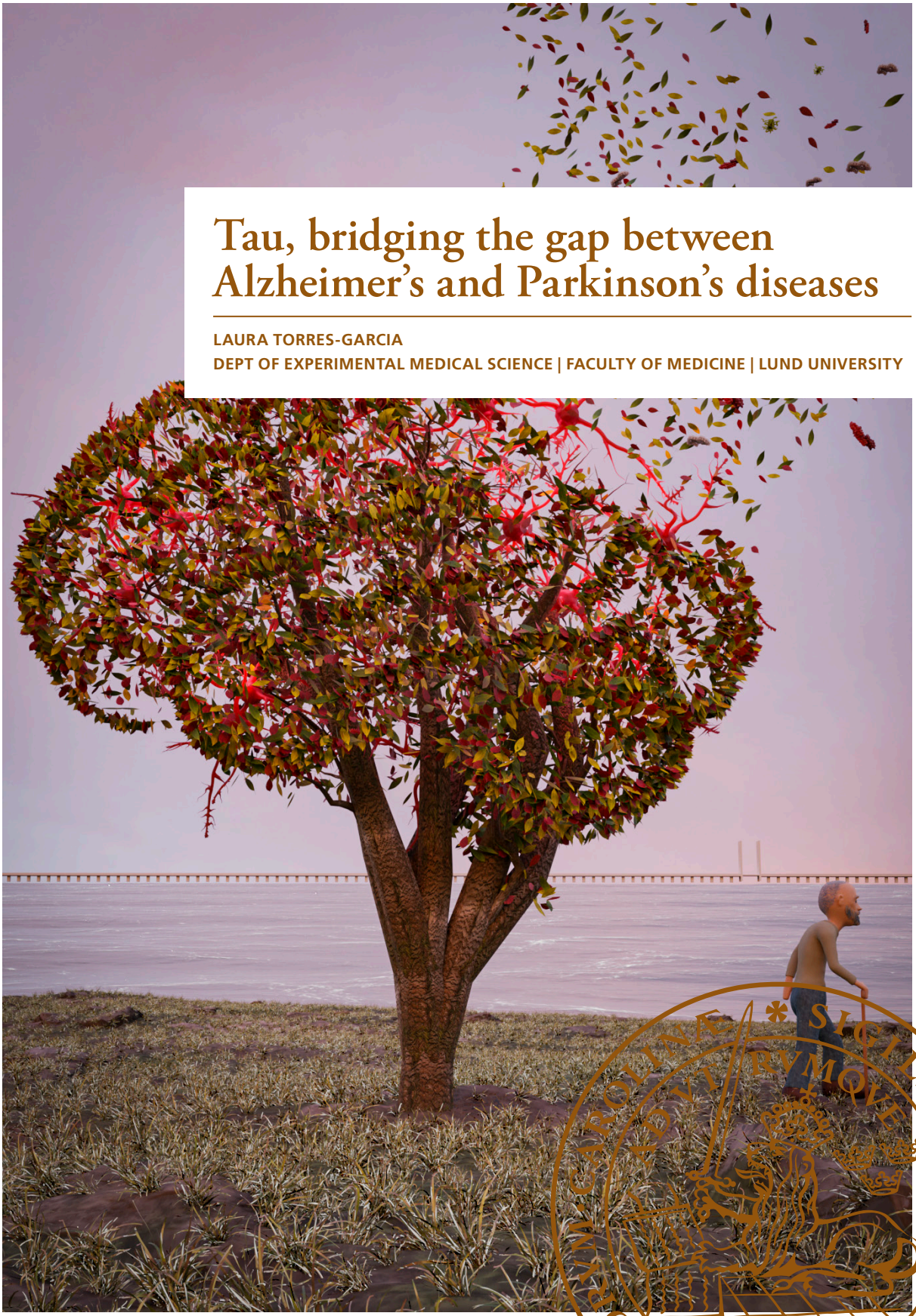

Tau, bridging the gap between Alzheimer's and Parkinson's diseases

Laura Torres-Garcia

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Abstract

Alzheimer's (AD) and Parkinson's diseases (PD) are the most common neurodegenerative diseases. They are both characterized by the pathological accumulation of amyloidogenic aggregates: amyloid-beta (Aβ) and Tau in AD, and alpha-synuclein (aSyn) in PD; and by progressive neuronal degeneration. However, mixed types of protein aggregates are frequently found in human post-mortem brains, and synergistic interactions between amyloids have been suggested.

In this thesis, our overall goal was to evaluate the effect that the direct and/or indirect interaction between amyloidogenic proteins have on the pathophysiology of AD and PD. For that we took different approaches: (1) we tested the ability of aSyn and Tau to physically interact with each other by bimolecular fluorescence complementation, (2) we assessed the impact that the co-expression of amyloidogenic proteins has on the conformation and secondary structure of amyloids by synchrotron-based Fourier Transform Infrared microspectroscopy (μ-FTIR), (3) we evaluated the impact that astrocytic and neuronal Apolipoprotein E (ApoE) isoforms have on neuronal excitability, also in relation to elevated Aβ; and (4) we determined the effects of Tau (WT and P301L) on neuronal excitability in the presence and absence of elevated Aβ.

In paper I, we showed the ability of aSyn and Tau to interact with each other in a biologically relevant context: in neuronal cells, and in the substantia nigra pars compacta (SNpc) of mice and rats. Moreover, in paper II, we observed how the co-occurrence of these proteins within cells resulted in changes in the conformation and secondary structure of the amyloidogenic proteins. Specifically, aSyn showed the potential to reduce β-sheet load, random coil (unordered) structures, and total protein folding when expressed in a cell line overexpressing human Aβ.

In papers III and IV we investigated changes in neuronal activity in different pathologically relevant contexts. In paper III, the impact of different isoforms of astrocytic vs. neuronal ApoE on neuronal excitability was evaluated. We observed a differential effect of the ApoE isoforms when they were obtained from astrocytic or endogenously expressed by the neurons. Lastly, in paper IV, the effects of Tau (WT and P301L) on neuronal excitability were evaluated in primary neuronal cultures, aiming to unravel the molecular mechanisms leading to Tau-induced changes in activity. Additionally, we evaluated how the presence of elevated Aβ might modulate these effects. We concluded that both Tau WT and Tau P301L lead to an increase in neuronal activity, but only Tau WT showed this effect when Aβ was overexpressed. Moreover, the impact of Tau WT on neuronal excitability seemed to be associated with a reduction in inhibitory synaptic input.

Overall, this thesis demonstrated (1) the ability of some amyloidogenic proteins to interact with each other, (2) a synergistic effect of co-occurrence of proteins on the structure and morphology of amyloidogenic aggregates, (3) a differential role of astrocytic vs. neuronal ApoE in neuronal excitability, and (4) a role for Tau in hyperexcitability. More work needs to be done to understand how the interaction of amyloidogenic proteins at the molecular level impacts on neurodegeneration.

Key words: Alzheimer's disease; Parkinson's disease; Tau; alpha-synuclein; amyloid-beta; neurodegeneration

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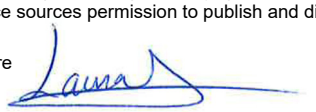
Signature  Date 2022-05-13

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To everyone who helped me to
grow during this journey

"The important thing is
to never stop questioning"

– Albert Einstein

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II. Paulus, A., Engdahl, A., Yang, Y., Boza-Serrano, A., Bachiller, S., **Torres-Garcia, L.,** Svanbergsson, A., Garcia, M. G., Gouras, G. K., Li, J.-Y., Deierborg, T., & Klementieva, O. (2021). Amyloid Structural Changes Studied by Infrared Microspectroscopy in Bigenic Cellular Models of Alzheimer's Disease. *International Journal of Molecular Sciences*, 22(7), 3430. https://doi.org/10.3390/ijms22073430

III. Konings, S. C., **Torres-Garcia, L**., Martinsson, I., & Gouras, G. K. (2021). Astrocytic and Neuronal Apolipoprotein E Isoforms Differentially Affect Neuronal Excitability. *Frontiers in Neuroscience*, 15, 734001. https://doi.org/10.3389/fnins.2021.734001

IV. **Torres-Garcia, L**., Martinsson, I., Comella-Bolla, A., Li, J.-Y., & Gouras, G. K. *Human Tau modulates synaptic activity in primary neuronal cultures*. (Manuscript in progress)

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VI. Brandi, E., **Torres-Garcia, L**., Svanbergsson, A., Haikal, C., Liu, D., Li, W., & Li, J.-Y. (2022) *Brain region-specific microglial activation in response to systemic LPS exposure*. [Preprint]. In Revision.

VII. Martinsson, I., Quintino, L., Garcia, M. G., Konings, S. C., **Torres-Garcia, L.**, Svanbergsson, A., Stange, O., England, R., Deierborg, T., Li, J.-Y., Lundberg, C., & Gouras, G. K. (2022) *Aβ/APP-Induced Hyperexcitability and Dysregulation of Homeostatic Synaptic Plasticity in Models of Alzheimer's Disease* [Preprint]. In Review. https://doi.org/10.1101/2022.01.25.477711

VIII. Svanbergsson, A., Ek, F., Martinsson, I., Rodo, J., Liu, D., Brandi, E., Haikal, C., **Torres-Garcia, L**., Li, W., Gouras, G., Olsson, R., Björklund, T., & Li, J.-Y. (2021). FRET-Based Screening Identifies p38 MAPK and PKC Inhibition as Targets for Prevention of Seeded a-Synuclein Aggregation. *Neurotherapeutics*, 18(3), 1692–1709. https://doi.org/10.1007/s13311-021-01070-1

IX. Navarro-Lobato, I., Masmudi-Martín, M., López-Aranda, M. F., Quiros-Ortega, M. E., Carretero-Rey, M., Garcia-Garrido, M. F., Gallardo-Martínez, C., Martín-Montañez, E., Gaona-Romero, C., Delgado, G., **Torres-Garcia, L**., Terrón-Melguizo, J., Posadas, S., Muñoz, L. R., Rios, C. V., Zoidakis, J., Vlahou, A., López, J. C., & Khan, Z. U. (2021). RGS14414-Mediated Activation of the 14-3-3ζ in Rodent Perirhinal Cortex Induces Dendritic Arborization, an Increase in Spine Number, Long-Lasting Memory Enhancement, and the Prevention of Memory Deficits. *Cerebral Cortex*, bhab322. https://doi.org/10.1093/cercor/bhab322

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Popular science summary

Neurodegenerative diseases are a group of diseases that are characterized by the progressive death of neurons (brain cells). Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative diseases. The typical feature of AD is memory loss, while PD is commonly associated with mobility impairment, reflecting that the brain regions affected by the diseases are different. However, both diseases share common features.

In both AD and PD, the accumulation of specific proteins (amyloidogenic proteins) in the brain leads to the development of the pathology. In AD, these proteins are amyloid-beta and Tau, while in PD the protein that aggregates is alpha-synuclein. However, it is common to find the three types of aggregates in AD and PD patients. The reason why these proteins aggregate is still unknown. Moreover, it is not clear whether these proteins can interact/communicate with each other and if so, what the subsequent consequences are.

Therefore, in this thesis, we aimed to investigate whether these proteins can interact with each other within cells and in the brain, and the impact their interaction has on pathology. For that, two different techniques were used: bimolecular fluorescence complementation (BiFC) and synchrotron-based Fourier Transform Infrared microspectroscopy (μ-FTIR). Both techniques provide the possibility to study our proteins of interest in cells, giving better information about what is happening in the brain during disease progression.

In paper I, we observed that alpha-synuclein (accumulated in PD) and Tau (accumulated in AD) could interact with each other in neurons. Moreover, in paper II, we demonstrated that the pathological forms of amyloidogenic proteins (amyloid-beta, Tau and alpha-synuclein), when expressed together within the same brain cell, led to changes in the composition and structure of aggregates.

Lifestyle and environmental factors are thought to have an impact on the development of both AD and PD. However, the propensity to develop both diseases is also influenced by genetics. For example, variants of the Apolipoprotein E gene, which forms a protein responsible for transporting lipids in the body, can increase the risk of developing AD by 10-fold. Still, how this protein increases the probability of suffering from neurodegeneration is not well understood.

In neurodegenerative diseases, one of the first changes observed in the brain are changes in neuronal activity, which impact how neurons can communicate with each other, hence, altering brain communication. In paper III, we showed that variants of Apolipoprotein E from different types of brain cells (neurons vs. glia) have a different impact on neuronal activity and brain communication.

Lastly, in paper IV, we investigated how normal and mutated forms of Tau can induce changes in neuronal activity and brain communication. In addition, because Tau alterations are present together with alterations in amyloid-beta in AD, we determined how the effect of Tau on neuronal activity might vary when amyloid-beta alterations are already present. We observed that both the normal and the mutated form of Tau could trigger changes in neuronal activity, and at least in the case of normal Tau, this effect was due to alterations in synapses (places of communication between neurons).

Therefore, the main conclusions reached during this thesis are (1) alpha-synuclein (PD) and Tau (AD) can interact with each other within neurons, (2) the pathological alteration of more than one amyloidogenic protein in the same neuron leads to changes in protein aggregates, (3) the different variants of the AD risk factor Apolipoprotein E impact brain activity and communication in different ways, and (4) the normal and the mutated variants of Tau seem to have a detrimental effect on neuronal activity, leading to alterations in brain communication and function.

Still, more work needs to be done to (1) understand what the consequences are of the interaction between alpha-synuclein and Tau in disease development and progression, (2) understand the meaning of the changes in protein aggregation: are they protective or harmful; and (3) decipher which pathways within the brain cells are causing the changes in neuronal communication, with the aim to find specific mechanisms that could be modulated to control disease development and/or progression.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom och Parkinsons sjukdom tillhör de så kallade neurodegenerativa sjukdomarna som kännetecknas av en progressiv förlust av nerver i hjärnan. Trots att de skiljer sig i vilka symtom som de kännetecknas av: minnesförlust och kognitiv försämring i Alzheimers sjukdom, och rörelserubbningar i Parkinsons sjukdom, så har de ändå vissa gemensamma drag.

I båda sjukdomarna uppstår onormala proteinlagringar i hjärnan. I Alzheimers sjukdom består dessa lagringar av proteinerna amyloid-beta och Tau, medans de i Parkinsons sjukdom består av alfa-synuklein. Det är dock inte ovanligt att alla tre proteiner ackumulerar i hjärnan i sjukdomsfall. Vad som orsakar denna lagring av proteiner och huruvida dessa tre proteiner påverkar varandra är ännu oklart. Detta har vi studerat i denna avhandling. Vi har kunnat visa att alfa-synuklein och Tau interagerar i nerver och när alla tre protein uttrycks i hjärnceller så ändras kompositionen av proteinlagringarna som uppstår.

Genetiska riskfaktorer kan öka risken för att insjukna i Alzheimers sjukdom. Den mest kända genetiska riskfaktorn är Apolipoproteinet E, som finns i 3 former, varav en ökar risken signifikant. Apolipoprotein E deltar i ämnesomsättningen av fetter men hur den ökar risken för Alzheimers sjukdom är ännu okänt. Vi har kunnat visa att olika varianter av Apolipoproteinet E från olika celltyper i hjärnan påverkar aktiviteten av nerver olika. Ändringar i nervers aktivitet är bland de första störningarna som uppstår i neurodegenerativa sjukdomar.

Vi har även studerat hur Tau påverkar nervers aktivitet. När amyloid-beta och Tau båda uttrycks i nerver, så kan Tau utlösa ändringar i nervernas aktivitet, detta genom att orsaka förändringar vid synapser: där nerver möter andra nerver.

Ännu behövs mer forskning för att svara på (1) vad interaktionen mellan Tau och alfa-synuklein innebär för utvecklingen av Alzheimers och Parkinsons sjukdomar, (2) huruvida proteinlagringarna som uppstår är patologiska eller skyddande, och (3) vilka mekanismer som påverkas av Apolioproteinet E och Tau för att orsaka en ändring in nervaktivitet. En djupare förståelse för dessa frågor skulle kunna bana vägen för nya terapier som förhindrar uppkomsten och motverkar fortskridningen av Alzheimers och Parkinsons sjukdomar.

Resumen de divulgación científica

Las enfermedades neurodegenerativas son un grupo de enfermedades que se caracterizan por la muerte progresiva de neuronas (células del cerebro). La enfermedad de Alzheimer y la enfermedad de Parkinson son las enfermedades neurodegenerativas más frecuentes. La característica típica de la enfermedad de Alzheimer es la pérdida de memoria, mientras que la enfermedad de Parkinson se caracteriza por la presencia de alteraciones del movimiento, esto refleja que en ambas enfermedades la región del cerebro afectada por muerte neuronal es diferente. Sin embargo, ambas enfermedades tienen características en común.

Tanto en la enfermedad de Alzheimer como en la de Parkinson, la acumulación de proteínas específicas (proteínas amiloidogénicas) en el cerebro da lugar al desarrollo de la enfermedad. En la enfermedad de Alzheimer, estas proteínas son beta-amiloide y Tau, mientras que en la enfermedad de Parkinson la proteína que se acumula es alfa-sinucleína. Sin embargo, es frecuente encontrar agregados de los 3 tipos de proteína en pacientes tanto de Alzheimer como de Parkinson. La razón por la que estas proteínas se acumulan en el cerebro no se conoce. Además, tampoco está aún claro si estas proteínas pueden interactuar/comunicarse entre ellas y en tal caso, las consecuencias de esa interacción.

Por tanto, en esta tesis nuestro objetivo era investigar si las proteínas amiloidogénicas pueden interactuar entre sí dentro de las células y en el cerebro, y el impacto que esa interacción tiene en enfermedades neurodegenerativas. Para ello, se usaron dos técnicas diferentes: complementación de fluorescencia bimolecular y microespectrometría infrarroja con transformada de Fourier (FTIR). Ambas técnicas posibilitan el estudio de nuestras proteínas de interés en células, dando información de qué es lo que ocurre en el cerebro durante el desarrollo y progreso de la enfermedad.

En el primer artículo de la tesis (paper I), pudimos observar como alfa-sinucleína (que se acumula en la enfermedad de Parkinson) y Tau (que se acumula en la enfermedad de Alzheimer) podían interactuar entre sí en neuronas. Además, en el segundo artículo de esta tesis, demostramos que las formas patológicas de las proteínas amiloidogénicas (beta-amieloide, Tau y alfa-sinucleína), cuando se expresaban juntas dentro de la misma célula, daban lugar a cambios en la composición y en la estructura de los agregados proteicos.

El estilo de vida y los factores ambientales tienen un gran impacto en el desarrollo de ambas enfermedades. Sin embargo, la propensión a sufrir de Alzheimer o Parkinson está también influencia por la genética. Por ejemplo, variaciones en el gen de la Apolipoproteína E, que da lugar a una proteína responsable del transporte de lípidos en el cuerpo, pueden aumentar el riesgo de padecer la enfermedad de Alzheimer hasta 10 veces. Aún así, todavía no se sabe cómo esta proteína aumenta el riesgo de desarrollar enfermedades neurodegenerativas.

En las diferentes enfermedades neurodegenerativas, uno de los primeros cambios que ocurren en el cerebro son cambios en la actividad neuronal, que influyen en cómo las neuronas pueden comunicarse entre sí, de esa manera, alterando la comunicación en el cerebro. En el tercer artículo de esta tesis (paper III), demostramos como las variantes de la Apolipoproteína E que provienen de diferentes tipos celulares (neuronas o glía) influyen de manera diferente en la actividad neuronal

Por último, en el cuarto artículo de esta tesis (paper IV), investigamos como Tau, en su forma normal y mutada, puede dar lugar a cambios en la actividad neuronal y en la comunicación en el cerebro. Además, debido a que la acumulación de Tau se produce cuando beta-amieloide se acumula en la enfermedad de Alzheimer, analizamos cómo el efecto de Tau en actividad neuronal puede estar modulado por la presencia de agregados de beta-amieloide. Observamos que tanto la Tau normal como la mutada alteraban la actividad neuronal. En el caso de la forma normal de Tau, los cambios que se produjeron en la actividad neuronal parecían ser debidos a alteraciones en sinapsis (sitios de comunicación entre neuronas).

De esta manera, las conclusiones principales a las que hemos llegado durante esta tesis son: (1) que alfa-sinucleína y Tau puede interactuar entre sí dentro de neuronas, (2) que alteraciones patológicas de más de una proteína amiloidogénica en la misma neurona dan lugar a cambios en la composición y estructura de los agregados proteicos, (3), que las diferentes variantes de la Apolipoproteínas E influyen de forma diferente en la actividad neuronal y la comunicación en el cerebro y (4) que las formas normal y mutada de la proteína Tau parecen tener un efecto perjudicial en la actividad neuronal, dando lugar a alteraciones en la comunicación y funciones del cerebro.

Todavía más investigación necesita ser llevada a cabo para (1) entender cuáles son las consecuencias de que alfa-sinucleína y Tau pueden interactuar en el desarrollo y progresión de enfermedades neurodegenerativas, (2) el significado que los cambios en la composición y estructura de los agregados proteicos: son protectoras o perjudiciales?, y (3) descifrar que cambios dentro de las neuronas dan lugar a cambios en comunicación neuronal, con el objetivo de encontrar mecanismos específicos que puedan ser modulado para controlar el desarrollo o la progresión de las enfermedades neurodegenerativas.

Abbreviations

μ-FTIR	Synchrotron-based Fourier Transform Infrared microspectroscopy
Aβ	Amyloid-beta
AAV	Adeno-Associated Virus
AD	Alzheimer's disease
AGD	Argyrophilic grain disease
AICD	APP intracellular domain
APLP1/2	APP-like protein 1 and 2
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
aSyn	Alpha-synuclein
BACE1	β-secretase
BBB	Blood brain barrier
BiFC	Bimolecular fluorescence complementation
CBD	Corticobasal degeneration
CNS	Central nervous system
DIV	Days in vitro
DLB	Dementia with Lewy bodies
EOAD	Early onset AD
FTD	Frontotemporal dementia
FTDP-17	Frontotemporal dementia with Parkinsonism associated to chromosome-17
GABA	Gamma-aminobutyric acid
GGT	Globular glial tauopathy
GWAS	Genome wide association studies

HDL	High-density lipoproteins
KI	Knock-in
LB	Lewy bodies
LN	Lewy neurites
LOAD	Late onset AD
LV	Lentiviral vector
MAO-B	Monoamine oxidase B
MCI	Mild cognitive impairment
MSA	Multiple system atrophy
MTBD	Microtubules binding domain
MVBs	Multivesicular bodies
N2a	Neuro 2a
NAC	Non-amyloid beta component
NFT	Neurofibrillary tangle
PART	Primary age-related tauopathy
PD	Parkinson's disease
PiD	Pick disease
PRD	Proline-rich domain
PSEN1/2	Presenilin 1 and 2
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PSP	Progressive supranuclear palsy
PTMs	Post-translational modifications
TBI	Traumatic brain injury
VGAT	Vesicular GABA transporter
vGlut1	Vesicular glutamate transporter 1
VLDL	Very low-density lipoproteins
VMAT2	Vesicular monoamine transporter 2
WT	Wild type

Introduction

Neurodegenerative diseases

Neurodegenerative diseases are a group of disorders that are characterized by the progressive loss of function of brain cells that can lead to movement and/or cognitive impairment. Millions of people worldwide are affected by these diseases, that are currently the leading cause of disability globally. Alzheimer's and Parkinson's diseases are the most common neurodegenerative disorders.

Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia. In 2020, over 58 million people are living with dementia worldwide, and this number is expected to increase to 88 million people in 2050.

AD presents different stages: an early preclinical stage, in which protein accumulation and neurodegeneration start without presenting clinical symptoms; a stage of mild cognitive impairment (MCI) in which some memory loss is detected but this does not interfere with everyday tasks; and a dementia stage, in which the memory loss and visual and spatial impairment do not permit the person suffering from AD to function independently.

At the clinic, AD is diagnosed by means of cognitive tests that measure alterations in attention, episodic memory, visual and spatial skills, language, and executive functions, together with fluid and imaging biomarkers. However, AD starts about 20 years before the clinical manifestations appear. Nowadays, the development of more accurate biomarkers seems to be promising for the detection of AD pathology during its preclinical phase; however, there are still no treatments to prevent or stop the emergence of AD pathology.

At the neuropathological level, AD is characterized by the formation of extracellular amyloid plaques, which however are preceded by intracellular amyloid-beta (Aβ) accumulation[1], and by the accumulation of hyperphosphorylated Tau in the form of Neurofibrillary tangles (NFT). This aggregation of amyloidogenic proteins happens in association with neuroinflammation and the loss of synapses and neurons, following the "amyloid cascade hypothesis"[2]. Synaptic loss correlates the best with the cognitive decline observed in AD[3].

In AD, Aβ and Tau pathology follow a stereotypical anatomical distribution in the brain. Tau accumulation goes from the locus coeruleus, entorhinal cortex and hippocampus (Braak stages I-II) to limbic structures, amygdala, and thalamus (Braak stages III-IV), and to cortical areas (Braak stages V-VI)[4,5]. While Aβ deposition starts in the neocortex (Phase I), followed by hippocampus, amygdala, diencephalon and basal ganglia (Phases 2 and 3), and reaches mesencephalon, brainstem and cerebellum (Phase 4 and 5).

Multiple efforts have been made to tackle AD. Initially, drug development against AD focused on reducing/stopping Aβ production by inhibition of the secretases involved in its production, but these approaches were shown to have a strong detrimental effect. Currently, new strategies are pursuing the reduction of pathological Tau, as its accumulation correlates better with cognitive decline than Aβ deposition[4]. Anti-Tau therapies inhibiting Tau kinases, Tau aggregation or stabilizing microtubules have so far been toxic or ineffective. Thus, new approaches such as Tau immunotherapy have been pursued. The reduction of specific/pathological Tau forms by passive Tau immunization are leading to promising results[6], and could potentially be used not only for AD treatment but also for other tauopathies.

The main risk factor for the development of AD, as for other neurodegenerative diseases, is aging. Besides that, alterations in genes associated with the synthesis of Aβ have been associated with familial early-onset AD (EOAD). EOAD comprises 5% of the AD cases, the remaining 95% are sporadic or late-onset cases (LOAD). Multiple genes have been associated with an increase in the risk of developing LOAD. Among these, Apolipoprotein E4 has been reported to be the major genetic risk factor for the development of AD[7,8].

Parkinson's disease

Parkinson's disease (PD) is the most common movement disorder. By 2020, 9.4 million people were estimated to live with PD worldwide, and those numbers are increasing fast as the population is aging. In fact, the World Health Organization has appointed PD as the fastest growing neurological disorder worldwide.

There are no specific tests for PD diagnosis. The examination of signs and symptoms of the patient, together with a dopamine transporter scan help diagnose the disease. PD signs and symptoms include motor alterations: resting tremor, bradykinesia, muscular rigidity, postural imbalance, freezing, and speech changes; but also, non-motor symptoms such as depression, anxiety, sleeping and mood disturbances, olfactory disorders, constipation, pain, and cognitive impairment have been associated to PD.

Environmental and genetic factors lead to the development of PD. Only 3-5% of the cases of PD are inherited or familial[9], the rest are sporadic cases in which the main risk factor is aging. Besides aging, genome-wide association studies (GWAS) have pointed to MAPT and SNCA genes, which encode for Tau and aSyn proteins respectively, as the main risk factors for the development of PD[10,11].

In the brain, PD is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta, and the accumulation of alpha-synuclein (aSyn) in the form of Lewy bodies (LB) and/or Lewy neurites (LN)[12]. aSyn deposition follows a specific pattern through anatomically connected regions and enables to characterize different stages of the disease. In PD, aSyn starts accumulating in the olfactory bulb and brainstem (Braak stages I-II), followed by pons, midbrain and basal forebrain (stages III-IV), and neocortex (Braak stages V-VI)[4]. Although the pattern followed by the pathological accumulation of aSyn differs from Aβ and Tau, the propagation of the different amyloidogenic proteins between trans-synaptically connected areas suggest a prion-like propagation of these diseases.

There is no cure for PD. However, there are a number of symptomatic treatment options. Pharmacological options focus on increasing dopamine levels in the brain, by promoting its production (L-DOPA) or inhibiting its degradation (MAO-B). Furthermore, brain stimulation, experimental trials with Adeno-Associated Virus (AAVs), and grafting of fetal dopaminergic neurons[13], embryonic stem cell-derived dopaminergic progenitors, and induced pluripotent stem cells (iPSCs) are currently used or under consideration. Moreover, new approaches are focused on reducing aSyn production and aggregation or promoting aSyn degradation. Still, aSyn immunotherapy seems to be the most promising therapeutic approach[14].

Tauopathies

AD is one of the tauopathies, which are a group of neurodegenerative disorders that are characterized by Tau aggregation. They can be subclassified into primary, in which the predominant pathological aggregates are made up of Tau; and secondary tauopathies, in which co-occurrence of Tau aggregates with other amyloidogenic aggregate define the diseases. AD is an example of a secondary tauopathy.

While all the tauopathies are defined by Tau aggregation, isoform composition, morphology and anatomical distribution of the aggregates varies between tauopathies. For example, Pick disease (PiD) presents with aggregates comprised of 3-repeats (3R) Tau, while progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) contain Tau aggregates made of 4-repeats (4R) Tau. Further, AD and primary age-related tauopathy (PART) present with mixed 3R/4R aggregates. Besides that, these aggregates can be mainly localized in neurons or glial cells, as in globular glial tauopathy (GGT) and argyrophilic grain disease (AGD). Mutations in the MAPT gene, such as the P301L mutation, lead to familial

frontotemporal dementia with Parkinsonism associated to chromosome-17 (FTDP-17), that can exhibit 3R, 4R, and mixed 3R/4R Tau aggregates[15].

A wide range of symptoms are displayed in tauopathies, from cognitive to motor symptoms, in that way complicating an accurate diagnosis of the pathology. Moreover, as for other neurodegenerative diseases, treatments for slowing down and/or stopping Tau diseases are lacking[16].

Apolipoprotein E

Apolipoprotein E (ApoE) is a 299 amino acid lipoprotein, which comprises two different regions separated by 20 – 30 amino acids: The N-terminal, containing the receptor binding region, consists of a four-helix bundle; and the C-terminal, containing the lipid binding region, and which forms amphipathic α-helices, is responsible for the binding to lipids (Fig. 1). ApoE is responsible for the transport of lipids within and between multiple tissues, and it is the main lipid transport vehicle in the brain[17]. ApoE is mainly synthetized in hepatocytes, but it is highly produced in the brain, which is the second major source of ApoE in the body. In the brain, ApoE is mainly produced by astrocytes but can also be synthetized by neurons and microglia[18]. It has been suggested that ApoE from different sources can have a different physiological and/or pathological role(s).

ApoE is released unlipidated from the cells, but it is quickly lipidated due to its avidity for lipids[17]. Lipidated ApoE presents high affinity for binding of cell-surface lipoprotein receptors and heparan sulfate proteoglycans (HSPGs), which facilitates the interaction of ApoE with lipoprotein receptors, allowing the internalization of the ApoE-lipid complexes into cells.

The APOE gene, which encodes for ApoE, presents three allelic variants: APOE2, APOE3, and APOE4, giving rise to three different isoforms: ApoE2, ApoE3, and ApoE4. Carriers of one copy of the APOE4 allele have 4-fold increased risk of developing AD, while the presence of two copies of APOE4 leads to a 14-fold higher probability of having AD[7,8]. The ApoE isoforms differ in their ability to bind lipoprotein receptors, HSPGs and/or lipids. For example, ApoE2 binds with reduced affinity to lipoprotein receptors, leading to type III hyperlipoproteinemia[19], but has a protective effect against the development of AD[20]. Moreover, the different ApoE isoforms prefer specific classes of lipoproteins: ApoE4 binds preferentially to large, triglyceride-rich very low-density lipoproteins (VLDL), while ApoE3 and ApoE2 are found more frequently associated to small, phospholipid-rich high-density lipoproteins (HDL)[17,19].

Apart from being the major risk factor for the development of AD, ApoE4 has also been associated to the progression and/or development of other neurodegenerative

diseases: PD [21–24], Frontotemporal dementia (FTD)[25,26], traumatic brain injury (TBI)[27], stroke[28], multiple sclerosis[29,30], and dementia with Lewy bodies (DLB)[31].

In addition to its role in disease, ApoE has a physiological role in peripheral nerve injury repair by redistributing lipids to the lesion site[32,33]. Further, several pathological roles for ApoE4 in AD have been suggested. ApoE seems to have cell-type and isoform-dependent effects on Aβ metabolism, and it exacerbates Aβ pathology and cognitive decline[34–38]. Between isoforms, ApoE4 has shown a reduced ability to stimulate Aβ clearance in comparison to ApoE2 and ApoE3[35]. Additionally, Bales et al. (1999)[39] showed that ApoE is required for Aβ plaque formation, suggesting that a reduction in ApoE levels could be protective against Aβ pathology[40].

Additionally, several Aβ-independent effects of ApoE in neurodegeneration have been postulated. ApoE4 leads to behavioral and cognitive deficits in mice without Aβ pathology[41]. Furthermore, ApoE4 is more susceptible to proteolytic cleavage than ApoE3[42,43], and these ApoE4 fragments seem to disrupt cytoskeletal structures[44] and mitochondrial function[45], accumulate in Aβ plaques and NFT[46,47], and are neurotoxic[43]. This neurotoxicity seems to be avoided when endogenous Tau is removed[48]. Besides that, ApoE4 impairs the blood-brain barrier (BBB) integrity[49], is associated with a reduction in the metabolism of glucose in the brain[50], impaired synaptogenesis[51], and has an age- and tau-dependent detrimental effect on GABAergic interneurons[48,52].

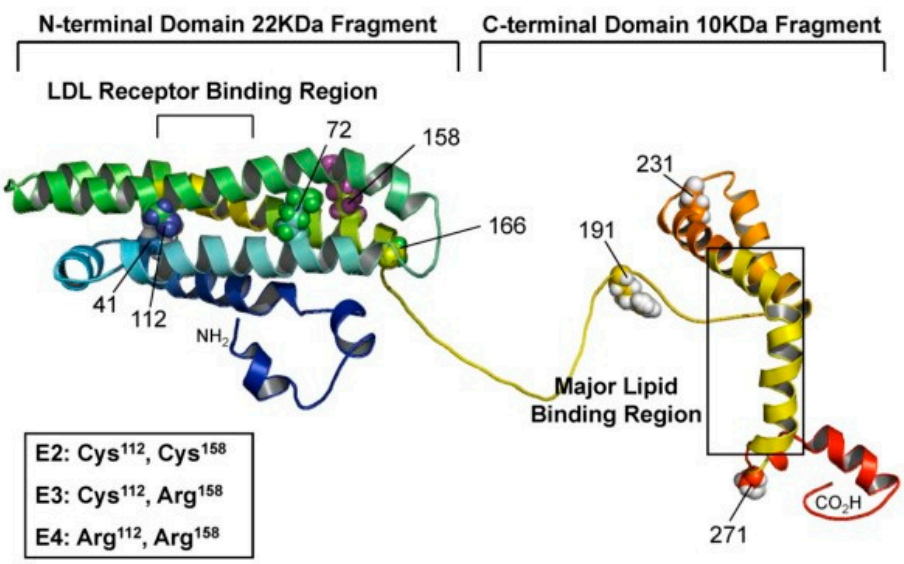

Figure 1. Structure of the human ApoE protein. Model of full-length ApoE in which the N-terminal domain (receptor binding) and C-terminal (lipid binding) can be distinguished. Amino acidic difference between isoforms: ApoE2 (E2), ApoE3 (E3), and ApoE4 (E4), are specified (black box). Figure from Hsieh et al. (2011)[53].

Amyloidogenic proteins

AD and PD are characterized by the accumulation of Aβ and Tau and aSyn, respectively. Aβ, Tau and aSyn are proteins that can form amyloids, which are pathological aggregates of proteins with a β-sheet secondary structure which lead to the disruption of the physiological function of these proteins and to the development of diverse pathologies.

Amyloid-beta

Amyloid-beta (Aβ) is produced by the sequential cleavage of the human amyloid precursor protein (APP). APP is a type I transmembrane protein, and one of the members of the APP family, which includes APP-like protein 1 and 2 (APLP1 and APLP2). However, APP is the only member of this family that contains the Aβ sequence. Differential splicing of the APP gene gives rise to different APP isoforms, with APP(695) predominantly expressed in neurons[54].

APP can be processed by two different cleavage pathways: the α- or non-amyloidogenic pathway, or the β- or amyloidogenic pathway (Fig. 2). In the non-amyloidogenic pathway, the cleavage of APP by α-secretase occurs within the Aβ sequence[55,56], and the subsequent cleavage by γ-secretase[57,58] results in the extracellular release of p3 and the formation of the APP intracellular domain (AICD). On the other hand, the cleavage of APP by β-secretase (BACE1)[59], followed by intramembrane γ-secretase cleavage generates Aβ, that accumulates in the endosomal lumen, and AICD that is released to the cytosol. Sequential γ-secretase cleavage of Aβ in its C-terminus results in the production of Aβ comprised of 37 – 44 amino acids. Aβ40 is the most abundant product, while Aβ42 is believed to be the responsible for the formation of toxic oligomers.

Both APP and BACE1 are ubiquitously expressed, but the concomitant high levels of both proteins in neurons make them the preferred tissue for amyloidogenic cleavage of APP[60]. The non-amyloidogenic APP cleavage occurs preferentially at the cell surface while the amyloidogenic cleavage occurs in endosomes. In fact, endocytosis is required for Aβ generation[61]. Specifically, endosomal production of Aβ and its pathological accumulation in multivesicular bodies (MVBs) at synaptic terminals in AD has been demonstrated[62].

Mutations in the APP gene and in genes promoting the amyloidogenic cleavage of APP (PS1 and PS2) cause an increased production of Aβ42, giving rise to the familial forms of AD. Since the discovery of this connection between increased Aβ levels and AD, a lot of work has been done to understand the pathological role(s) of APP and Aβ; however, less is known about their physiological function(s). Potential

physiological roles for the APP family and its soluble products (sAPP) have been suggested: involvement in neuronal development, axonal transport, intracellular signaling, and cellular homeostasis[63–67].

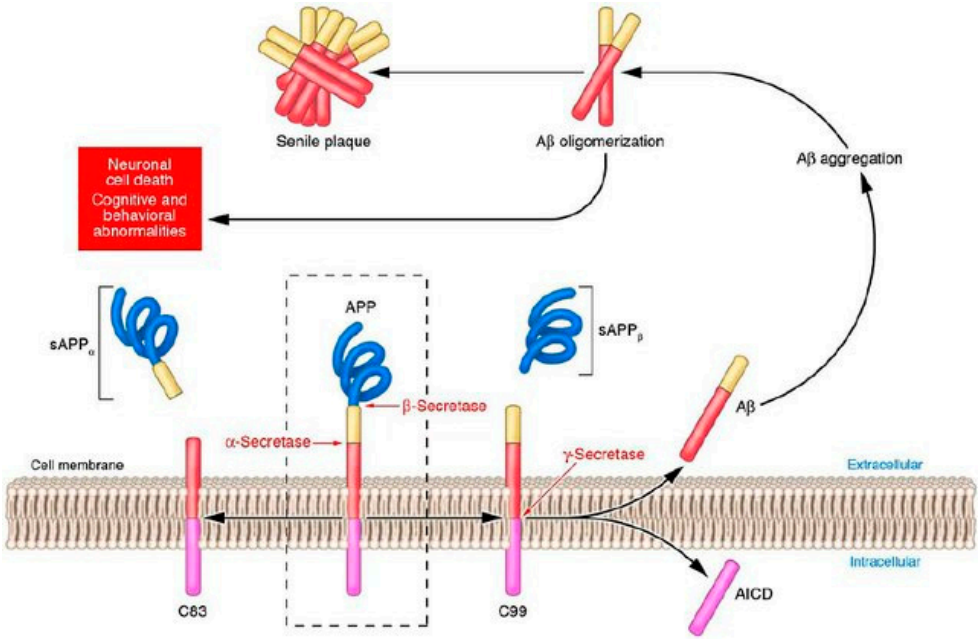

Figure 2. Proteolytic APP processing following the α- or non-amyloidogenic pathway, or the β- or amyloidogenic pathway. The non-amyloidogenic cleavage happens preferentially at the cell surface and involves cleavage of APP by α- and γ-secretase. The amyloidogenic cleavage occurs in endosomes, the sequential cleavage of APP by β- and γ-secretase results in the production of 37 – 44 amino acid Aβ. Aβ accumulation leads to the formation of oligomeric and fibrillar forms that drive AD pathology. Figure from Gandy et al. (2005)[68] ©.

Microtubule-associated protein Tau

The microtubule-associated protein Tau is a soluble and natively unfolded protein[69], which is encoded by the MAPT gene. The alternative splicing of the MAPT gene results in different Tau isoforms. In the central nervous system (CNS), 6 Tau isoforms can be found, which can be differentiated by the presence of 0, 1 or 2 inserts in the N-terminal (0N, 1N, or 2N, respectively) and by the presence of 3 repeats (3R) or 4 repeats (4R) in the microtubule binding region[70]; resulting in the isoforms: 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R. These isoforms are present in the brain in different proportion, with 0N, 1N, and 2N isoforms comprising 37, 54, and 9% of the total Tau in human CNS[71]. In the adult human brain, equal amounts of 3R and 4R Tau are found[72].

Tau is mainly expressed in neurons, but also can be produced by astrocytes and oligodendrocytes. It is mainly localized in axons, but its localization in

somatodendritic compartments[73] and presynapses[74,75] has been also documented. Tau protein consists of 4 major domains: N-terminal domain, proline-rich domain (PRD), microtubule binding domain (MTBD), and the C-terminal domain (Fig. 3). Tau is known by its canonical role in microtubule stabilization. However, other potential physiological roles for Tau have been proposed.

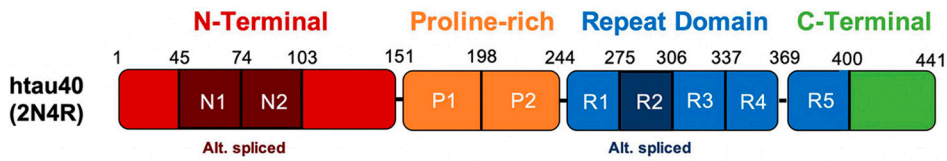

Figure 3. Scheme of the Tau protein, 2N4R isoform. In the human CNS, Tau can be found in 6 different isoforms depending on the expression of 0N, 1N or 2N in its N-terminal, and the presence of 3-repeats (3R) or 4-repeats (4R) in its repeat domain. Tau consists of 4 major domains: N-terminal domain, proline-rich domain (PRD), microtubule binding domain (MTBD), and the C-terminal domain. Figure from Sallaberry et al. (2021)[76].

The N-terminal domain projects away from microtubules, but it is involved in regulating the spacing between them[77]. Moreover, the presence of 0N, 1N, or 2N inserts in the N-terminal of Tau could potentially determine Tau localization in the brain, as an isoform-specific localization pattern has been observed in mouse brain[78]. Additionally, different number of inserts in the N-terminal seem to promote the interaction of Tau with different proteins, for example, apolipoprotein A1 binds mainly 2N Tau, whereas aSyn and synaptophysin interact preferentially with 0N Tau isoforms[79].

The PRD presents with seven Pro-X-X-Pro (PXXP) motifs, that can be recognized by Src homology-3 (SH3)-containing proteins, like Fyn and bridging integrator 1 (Bin1)[80]. The potential interaction of Tau with SH3-containing proteins suggests a signaling role for Tau. Tau seems to be able to interact with DNA and RNA in the nucleus[81,82], where Tau has been shown to localize after N-terminal truncation[83]. Moreover, the proline-rich regions are targeted by several protein-interacting motifs, thus, suggesting multiple potential interaction partners for Tau. Besides that, the PRD is involved in microtubule assembly[84] and actin binding[85,86]. Thus, supporting Tau roles in neuronal signaling, nuclear function, and in maintenance of the neuronal cytoskeleton.

The MTBD is the region responsible for microtubule binding. Besides that, aSyn [87,88] and several other proteins have been shown to bind this region: F-actin[85], ApoE[89], and presenilin[90] are some of them. MTBD can bind both the actin and the microtubule cytoskeletons[91], and it has been suggested that it associates with lipid membranes, DNA, and RNA[82,92]. Lastly, less is known about the interaction partners and/or functions of the C-terminal region.

Furthermore, Tau undergoes several post-translational modifications (PTMs): phosphorylation, Tau presents with 85 phosphorylation sites[93]; acetylation, oxidation, nitration, glycation, isomerization, ubiquilylation, sumoylation, polyamination, O-GlcNAcylation, and proteolysis[94,95], which have been shown to impact the ability of Tau to oligomerize. Additionally, these PTMs potentially impact the neuronal localization and the propensity with which Tau interacts with specific interaction partners.

Therefore, Tau is present in 6 different isoforms in the brain that preferentially bind to specific interaction partners. Moreover, due to the versatility of its PRD and MTBD, multiple Tau-interacting partners have been suggested, some of them associated to neurodegenerative diseases: aSyn, ApoE, presenilin, 14-3-3, FUS, or TIA1[77]. That, together with the ability of Tau to be modified by multiple PTMs, makes Tau a complex protein whose physiological and pathological roles still need to be deciphered.

Alpha-synuclein

Alpha-synuclein (aSyn) is a small (140 amino acids), soluble protein encoded by the SNCA gene. Mutations, duplications, and triplications of the SNCA gene have been associated to PD and other synucleinopathies, such as DLB, multiple system atrophy (MSA), and Lewy body variant of Alzheimer's disease[96–99], highlighting the importance of aSyn pathology in neurodegeneration.

Although aSyn is enriched in neurons[100], it is ubiquitously expressed. aSyn binds to phospholipid membranes[101] and is mainly localized at presynaptic terminals[102], although it has been also detected in the nucleus[103]. Due to its predominant presynaptic localization, a synaptic function for aSyn has been hypothesized, but still the role of aSyn in neurons is not clear.

aSyn presents three different regions: the N-terminal region, the NAC (non-Aβ component) region, and the C-terminal region (Fig. 4). In its N-terminal region, aSyn contains a distinctive 11-residue sequence (consensus XKTKEGVXXXX) repeated 7 times, which forms the amphipathic α-helix that allows aSyn to bind membranes; therefore, truncations of the N-terminus of aSyn reduces binding of lipids. The NAC region is hydrophobic and prone to aggregate, while the C-terminal region is acidic and unstructured and is involved in the interaction with many other proteins. The C-terminal region seems to be protective against aSyn aggregation.

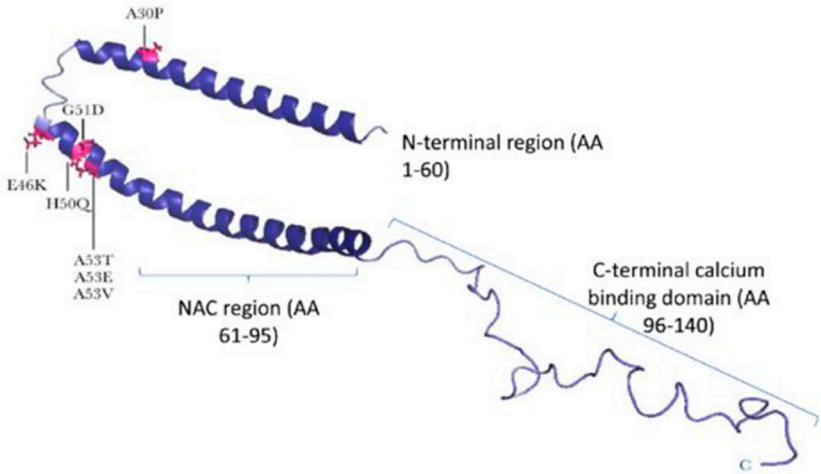

Figure 4. Alpha-synuclein structure. Model of alpha-synuclein (aSyn). aSyn presents with 3 different regions: N-Terminal region, non-Aβ component (NAC) region, and C-terminal domain. In pink are highlighted the aSyn mutations that have been described. Figure from Whittaker et al. (2017)[104].

Moreover, aSyn can undergo several PTMs: phosphorylation, acetylation, nitration, ubiquitination, glycation, glycosylation, oxidation, sumoylation, and proteolysis. These PTMs occur mainly in the C-terminal region of aSyn and are believed to impact the propensity with which aSyn interacts with lipids and its different interaction partners. Moreover, the PTMs have been shown to impact the ability of aSyn to oligomerize.

The physiological function of aSyn is still under debate. Its presynaptic localization, due to its preference for binding to synaptic vesicles[102] and/or to its ability to interact with synaptobrevin-2 vesicular SNARE protein and the chaperoning SNARE-complex assembly[105], suggests a potential presynaptic role of aSyn in neurotransmitter release and synaptic plasticity. In addition, several other potential functions have been attributed to aSyn: metabolism and/or transport of lipids, due to its similarity to class A2 apolipoproteins[106,107]; molecular chaperone activity, due to its biochemical structure and its homology with the 14-3-3 family of molecular chaperone proteins[108]; a role in dopamine synthesis and transport: aSyn can stabilize inactive tyrosine hydroxylase (TH)[109–111] and bind to the vesicular transporter VMAT2, thus, reducing dopamine synthesis and interfering in dopamine homeostasis by increasing the cytosolic dopamine levels[112]; and the potential ability of regulating microtubule formation and dynamics due to aSyn interaction with Tau[87,88], tubulin[113,114], and other motor proteins. Moreover, aSyn can enter the nucleus, where it has been shown to inhibit histone acetylation[115]; and it seems to bind mitochondria[116]. A pathological role for aSyn in promoting mitochondria fission has been proposed.

Under pathological circumstances, aSyn adopts a β-sheet conformation that promotes aSyn aggregation and neurotoxicity. Currently, a neurotoxic role for the oligomeric forms of aSyn is hypothesized, while larger aggregates (LB and LN) seem to have a benign or even protective role[117]. Together with this neurotoxic effect, a loss of function associated to the inability of aSyn to carry on with its physiological role(s) would be predicted.

Bridging the gap

Amyloid-beta and Tau

In AD, the association between Aβ and Tau becomes clear. The accumulation of both proteins is required for the diagnose of AD pathology. Moreover, the well-established "amyloid cascade hypothesis" postulates that alteration in Aβ will trigger Tau pathology, then leading to the accumulation of both aggregates in the AD brain. This has been supported by several publications showing that Aβ can induce Tau phosphorylation and worsen Tau pathology[118], placing Tau alterations downstream of Aβ pathology.

Nevertheless, Tau seems to mediate Aβ toxicity[119,120], postsynaptic Tau is required for the NMDAR-mediated excitotoxicity induced by Aβ[120]; and the MAPT H1 haplotype is associated with an increased risk of developing AD[121], highlighting the importance of Tau in AD. Additionally, extracellular human Tau increases Aβ levels, possibly by increasing neuronal excitability[122]. Moreover, Tau reduction and Tau passive immunization was shown to reduce Aβ pathology[123–125], but this effect has not been consistent[119,126].

In AD, a synergistic effect between Aβ and Tau has been proposed[127,128]. However, Aβ and Tau do not appear to directly interact at the ultrastructural level[129]. Still, the cellular and molecular mechanisms whereby Aβ and Tau either directly or indirectly interact and influence each other remain unclear.

Alpha-synuclein and Tau

The MAPT gene that encodes for Tau is one of the major risk factors for the development of PD[11,130], in which pathological accumulation of aSyn is one of the hallmarks of the pathology. Besides that, a growing body of evidence suggests an important role for Tau in synuclein pathology.

In human post-mortem brain, co-occurrence of aSyn and Tau pathology has been reported. In particular, co-occurrence of both aggregates has been observed in

amygdala in AD, PD, Down syndrome, and DLB[131–134]. Moreover, animal work has shown that Tau reduction improves synuclein pathology[135], and that mutant aSyn drives the phosphorylation and the missorting of Tau to dendritic compartments, where it has a detrimental effect[136]. Additionally, the physical interaction between aSyn and Tau has been suggested[87,88]. However, how the aSyn-Tau interaction impacts on neurodegeneration is still poorly understood.

Amyloids at the synapses

The proteins involved, the neuronal type affected, and the anatomical paths by which the propagation occurs make AD and PD two different neurodegenerative diseases. However, they both have several aspects in common, and are somehow interconnected. Changes in neuronal activity, synaptic alterations, axon degeneration, mitochondrial dysfunction, and increased ROS production are some of the pathological changes that have been reported in both diseases.

Synaptic alterations are reported before, and seem to be the basis of, the cognitive decline observed in the different neurodegenerative diseases. Furthermore, Aβ, Tau, and aSyn have been shown to be released and taken up by neurons, acting as pathological seeds in their oligomeric forms. This release occurs at synapses and is associated to neuronal activity[60,137,138], thus, pointing out synapses as one of the anatomically relevant places in which both diseases can be interconnected. In fact, as discussed above, aSyn and Aβ are predominantly located at synapses, aSyn due to its avidity for binding synaptic vesicles and Aβ because of its production and accumulation in endosomes at the synapse. Moreover, Tau has been reported to be physiologically present in pre- and post-synaptic compartments[73–75].

Pathological aSyn and Tau have been reported to lead to defects in vesicle trafficking[75,139], and vesicle release is required for the non-amyloidogenic processing of APP[60]. Therefore, impairment in vesicle trafficking might lead to a shift of the APP processing towards the amyloidogenic pathway.

Furthermore, the accumulation of Aβ in endosomes results in their leakage[62]. Under pathological circumstances, the hyperphosphorylation of Tau leads to an increase in the concentration of the protein at synapses. Recently, Polanco et al. (2021)[140] showed that endolysosomal permeabilization triggers Tau accumulation, thus, the permeabilization induced by Aβ accumulation could potentially enhance Tau aggregation at the synapses.

Moreover, changes in neuronal excitability have been reported in both AD and PD. Increased activity leads to greater release of the pathological forms of Aβ, Tau and aSyn, thus, accelerating the propagation and progression of the disease. However, how the interaction and co-occurrence of these proteins take place at synapses to lead to neurodegeneration requires extensive investigation.

Aims of the thesis

The overall aim of this thesis was to evaluate the effect of the direct and/or indirect interactions between amyloidogenic proteins in the pathophysiology of Alzheimer's and Parkinson's disease. The specific aims were:

I. To test the ability of alpha-synuclein and Tau to physically interact with each other in vivo, and to evaluate whether bimolecular fluorescence complementation was a suitable technique for the study and visualization of these interactions.

II. To evaluate the impact that the co-expression of amyloidogenic proteins have over the secondary structure of pathological amyloid aggregates by synchrotron-based Fourier Transform Infrared microspectroscopy (μ-FTIR)

III. To understand the role of astrocytic and neuronal ApoE isoforms in Alzheimer's disease pathophysiology, specifically their impact on neuronal excitability.

IV. To unravel the effects of Tau (WT and P301L) on neuronal excitability, the mechanism(s) underlying these effects, and to evaluate how the presence of elevated amyloid-beta might modulate these effects.

Key methods

Experimental models

To study the effect of interaction between different amyloidogenic proteins have in neurodegeneration we used the following cellular and rodent models.

Human embryonic kidney 293 (HEK293) cells and a neuronal-like cell line, **SH-SY5Y cells**, were used to study the interaction between aSyn and Tau in paper I. Another neuronal-like cell line, **Neuro 2a (N2a)** was used to investigate the pathological β-sheet formation of amyloids in paper II, where N2a cells were used in their naïve state or stably transfected with the human amyloid precursor protein (APP) protein carrying the Swedish mutation (**N2a-APPSwe**), that leads to the generation of high Aβ levels.

Neuronal excitability was studied by calcium imaging in **primary neuronal cultures**. These cultures were obtained from WT and APP/PS1 AD transgenic (Tg) mice in paper III and IV, and from WT, ApoE KO, ApoE3 KI, and ApoE4 KI in paper III. Primary neurons have shown to recapitulate the maturation process occurring to neurons in the brain in a shorter period of time, being mature after 21 days in vitro (DIV). Thus, they provide an excellent tool for studying the mechanisms underlying the neurodegenerative processes occurring in AD and PD.

Primary astrocytic cultures from ApoE KO, ApoE3 KI, and ApoE4 KI mice were generated for paper III, and their conditioned media was used to assess the neuronal activity induced by human astrocytic ApoE in WT and APP/PS1 primary neuronal cultures.

Moreover, in paper I, expression of aSyn and Tau in the substantia nigra pars compacta (SNpc) of **WT mice and rats** allowed us to study the biologically relevant interaction of aSyn and Tau.

Generation of constructs and viral transduction

Cloning was used for the generation of the different BiFC constructs used in paper I, the generation of the CMV_GFP, CMV_aSyn(A53T)-GFP, and CMV_Tau(P301L)-GFP that were used to transfect N2a-APPSwe in paper II, and for the development of the Syn1_GFP, Syn1_Tau WT and Syn1_Tau P301L constructs that were used in paper IV.

Lipofectamine transfection was used to express the constructs in cell lines in papers I and II. Additionally, in paper I, **AAV6** was generated carrying the different BiFC constructs, thus, allowing the injection and expression of the constructs in the SNpc of mice and rats. Moreover, in paper IV, **lentiviral vectors (LVs)** were generated to induce the expression of GFP, Tau WT and Tau P301L in primary neuronal cultures.

Cytotoxicity and protein assessment

The **Lactate dehydrogenase (LDH) cytotoxicity assay** was performed in paper IV to evaluate the toxicity induced by GFP, Tau WT and Tau P301L in primary neuronal cultures.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), blue-native PAGE (BN-PAGE), and Western blot (WB) were used to quantify protein levels in paper I, III and IV. Specifically, in paper I, SDS-PAGE was used to evaluate the different levels of aSyn and Tau when they were expressed linked to one of the Venus halves. Moreover, in paper III, SDS-PAGE and WB were used to measure ApoE levels in astrocytic conditioned media and neuronal cultures, while BN-PAGE and WB were used to characterize the lipidation state of ApoE. Lastly, in paper IV, SDS-PAGE and WB were used to quantify the increase in Tau expression after LV transduction of primary neuronal cultures with Tau WT and Tau P301L.

Immunofluorescence was performed to measure protein levels in paper I, II, III and IV. In paper I, immunohistochemistry allowed for the observation of aSyn and Tau, and their phosphorylated forms (pS129 and AT8), after AAV6 injection in SNpc; also, Tyrosine hydroxylase (TH) levels were assessed. In paper II, immunocytochemistry enabled the observation of total and fibrillar amyloid-beta (Aβ). In paper III, immunofluorescence was used to assess ApoE localization at or close to synaptic terminals and to evaluate changes in excitatory and inhibitory synaptic densities. Likewise, in paper IV, immunofluorescence was performed to quantify changes in inhibitory synaptic densities and the percentage of GABAergic neurons in culture.

Bimolecular fluorescence complementation

Amyloids have shown to be able to influence each other's pathology. However, the relevance of these interactions is under debate. Moreover, it is still not clear whether these synergistic effects between amyloidogenic proteins are due to direct and/or indirect interaction between them.

Previous literature supports the potential of aSyn and Tau to not only influence each other's pathology but also to interact with each other physically. Thus, in paper I, we wanted to evaluate the potential interaction between both proteins in a biological context. In particular, in paper I, bimolecular fluorescence complementation (BiFC) was used to evaluate the ability of aSyn and Tau to interact with each other. BiFC is a technique that enables direct visualization of **protein-protein interactions** within the live cell, providing new insights into the ability of proteins to interact with each other in a biologically relevant context.

Unlike other techniques to visualize interaction and/or co-localization, BiFC does not require fixation or lysis steps that could alter the physiological interaction of the proteins in vivo. Thus, it could potentially be used not only to analyze protein interaction but also to evaluate how different processes/treatments can increase or reduce the interaction between specific proteins.

Infrared microspectroscopy

In paper II, synchrotron-based Fourier Transform Infrared microspectroscopy (μ-FTIR) was used to evaluate changes in the pathological conformation of amyloids when they were co-expressed.

Under pathological circumstances, amyloidogenic proteins aggregate, forming oligomeric and/or high-molecular aggregates that potentially lead to the neurotoxic effect observed in neurodegenerative diseases. It is still under debate which molecular conformation of these proteins is the most neurotoxic one, but recent research points out a gain of toxic effect of the oligomeric forms of Aβ, Tau, and aSyn; while their fibrillary forms seem to be harmless or have a reduced effect on toxicity. Moreover, these oligomeric aggregates seem to have the ability to adopt different conformations, that potentially lead to a different degree of toxicity.

Multiple techniques have been used to characterize the different oligomeric forms of amyloids. However, most of them require harsh pre-processing steps that might alter the native conformation of those aggregates in vivo.

μ-FTIR is a technique that allows to investigate **structural changes in proteins and lipids**, and to characterize the molecular conformation of amyloids directly in

cells. It is a label-free technique that does not require pre-processing of the samples, thus, allowing the study of subtle structural/conformational changes in the native state of the proteins in the cell.

Neuronal excitability by calcium imaging

Changes in neuronal excitability prior to neurodegeneration has been observed in AD and PD, and the specific role of the different amyloidogenic proteins in this process is still under debate. Thus, in papers III and IV, we wanted to evaluate the role of ApoE and Tau, respectively, in neuronal excitability.

Specifically, neuronal excitability was evaluated by live-cell Ca2+ imaging. A calcium dye, Fluo-4, was added to the primary neuronal cultures and **calcium fluctuations** were measured as an indirect readout of neuronal excitability. While this technique provides an indirect readout in activity, it enables to study 100 – 1000 neurons at a time, providing information not only from single cells but also informing about changes in activity in the neuronal network.

Summary of key results

Paper I

In paper I, we aimed to investigate the potential interaction between aSyn and Tau in a physiologically relevant context and to test whether bimolecular fluorescence complementation (BiFC) would be a suitable tool to evaluate this interaction.

aSyn and Tau, each carrying one of the halves of the Venus fluorescence protein, were co-transfected in HEK293 cells to evaluate whether BiFC would allow observing aSyn-Tau interaction in a cellular model (Fig. 5A). Single transfection of Venus and aSyn-Venus was used as a positive control for the maximum fluorescence that we could expect from the Venus protein and to confirm that the Venus fluorescence could be emitted when linked to aSyn. Moreover, the co-transfection of the Venus N-terminal half (VN-) together with the Venus C-Terminal half (-VC) was used as a negative control, to prove that BiFC Venus complementation could happen when both Venus halves were attached to proteins that can interact with each other (Fig. 5B).

After co-transfection of the single proteins carrying each of the Venus halves, we detected self-interaction of aSyn, Tau WT and Tau P301L (Fig. 5B), proving that protein-protein interaction could be observed by BiFC. Furthermore, the co-transfection of aSyn (WT) and Tau (WT or P301L) with Venus halves showed the ability of the two proteins to interact with each other (Fig. 5C).

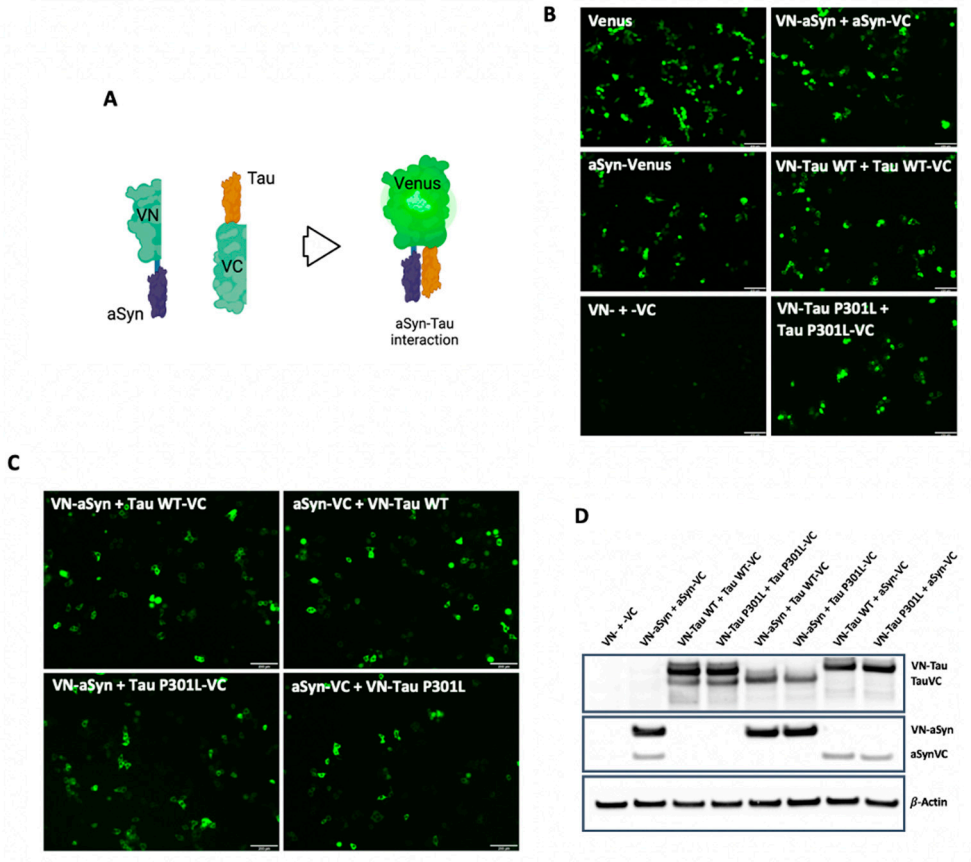

Figure 5. Bimolecular Fluorescence Complementation (BiFC) in HEK293 cells shows aSyn-Tau interaction. A) Scheme of the BiFC system in which aSyn and/or Tau were expressed together with half of the Venus fluorescence protein. Interaction between proteins (aSyn and/or Tau) results in BiFC Venus complementation. B) Positive control (Venus and aSyn-Venus), negative control (VN- + -VC) and self-interaction of aSyn, Tau WT and Tau P301L shows the suitability of the BiFC assay to observe protein-protein interaction. C) aSyn-Tau (WT or P301L) interactions are occurring at the cellular level and can be observed by BiFC Venus complementation. D) Western blot demonstrating an imbalance in the protein levels when aSyn or Tau are expressed linked to the Venus N-terminal half (VN-), in comparison to their levels in HEK293 cells when they are expressed attached to the Venus C-terminal (VC-).

Similar experiments were performed in SH-SY5Y cells, demonstrating that aSyn-Tau can interact with each other in a neuronal-like cell line, and that the BiFC assay was also appropriate to observe this interaction.

Once we confirmed that the BiFC assay could be used to observe aSyn-Tau interaction in cells, we co-expressed the BiFC constructs in the SNpc of mice by AAV6 injection. Fifteen weeks after injection, we observed BiFC Venus complementation when aSyn and Tau were co-expressed. As expected, this signal was lower than the one obtained when we expressed aSyn linked to full-length Venus (aSyn-Venus) in the same area, but higher than when VN- and -VC not linked to the interacting proteins were co-

expressed (Fig. 6); confirming that aSyn-Tau interaction could be tracked in vivo by BiFC in mouse SNpc.

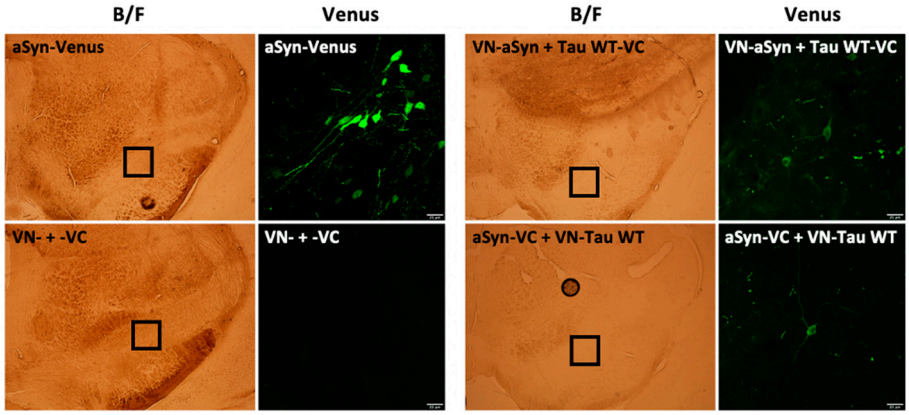

Figure 6. AAV6 injection of BiFC aSyn WT and BiFC Tau WT in mouse substantia nigra pars compacta (SNpc) shows aSyn-Tau interaction in vivo. By BiFC we observed that aSyn and Tau can interact with each other in the SNpc of the living mouse. aSyn-Venus was used as a positive control, and VN- + -VC as a negative control, demonstrating that the BiFC assay is appropriate to observe protein interaction in vivo..

Lastly, we injected VN-aSyn WT + Tau WT-VC in the rat SNpc. After 8 weeks, BiFC Venus complementation could be observed, confirming aSyn-Tau interaction also in the rat SNpc. Moreover, immunohistochemistry against phosphorylated aSyn (pS129) and Tau (AT8) showed the ability of aSyn and Tau to interact with each other even when phosphorylated (Fig. 7A). Further staining for total aSyn and Tau demonstrated a different pattern of propagation of aSyn and Tau in the rat brain after injection (Fig. 7B), with aSyn propagating from the SNpc to the striatum while Tau remained in the injection site.

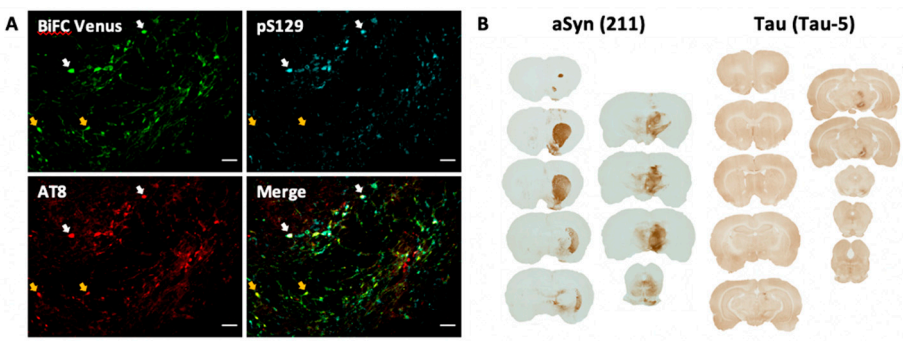

Figure 7. aSyn-Tau interaction is observed by BiFC in rat SNpc. A) BiFC Venus complementation after co-expression of VN-aSyn WT and Tau WT-VC in rat SNpc demonstrates the interaction between aSyn and Tau. Immunohistochemistry for phosphorylated Tau (AT8) and aSyn pS129 in phosphorylation sites associated to pathological Tau and aSyn. B) Expression of aSyn and Tau 8 weeks after AAV6 injection of the BiFC constructs in SNpc, showing propagation of aSyn from SNpc to striatum.

Paper II

In paper II we aimed to evaluate the impact that the co-expression of amyloidogenic proteins (Aβ-Tau and Aβ-aSyn) has on amyloid aggregates. For that, a mouse neuroblastoma cell line, Neuro 2a (N2a), stably expressing the human amyloid precursor protein (APP) carrying the Swedish mutation (N2a-APPSwe) was co-transfected with Tau or aSyn, and structural changes in amyloid content were assessed by synchrotron-based Fourier Transform Infrared microspectroscopy (μ-FTIR).

First, we confirmed the pathological accumulation of Aβ in our cell model (Fig. 8A). Naïve (untransfected) N2a cells did not show increased Aβ levels nor Aβ aggregates, as can be observed by the lack of signal after staining for total (82E1; Aβ specific antibody) and fibrillar (OC amyloid fibril-specific antibody) Aβ; in comparison to N2a-APPSwe cells. Surprisingly, the co-expression of Aβ-aSyn(A53T)-GFP (APPSwe-aSyn) and Aβ-Tau(P301L)-GFP (APPSwe-Tau) led to a two-fold reduction in the particle size of the Aβ aggregates (Fig. 8B). This reduction in particle size occurred together with a significant reduction (22%) in fibrillar Aβ in APPSwe-aSyn cells (Fig. 8C), demonstrating that aSyn A53T can impact the structure and morphology of Aβ aggregates.

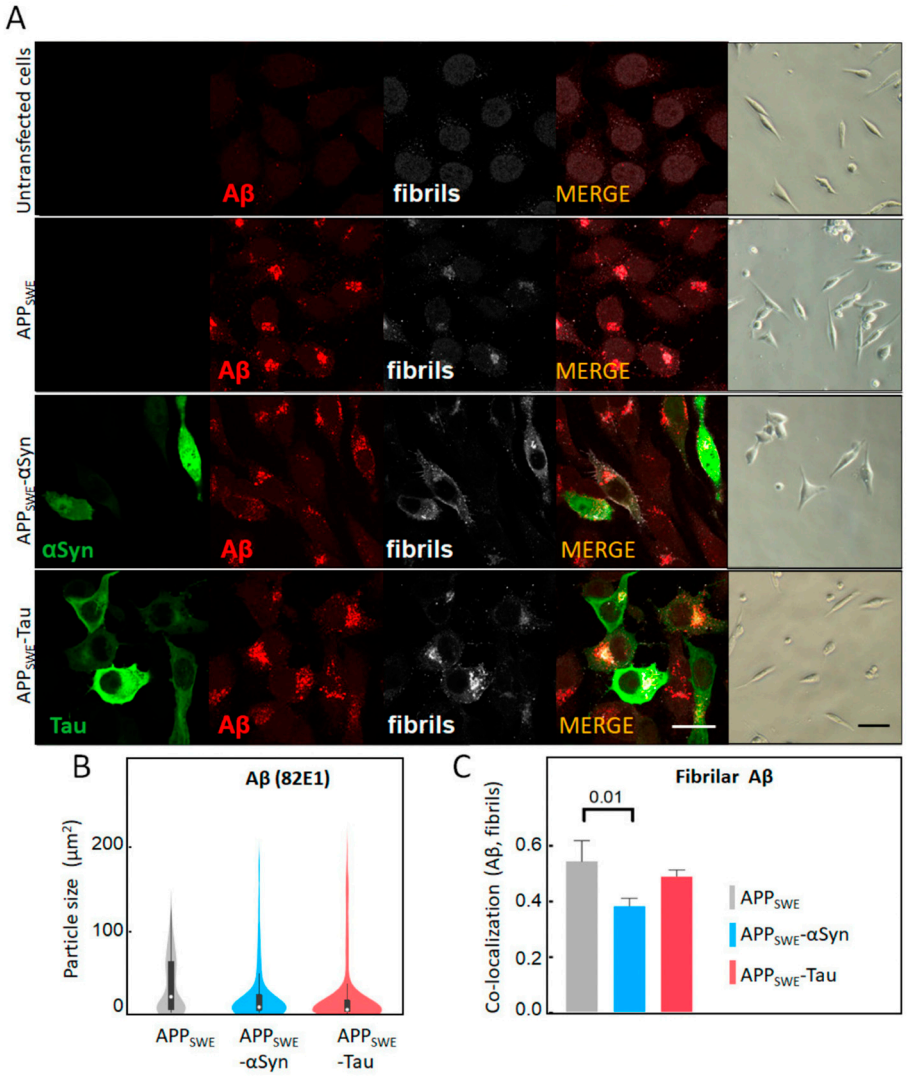

Figure 8. Aβ characterization of cellular models of Alzheimer's disease (AD) by immunofluorescence. A) Naïve, APPSwe, APPSwe transfected with aSyn(A53T)-GFP (APPSwe-aSyn), and APPSwe transfected with Tau(P301L)-GFP (APPSwe-Tau) N2a cells stained with antibody 82E1 (Aβ specific antibody) and OC (amyloid fibril-specific antibody) to evaluate structural and morphological changes in Aβ composition. B) APPSwe-aSyn and APPSwe-Tau showed a 2-fold reduction on Aβ particle size. C) APPSwe-aSyn cells, unlike APPSwe-Tau cells, resulted in a 22% reduction of fibrillar Aβ.

Next, we characterized the secondary structure of those aggregates. μ-FTIR measures changes in functional groups which are part of biological molecules, thus, providing information about the main components of amyloids, and their secondary structure, specifically their composition in β-sheets and random coil (unordered)

structures. In these experiments, control N2a-APPSwe were transfected with GFP (APPSwe) as the expression of GFP alone showed not to lead to changes in the composition of β-sheet structures observed by μ-FTIR.

The transfection of APPSwe with aSyn(A53T)-GFP (APPSwe-aSyn) resulted in a significant reduction in the levels of β-sheet and random coil structures in comparison to APPSwe and aSyn(A53T)-GFP (aSyn) cells. Additionally, a significant reduction in total protein folding was observed in APPSwe-aSyn cells (Fig. 9A and B). The reduction in total protein folding observed in APPSwe-aSyn is in agreement with the reduction in fibrillar Aβ detected by immunofluorescence (Fig. 9C).

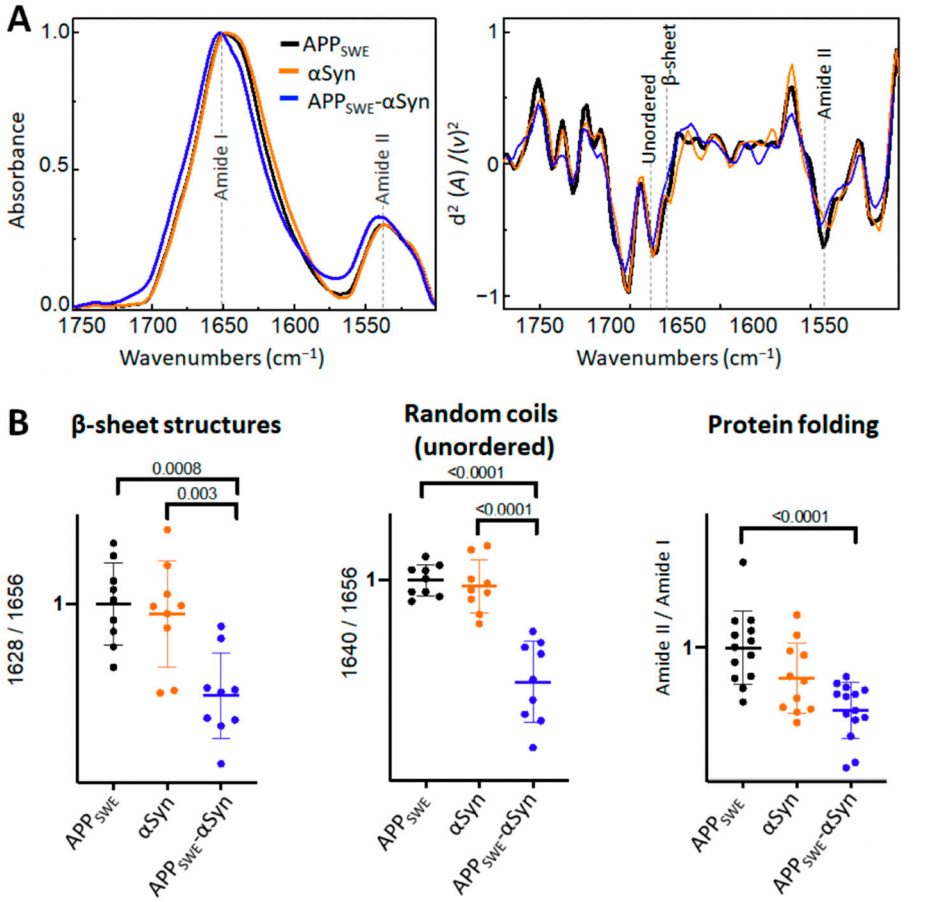

Figure 9. Impact of aSyn (A53T) on pathological amyloidogenic aggregation. Structural and conformational changes in amyloidogenic aggregates were evaluated in N2a cells overexpressing human APP (Swedish mutation) and GFP (APPSwe), or human aSyn (A53T) (aSyn), or both APP and aSyn (APPSwe-aSyn) by μ-FTIR. A) Averaged and normalized infrared absorbance spectra. B) A reduction in β-sheet structures and C) random coil structures was observed in APPSwe-aSyn compared to the other cell types. D) A reduction in total protein folding was observed in APPSwe-aSyn compared to APPSwe cells.

Later, the effect of Tau(P301L)-GFP in Aβ aggregation was assessed. No differences were detected when N2a-APPSwe and the bigenic APPSwe-Tau were compared. However, the single transfection with Tau(P301L)-GFP (Tau) resulted in an increase in β-sheet and random coil structures, together with an increase in lipid oxidation (Fig. 10A and B).

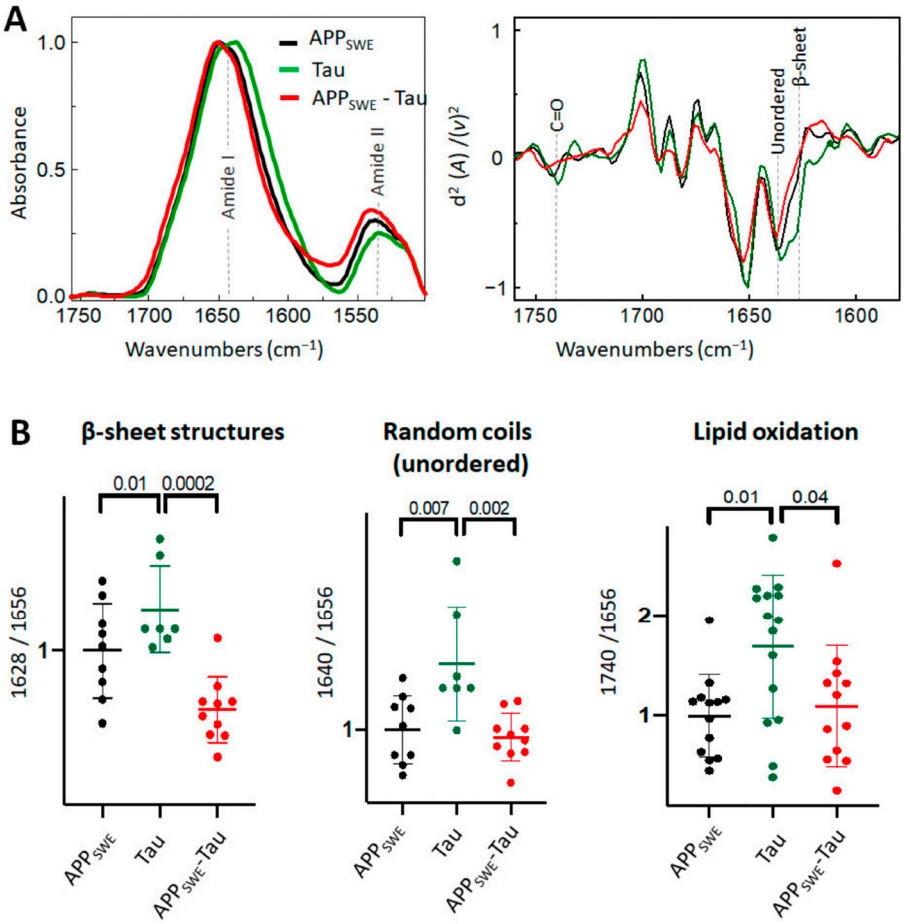

Figure 10. Impact of Tau (P301L) on pathological amyloidogenic aggregation. Conformational and structural changes in amyloidogenic aggregates were assessed in N2a cells overexpressing human APP (Swedish mutation) and GFP (APPSwe), or human Tau (P301L) (Tau), or both APP and Tau (APPSwe-Tau) by μ-FTIR. A) Averaged and normalized infrared absorbance spectra. B) An increase in β-sheet structures. C) random coil structures, and D) total protein folding was observed in Tau cells compared to the other cell types.

Furthermore, when comparing the bigenic cell lines between themselves, we could observe that the co-expression of N2a-APPSwe with either aSyn or Tau led to a reduction in β-sheet load. In APPSwe-aSyn an additional reduction of random coil

structures and total protein folding was observed, while APPSwe-Tau did not show any difference in these parameters when compared to APPSwe (Fig. 11A and B).

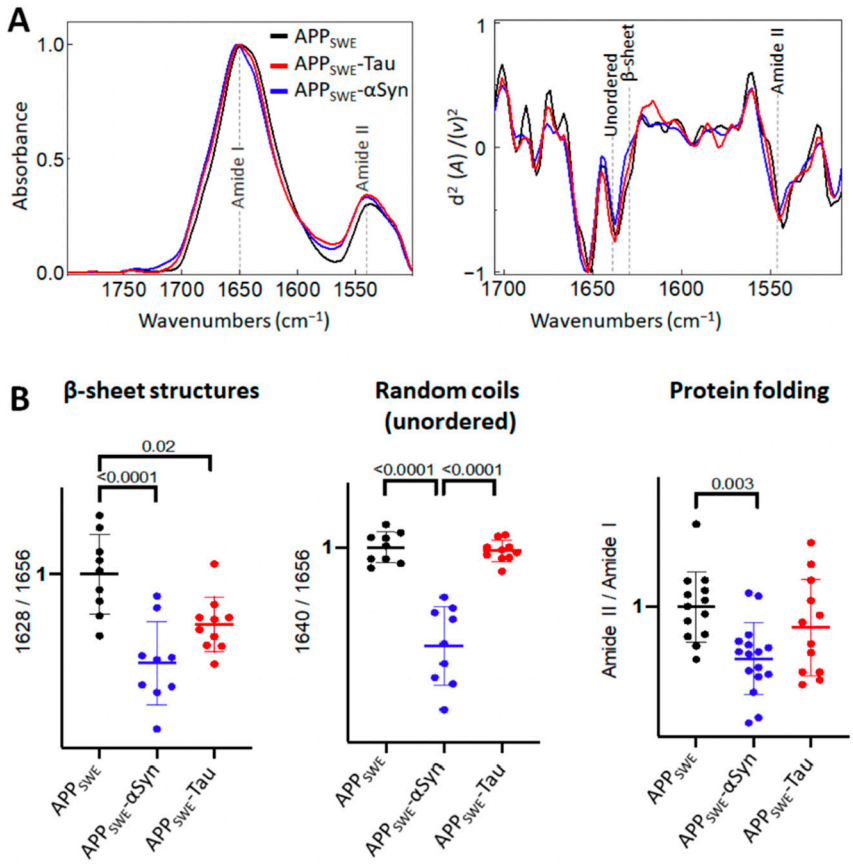

Figure 11. Conformation and structural changes in pathological amyloidogenic aggregates in bigenic AD cell lines. Comparison of the changes observed in amyloidogenic aggregates in N2a-APPSwe cells co-expressing GFP (APPSwe), aSyn (A53T) (APPSwe-aSyn), or Tau (P301L) (APPSwe-Tau). A) Averaged and normalized infrared absorbance spectra of the different bigenic cell lines. B) Reduction in β-sheet structures in APPSwe-aSyn and APPSwe-Tau cells compared to APPSwe. C) APPSwe-aSyn showed reduced random coil structures compared to APPSwe and APPSwe-Tau. D) Total protein folding is significantly lower in APPSwe-aSyn compared to APPSwe.

Paper III

ApoE in the central nervous system (CNS) is mainly expressed by astrocytes; however, under stress conditions, neurons are able to produce ApoE. Additionally, a role for ApoE in neuronal excitability has been reported. Thus, in paper III, we wanted to evaluate the effect of the different ApoE isoforms, from different sources (neuronal vs. astrocytic), on neuronal excitability.

To study the effect of neuronal human ApoE in activity, primary neuronal cultures from WT, ApoE KO, ApoE3 KI, and ApoE4 KI mice were used. First, the levels of human ApoE in the neuronal cultures were measured by Western blot. We confirmed that human ApoE in ApoE KI cultures is expressed at similar levels for ApoE3 KI and ApoE4 KI (Fig. 12A and B). Next, we noted that ApoE targeted synapses. Co-labelling of ApoE with the vesicular glutamate transporter 1 (vGlut1) (Fig. 12C) demonstrated that 40% of the ApoE in neurons was present at or close to vGlut1-positive synapses, where it could have an effect on neuronal excitability. No significant difference between ApoE3 and ApoE4 was observed (Fig. 12D and E).

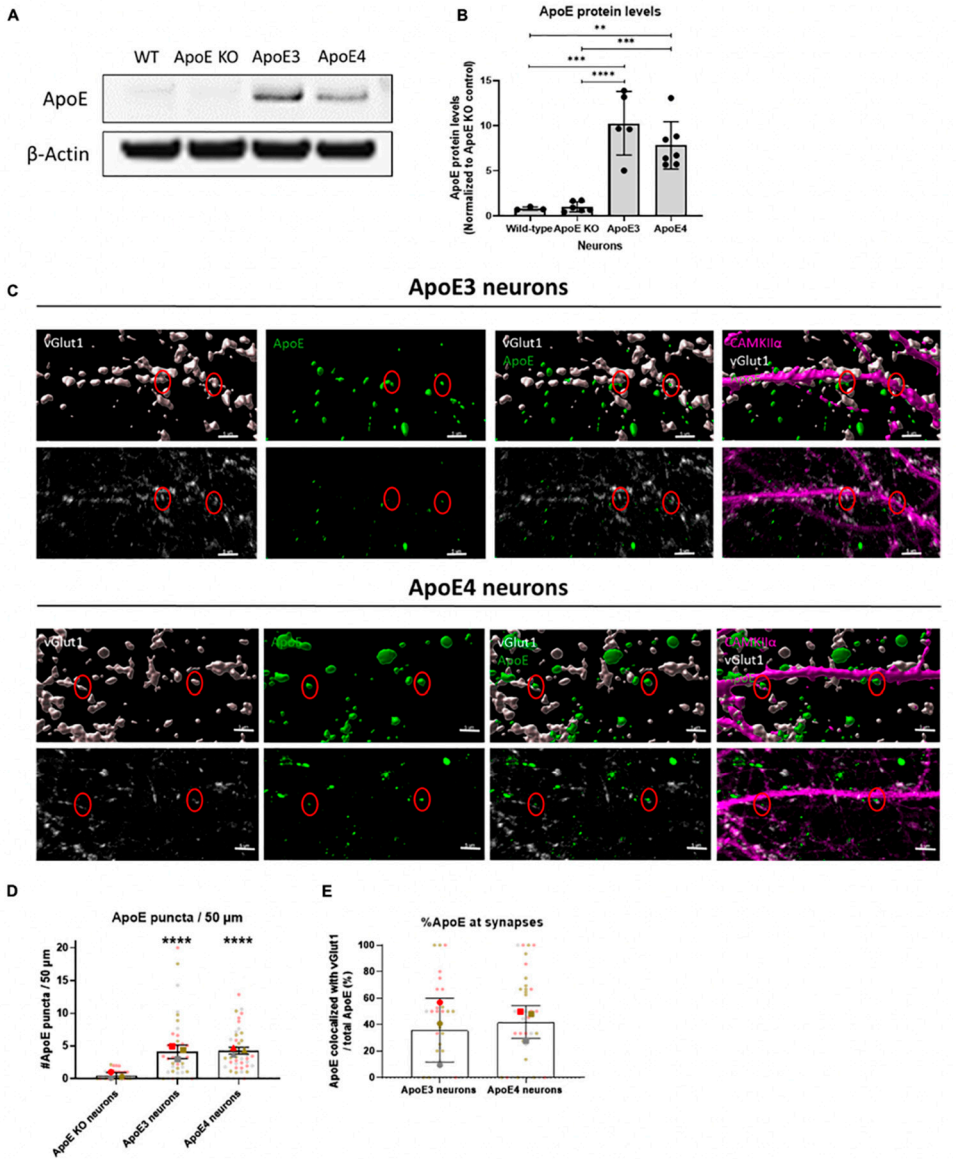

Figure 12. Synaptic localization of human ApoE. A) Western blot showing the levels of human ApoE in primary neuronal cultures from WT, ApoE KO, ApoE3 KI, and ApoE4 KI mice. B) Human ApoE was seen to be expressed at similar levels in both ApoE3 KI and ApoE4 KI primary cultures. C) Co-localization of ApoE with vGlut1 in ApoE3 KI and ApoE4 KI primary neurons. D) Number of ApoE puncta in neurites (ApoE puncta / 50 µm of neurite). D) Percentage of ApoE puncta at or close to vGlut1-positive synapses.

Later, the effect of ApoE isoforms on neuronal excitability was evaluated. Primary neuronal cultures from WT, ApoE KO, ApoE3 KI, and ApoE4 KI were prepared, and after 18-20 DIV, neuronal activity was measured by live-cell Ca2+ imaging. A

similar spike frequency distribution was observed between cultures, with ApoE3 presenting on average higher spike frequency than the other cultures (Fig. 13A and B). Besides an increase in spike frequency (Fig. 13B), ApoE3 cultures showed a higher percentage of active neurons than WT and ApoE4 cultures, and a reduction in the percentage of independent spikes in comparison with WT, ApoE KO and ApoE4 neurons (Fig. 13C). Greater variability between cultures was observed in relation to the amplitude, with ApoE3 neurons showing the highest amplitude and WT neurons the lowest. These data suggested an isoform-dependent effect of ApoE on neuronal excitability, with ApoE3 leading to increased neuronal activity.

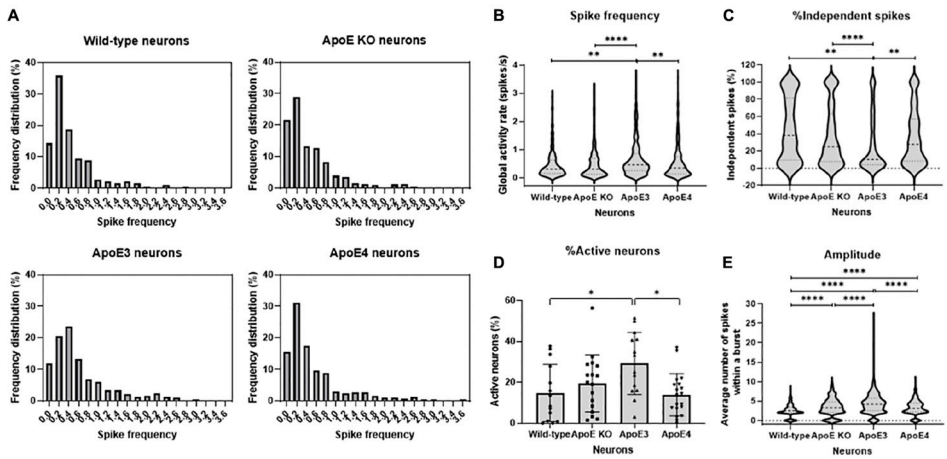

Figure 13. Neuronal ApoE3 increases neuronal activity. Live-cell Ca2+ imaging was used to evaluate changes in neuronal activity in WT, ApoE KO, ApoE3 KI, and ApoE4 KI primary cultures, different parameters were assessed: A) Spike frequency distribution: All the cultures showed similar distribution of spike frequency, but ApoE3 neurons showed higher average spike frequency; B) spike frequency: Increased spike frequency was observed in ApoE3 neurons in comparison with the other cultures; C) % independent spikes: ApoE3 neurons presented reduced percentage of independent spikes compared to the other cultures; D) % active neurons: ApoE3 showed an increased percentage of active neurons when compared to WT and ApoE4 neurons; and E) amplitude: High variability in amplitude was detected between cultures, with ApoE3 neurons showing the highest amplitude, and WT neurons the lowest.

Furthermore, to identify the potential mechanism(s) underlying the changes in neuronal excitability induced by ApoE, changes in neuronal morphology and/or dendritic branching were measured by Sholl analysis. Dendritic intersections were quantified, and no significant differences in the number of dendritic intersections or dendritic area were observed between WT, ApoE KO, ApoE3 and ApoE4 neurons (Fig. 14A, B and C). Next, synaptic changes were assessed. Changes in the number of excitatory (vGlut1-positive) and inhibitory (VGAT-positive) presynaptic inputs in the different cultures were quantified by co-labeling of neurons with vGlut1, VGAT and phalloidin. A reduction in excitatory and inhibitory presynaptic densities was observed in ApoE4 neurons, suggesting that the reduced neuronal activity observed in ApoE4 neurons compared to ApoE3 neurons could be due to the reduction in presynaptic inputs.

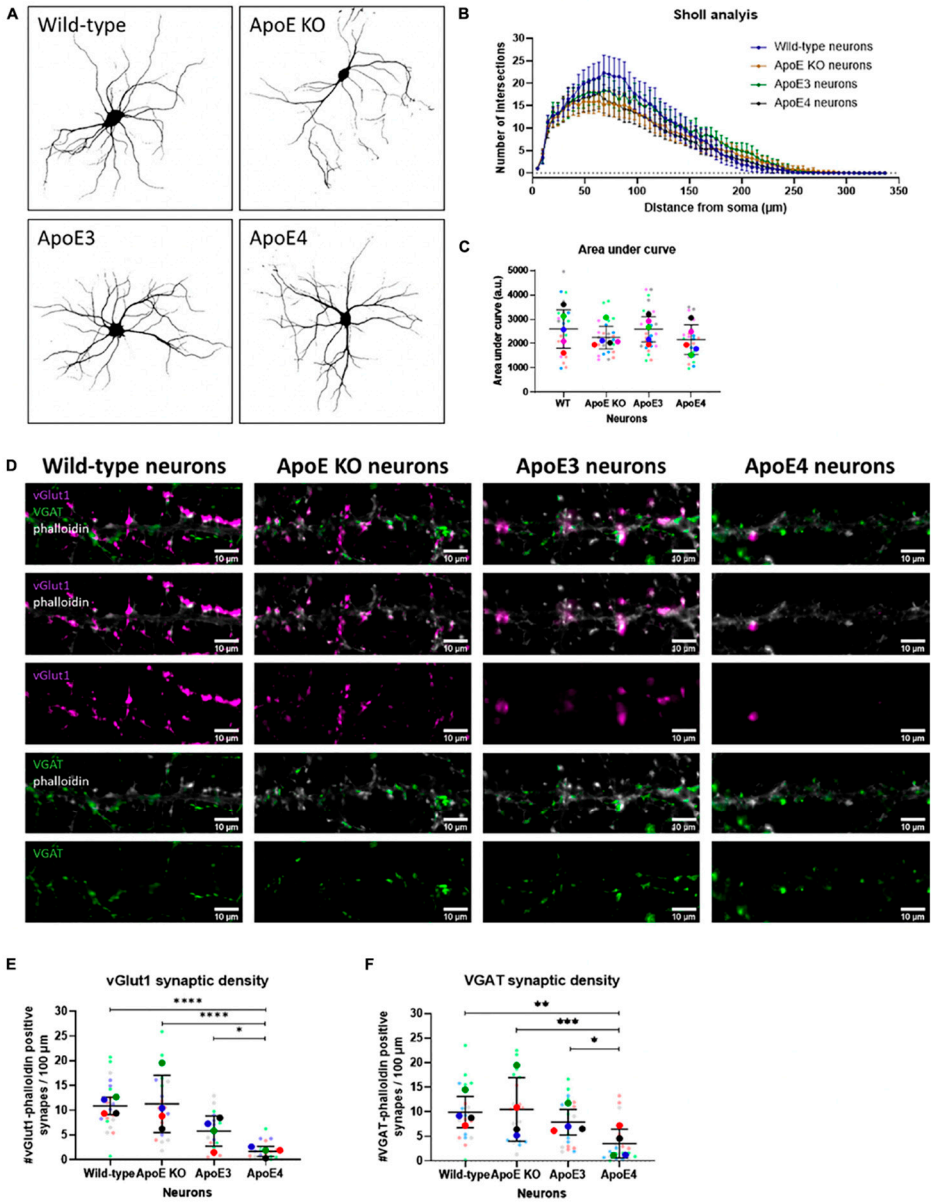

Figure 14. Reduction in synaptic inputs induced by neuronal ApoE4. A) Representative neurons from WT, ApoE KO, ApoE3 KI, and ApoE4 KI primary cultures. B and C) Sholl analysis was used to measure changes in dendritic branching and neuronal morphology. No differences in B) number of dendritic intersections or C) dendritic area were found. D) Representative images of the co-localization of ApoE with excitatory (vGlut1-positive) or inhibitory (VGAT-positive) synapses. E and F) ApoE4 neurons showed a significant reduction in E) excitatory and F) inhibitory presynaptic inputs.

To study the effect of astrocytic human ApoE on neuronal activity, primary neuronal cultures from WT and APP/PS1 AD transgenic (Tg) cultures were used. Conditioned media from astrocytic cultures (ApoE KO, ApoE3 KI, and ApoE4 KI) was collected and added to primary neuronal cultures. After 24 h (Fig. 15A), neuronal activity was assessed by measuring live-cell Ca2+ imaging.

As discussed in paper IV and other publications[141], APP/PS1 AD Tg primary neurons show an increase in firing/spike frequency and amplitude in comparison to WT neurons. Moreover, an increase in coordinated activity, or reduction in the percentage of independent spikes, is also characteristic of APP/PS1 neurons when compared to WT neurons. Interestingly, 24 h after the addition of astrocytic ApoE3 conditioned media, no significant difference between WT and APP/PS1 neuronal cultures in relation to spike frequency and percentage of independent spikes was observed (Fig. 15B and C). Moreover, astrocytic human ApoE3 led to a significant reduction in the amplitude of APP/PS1 neurons compared to WT neurons (Fig. 15D). However, the incubation of WT and APP/PS1 AD Tg cultures with ApoE KO or ApoE4 conditioned media resulted in similar differences in activity between WT and APP/PS1 neurons. After the addition of ApoE KO and ApoE4 astrocytic media, APP/PS1 neurons showed an increased spike frequency, amplitude and coordinated activity, reflected in the reduction of independent spikes, compared to WT neurons.

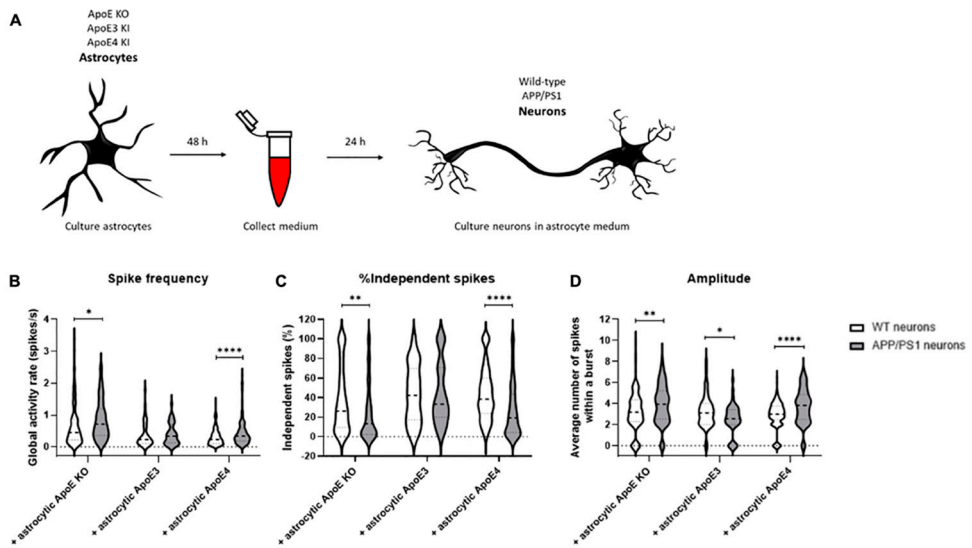

Figure 15. Astrocytic ApoE3 stabilizes the increase in neuronal activity characteristic of APP/PS1 AD Tg neurons. Conditioned media from ApoE KO, ApoE3 KI, and ApoE4 KI primary astrocytic cultures was collected, and added to WT and APP/PS1 AD Tg primary neuronal cultures. Neuronal activity was assessed 24 h later by Live-cell Ca2+ imaging. A) Schematic representation of the performed protocol. B) Spike frequency: Astrocytic ApoE3 led to no difference in spike frequency between WT and APP/PS1 AD Tg neurons. C) % independent spikes: Astrocytic ApoE3 led to no difference in percentage of independent spikes between WT and APP/PS1 AD Tg neurons. D) After addition of astrocytic ApoE3, APP/PS1 AD Tg neurons presented a reduction in amplitude compared to WT neurons; however, after addition of ApoE KO and ApoE4, APP/PS1 AD Tg neurons presented an increase in amplitude compared to WT neurons.

Paper IV

In paper IV, our goal was to determine the effects of human Tau (WT and P301L) on neuronal excitability in primary neuronal cultures, which would allow us to begin to unravel the molecular mechanisms underlying these effects. Additionally, we wanted to determine how elevated Aβ might modulate Tau effects. Primary neuronal cultures from WT and APP/PS1 AD Tg mice were prepared and, at 12-13 DIV, transduced with lentiviral vectors (LVs) carrying constructs for the specific neuronal expression of GFP, human WT Tau or human P301L Tau. After 3 and/or 7 days of expression, data was collected.

First, to characterize our model, we evaluated Tau levels 3 and 7 days after LV transduction. We could observe an increase of 40-60% in the levels of total Tau 7 days after transduction, but no increase after 3 days (Fig. 16A). Additionally, cell toxicity was evaluated 7 days after LV transduction. We could observe that the expression of WT Tau, but not control GFP or P301L Tau, led to an increase in cytotoxicity in both WT and APP/PS1 AD Tg cultures (Fig. 16B).

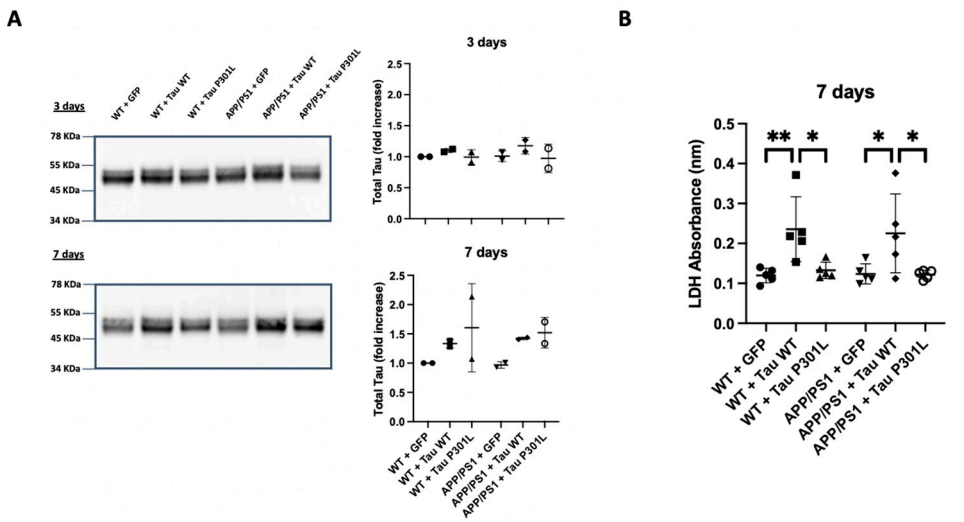

Figure 16. Characterization of the system used to study Tau-induced changes in neuronal activity. WT and APP/PS1 AD Tg primary neuronal cultures were transduced with GFP, Tau WT or Tau P301L. A) Western blot measuring total Tau levels 3 and 7 days after the transduction of WT and APP/PS1 AD neurons showed increased expression from the Tau constructs 7 days after transduction. B) Seven days after transduction, Tau WT, but not Tau P301L or control GFP, lead to a 2-fold increase of cytotoxicity in the cultures.

Furthermore, we assessed the effect of Tau (WT and P301L) on the activity of WT and APP/PS1 AD Tg neuronal cultures by live-cell Ca2+ imaging (Fig. 17A). At 19-20 DIV, we could observe that non-transduced APP/PS1 AD Tg neurons presented with an increase in firing frequency, amplitude and bursting rate, together

with increased coordinated activity, in comparison to WT neurons (Fig. 17B – E). Moreover, 7 days after transduction, the expression of WT Tau led to an increase in the firing frequency and bursting rate in both WT and APP/PS1 cultures. WT Tau strongly reduced the amplitude exclusively in APP/PS1 AD Tg cultures (Fig. 17B – E). On the other hand, an increase in frequency and bursting rate induced by P301L Tau was observed in WT, but not in APP/PS1 AD Tg neuronal cultures. In contrast to WT Tau, P301L Tau strongly reduced the number of independent spikes in APP/PS1 AD Tg cultures (Fig. 17B – E).

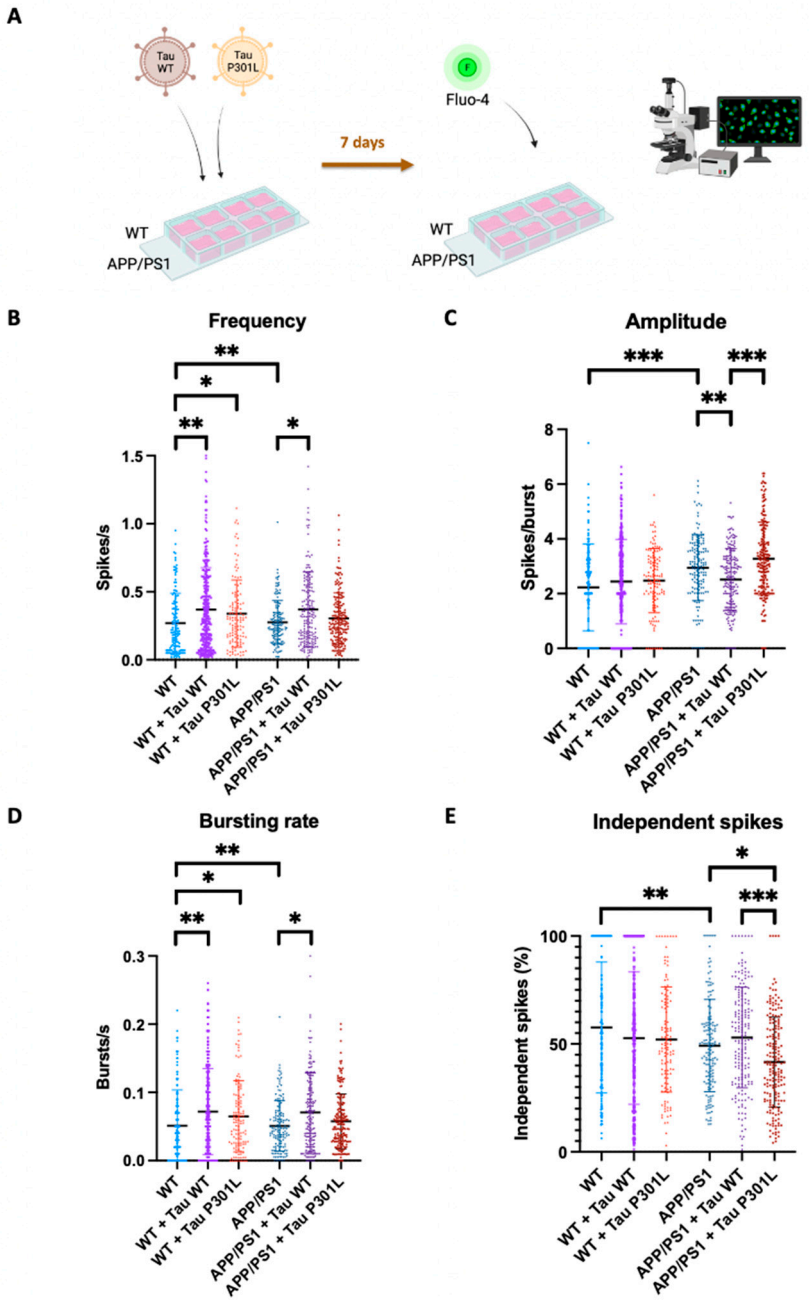

Figure 17. Impact of Tau WT and P301L on neuronal excitability. WT and APP/PS1 AD Tg primary neuronal cultures were transduced at 12-13 DIV with LVs for Tau WT or Tau P301L, 7 days later neuronal excitability was evaluated by Live-cell Ca2+ imaging. A) Schematic representation of the procedure. B-D) Significant differences between untransfected WT and APP/PS1 AD Tg cultures were observed, together with divergent effects of Tau WT and Tau P301L; in B) firing frequency (frequency), C) amplitude, D) bursting rate, and E) % independent spikes were observed.

Next, to identify the molecular mechanisms leading to these changes in activity, we evaluated neuronal and synaptic changes in our cultures. Quantification of the percentage of GAD67-positive neurons in the different cultures did not show any difference (Fig. 18B), demonstrating that the changes in activity induced by Tau were not due to alterations in the inhibitory GABAergic population. However, after co-immunolabelling for the pre- and postsynaptic inhibitory markers VGAT and gephyrin, respectively (Fig. 18A), a reduction in inhibitory synaptic densities was observed when WT Tau, but not P301L Tau, was overexpressed in both WT and APP/PS1 AD Tg neurons (Fig. 18C), suggesting that the increase in neuronal excitability induced by WT Tau could be due to a reduction in inhibitory input.

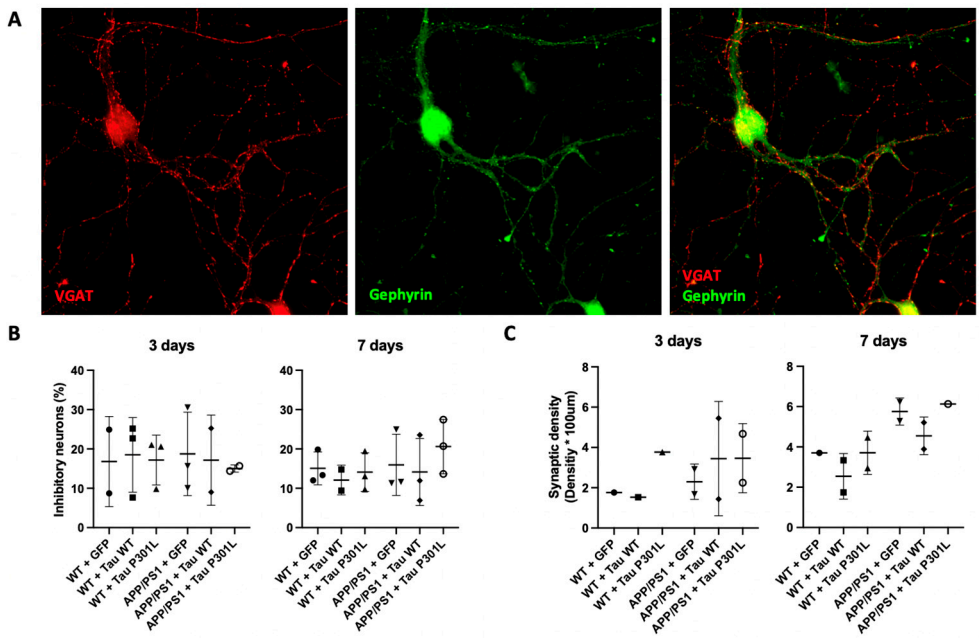

Figure 18. Human WT Tau reduces the synaptic inhibitory input. Changes in the percentage of inhibitory neurons or inhibitory synaptic inputs were quantified in WT and APP/PS1 AD Tg neurons upon transduction with WT and P301L Tau. A) Representative image showing the co-localization of VGAT (presynaptic inhibitory marker) and gephyrin (postsynaptic inhibitory marker) in primary neurons. B) Quantification of the percentage of inhibitory neurons in the different cultures. No significant difference between cultures were observed. C) Quantification of inhibitory synaptic densities in the different experimental settings. Tau WT led to a significant reduction of inhibitory synaptic input in both WT and APP/PS1 AD Tg neurons.

Discussion

Alzheimer's disease (AD) and Parkinson's disease (PD) are the two most common neurodegenerative diseases. The accumulation of amyloidogenic proteins that characterize both diseases, amyloid-beta (Aβ) and Tau in AD and alpha-synuclein (aSyn) in PD, is not limited to those diseases since mixed types of protein aggregates are frequently found in the brain. Thus, the overall goal of this thesis was to investigate the direct and/or indirect effects that amyloidogenic proteins have on each other's pathology.

First, in paper I, we assessed the ability of aSyn and Tau to physically interact with each other. Previous literature had shown how aSyn and Tau can influence each other's pathology, where previous in vitro work has suggested their potential to physically interact with each other[87,88]; however, the physical interaction between the proteins was not shown in vivo. By bimolecular fluorescence complementation (BiFC) we could confirm that aSyn and Tau were able to interact with each other in the physiological environment of the cell, not only in cell lines, but also within neurons in the substantia nigra pars compacta of different rodent models. Additionally, this provides a new tool to study the physiological and/or pathological consequences that aSyn and Tau interaction might have during health and disease, and to test potential treatments that could modulate aSyn-Tau interaction in vivo.

In paper II, we assessed how the co-expression of amyloidogenic proteins could impact on the secondary structure of amyloid aggregates. Immunofluorescence and synchrotron-based Fourier Transform Infrared microspectroscopy (μ-FTIR) were used. By immunofluorescence we could observe that both expressions of aSyn(A53T) and Tau(P301L) led to a reduction in the particle size of Aβ aggregates. Moreover, aSyn(A53T) expression in N2a-APPSwe cells reduced the fibrillar Aβ load by 22%, thus, indicating the ability of aSyn A53T to impact the structure and morphology of Aβ aggregates. In agreement with this data, the overexpression of aSyn A53T in N2a-APPSwe cells led to a reduction of β-sheet load, random coil (unordered) structures, and total protein folding. On the other hand, co-expression of Tau P301L in N2a-APPSwe cells did not result in changes in the secondary structure of pathological aggregates when compared to N2a-APPSwe cells. However, the single transduction of Tau P301L in naïve N2a cells gave rise to an increase in β-sheet and random coil (unordered) structures, together with increased total protein folding. Therefore, our data confirmed a synergistic effect between

amyloidogenic proteins. μ-FTIR characterization of amyloidogenic aggregates showed a clear impact on co-occurrence of pathology in the conformation of amyloid aggregates. Still, the impact of those changes on neurotoxicity, and neurodegeneration, needs to be further studied.

Changes in neuronal excitability are observed early in AD pathology, before neurodegeneration. Apolipoprotein E (ApoE) is the major risk factor for the development of AD and has been shown to have an impact in PD[21–24]. However, it is still not understood how ApoE isoforms can impact disease progression. ApoE is mainly expressed by astrocytes in the brain, but under stress, neurons can also synthesize ApoE[142]. Moreover, differential roles for astrocytic and neuronal ApoE have been suggested. Thus, in paper III, we investigated the role of astrocytic and neuronal ApoE in neuronal excitability by live-cell Ca2+ imaging.

First, the ability of ApoE to target synapses was detected. Both ApoE3 and ApoE4 showed to be present at or close to vGlut1-positive synapses; specifically, 40% of the ApoE in neurons was observed at or close to synaptic terminals, raising the possibility that ApoE could have a direct impact on neuronal excitability. Next, the neuronal activity of ApoE3 KI and ApoE4 primary neuronal cultures was compared. An increase in the percentage of active neurons, firing/spike frequency, amplitude, and coordinated activity was observed in ApoE3 compared to ApoE4. Finally, to elucidate the isoform-specific potential mechanism(s) that confer differences between ApoE3 and ApoE4, neuronal and synaptic changes between isoforms were measured. While there were no differences in morphology and/or dendritic branching between ApoE3 and Apo4 neurons, a significant reduction in excitatory and inhibitory presynaptic inputs was observed in ApoE4 neurons compared to ApoE3 neurons. Thus, potentially explaining the reduced activity observed in ApoE4 neurons compared to ApoE3 neurons.

Further, the effect of astrocytic ApoE on neuronal activity was determined. WT and APP/PS1 AD transgenic (Tg) cultures were incubated in astrocytic ApoE KO, ApoE3 KI, and ApoE4 KI conditioned media, and after 24 h, live-cell Ca2+ imaging was performed. As discussed in paper IV and was previously reported[141], APP/PS1 AD Tg neurons present increased firing frequency, amplitude, and coordinated activity compared to WT primary neurons. Surprisingly, when APP/PS1 AD Tg neurons were incubated with astrocytic ApoE3 conditioned media, no difference in firing frequency and coordinated activity were observed between WT and APP/PS1 AD Tg cultures. Moreover, the presence of astrocytic ApoE3 conditioned media led to a strong reduction of the amplitude in APP/PS1 AD Tg neurons compared to WT, thus, counteracting the increased amplitude usually observed in APP/PS1 AD Tg neurons when compared to WT neurons. Therefore, our results demonstrated a differential effect of astrocytic versus neuronal ApoE on neuronal excitability.

Lastly, in paper IV, we wanted to determine the effects of Tau (WT and P301L) on neuronal excitability. In AD, there is a consensus on the ability of Aβ to drive

hyperexcitability; however, the role of Tau is not as clear. Opposing results have been reported, with Tau leading to reduced and increased neuronal activity. Thus, we wanted to evaluate the role of Tau in activity in primary neuronal cultures, thus, providing a system not only to study the effects of Tau on excitability but also to understand the mechanisms underlying these effects. Moreover, a potential synergistic effect between Aβ and Tau in pathology is being suggested[127,128] Therefore, we also wanted to assess the effect that elevated Aβ levels might have in the effects of Tau on excitability.

WT and APP/PS1 AD Tg primary neuronal cultures were transduced and after 7 days, we observed an increase in the levels of total tau after Tau WT and Tau P301L. Moreover, together with increased Tau expression, we observed an increase in cytotoxicity that was associated to the expression of Tau WT, but not Tau P301L or control GFP.

Once we characterized our system, neuronal excitability was assessed. First, we noticed an increase in firing frequency, bursting rate, amplitude, and coordinated activity in APP/PS1 AD Tg cultures compared to WT cultures. Both Tau WT and Tau P301L led to increased firing frequency and bursting rate in WT primary neurons; however, only Tau WT led to an increase in frequency and bursting rate in APP/PS1 AD Tg. Next, to understand the processes underpinning the changes in activity induced by Tau, neuronal and synaptic changes were quantified. No changes in the number of GABAergic neurons between cultures were observed, while a significant reduction in inhibitory synaptic inputs were observed associated with human Tau WT expression were quantified, thus, suggesting that the increase in activity observed after Tau WT expression can be explained by a reduction in inhibitory inputs. More work needs to be done to understand the differential effect that Tau P301L has in WT and APP/PS1 AD Tg neurons.

Overall, in this thesis we have demonstrated the ability of some amyloidogenic proteins (aSyn and Tau) to interact with each other, a synergistic effect of co-occurrence of pathology to impact in the structure and morphology of amyloidogenic aggregates, and the differential effects that proteins associated with increased risk of developing AD and PD, specifically ApoE and Tau, have over neuronal excitability, one of the earliest alterations observed in neurodegenerative diseases.

Future perspectives

In this thesis we demonstrated the potential interactions and synergistic effects between amyloidogenic proteins, which may further open the door to new questions. Where and in which context do amyloidogenic proteins interact? What are the physiological and/or pathological consequences of these interactions? Can changes in neuronal activity be modulated by the interplay between astrocytic vs. neuronal proteins? What are the molecular mechanisms leading to changes in activity, and how could we adjust them?

BiFC has shown to be a suitable technique to visualize protein interactions. Prior in vitro work has shown that the C-terminal of aSyn can bind the MTBD of Tau. Therefore, BiFC could be used to confirm which part of the proteins are required for their interaction, and where in the neuron this interaction is occurring. Additionally, the effects on cytotoxicity that promote or inhibit the interaction between proteins could be assessed.

Our results obtained by μ-FTIR demonstrated a synergistic effect between amyloidogenic proteins, but still the neurotoxic impact of this effect needs to be evaluated. Moreover, further characterization of the pathological state of the amyloidogenic proteins in the same set up needs to be done to understand which one is the association between the changes in infrared absorbance spectra (which is our main readout) and protein aggregates in the cell.

Similarly, more work needs to be done to understand how neuronal activity is modulated. Here, we observed a differential role for astrocytic and neuronal Apolipoprotein E (ApoE) in neuronal activity, but how is this process modulated in the brain? Under stress, the production of neuronal ApoE increases, but for how long do neurons keep producing ApoE once the stress is over? Additionally, we also observed a potential pathological role for Tau WT to induce hyperexcitability, what are the consequences of this? The H1 haplotype of Tau, that leads to higher levels of expression of the protein, is associated with increased risk of developing different neurodegenerative diseases; is Tau leading to a reduction in inhibitory synapses in these cases? How could the reduction of Tau levels in these people, by for example immunotherapy, impact neuronal/brain activity?

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